Archaeological, proteomic and isotopic approaches to investigating dietary change in Holocene Africa

Dissertation

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Declaration of Authorship

I hereby declare that I am the author of the present dissertation thesis.

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Table of Contents

1. Introduction

Achieving global food security is one of the biggest challenges for the future with an estimated one billion people suffering from malnutrition worldwide. In sub-Saharan Africa, undernourishment is on the rise and in 2018 an estimated 133 million people in eastern Africa and 45 million people in Central Africa were affected [1]. While a myriad of factors contribute to food insecurity, climate change can have severe long-term effects on food production. In recent years, conservation and policy efforts have focused on sustainably increasing agricultural yields of particular high-yield crops but there have also been calls to consider diversification as a sustainable solution [2]. Archaeology has the ability to explore how humans successfully responded to a range of environmental challenges to food systems over long time-scales. Investigations of which crops and animals people used through time, particularly in eastern and Central Africa, can provide insights into past and future agricultural resilience [3,4].

Africa is one of the most climatically and ecologically diverse continents on the planet and subsequently provides important case studies for considering the spatial and temporal context in which pastoralism and agriculture emerged. Significantly, pathways to early food production in Africa differ from most parts of the world as herding emerged before the spread of agriculture throughout most of sub-Saharan Africa [5,6]. Despite the importance of Africa in our global understanding of food production, historically, archaeological research in Africa has been dominated by studies concerning human origins and evolution. In many regions, the paucity of large-scale, dated zooarchaeological and archaeobotanical evidence from the Holocene means we still have a limited understanding about the context in which herding and farming developed, spread, and changed.

This thesis provides new dietary insights into past communities living in eastern and Central Africa: two key regions where it has been argued that changing climates and environments played a major role in shaping agricultural and pastoral expansion and adaptations. It is hypothesized that in arid northern and eastern Africa the emergence and development of pastoral lifeways, including dairying, were influenced by water availability with the desiccation of the Sahara and shifting monsoons pushing communities southwards [7,8]. In contrast, the closed, humid and wet conditions of the equatorial rainforest are thought to have presented barriers for the early expansion of herding and agriculture [9]. The three papers presented here provide new multidisciplinary evidence for milk and domesticated plant consumption in Holocene Africa and emphasise the need for a more nuanced, context-

specific approach to understanding past subsistence in eastern and Central Africa rather than sweeping broad models based on genetics, linguistics, or environmentally determinist assumptions.

In turn, the first paper critically assesses hypotheses for the emergence of dairying across Holocene Africa by reviewing current archaeological, isotopic, and genetic evidence (Manuscript A). This synthesis of data for the entire African continent highlights the need for direct and species-specific information to refine regional narratives for dairying. The second paper combines proteomic evidence obtained from human dental calculus with the isotopic data from human and faunal remains to investigate dairying in Sudan and Kenya (Manuscript B). It provides new, direct, species-specific evidence for milk consumption in northeastern Africa around 6,000 years ago and proposes that communities were consuming milk before the emergence of the genetic adaptation to digest milk. Finally, the third paper uses isotope analyses ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) of human and faunal remains from the Congo Basin to assess the consumption of wild sources (C_3) against incoming domestic cereals such as millet (C_4) from the first human arrival in this region (c. 2,000 years ago) through to recent history (c. 400 years ago) (Manuscript C). Collectively these manuscripts illustrate the strength of combining biomolecular methodologies with archaeological approaches and reveal heterogeneous regional trajectories for food production and pastoralism across Africa in response to changing environments.

2. Pastoral lifeways in eastern Africa

2.1 "Cattle before crops": the emergence of pastoralism in Africa

In Africa, pastoralist lifeways focused on mobile herding livestock emerged before agriculture in most regions [5]. Cattle (*Bos taurus/Bos africanus*) were the earliest domesticates in Africa. There has been significant debate about their origins with models for their independent domestication in Africa having been debated for the last forty years [10–13]. Archaeological evidence demonstrates that cattle herding was established in the eastern Sahara more than 8000 years ago [5]. The other key domestics that are now milked (goats, sheep and camel) are not indigenous to Africa. Domesticated goats (*Capra hircus*) and sheep (*Ovis aries*) are believed to have been introduced from Southwest Asia around 7800-7000 cal. BP [14–16] spreading into Africa via the Isthmus of Suez or Sinai Peninsula [17]. The one-humped camel (*Camelus dromedarius*) is believed to have been domesticated in the Arabian Peninsula [18] around the late second millennium BC [19,20] but were not present in Africa in great numbers until relatively recently [21], [22]. Finally, the donkey was domesticated in Africa from wild *Equus africanus* around 6,000 years ago and is present at some early herding sites in East Africa [23–25].

The emergence of pastoralism and dairying were argued to be heavily influenced by global climatic events and regional ecological shifts. Strategies based on cattle herding in northern Africa were clearly established by 8000 cal. BP (**Figure 1**) during the "African Humid Period" when conditions were relatively wet across the Sahara [15,26] and domestic goat and sheep were introduced into these economies. A shift in the Inter-Tropical Convergence Zone (ITCZ) in subsequent centuries pushed seasonal monsoons further south, resulting in the onset of hyper-aridity across northern Africa. Genetic and archaeological evidence indicates these conditions fuelled pastoralist migrations southward, into the Niger River Valley zones of western Africa, and to the highland and savanna ecotones of eastern Africa [27–29]. Archaeological sites with high proportions of domesticated fauna are evident in the Lake Turkana Basin of northern Kenya by 5000 cal. BP [30,31], southern Kenya by 3600 cal. BP [32], and central Tanzania by 3000 cal. BP [33] **(Figure 1)**. There are few well documented archaeological sites across south-central Africa that document the early spread of herding until it appears on the South African cape after 2000 cal. BP [34,35].

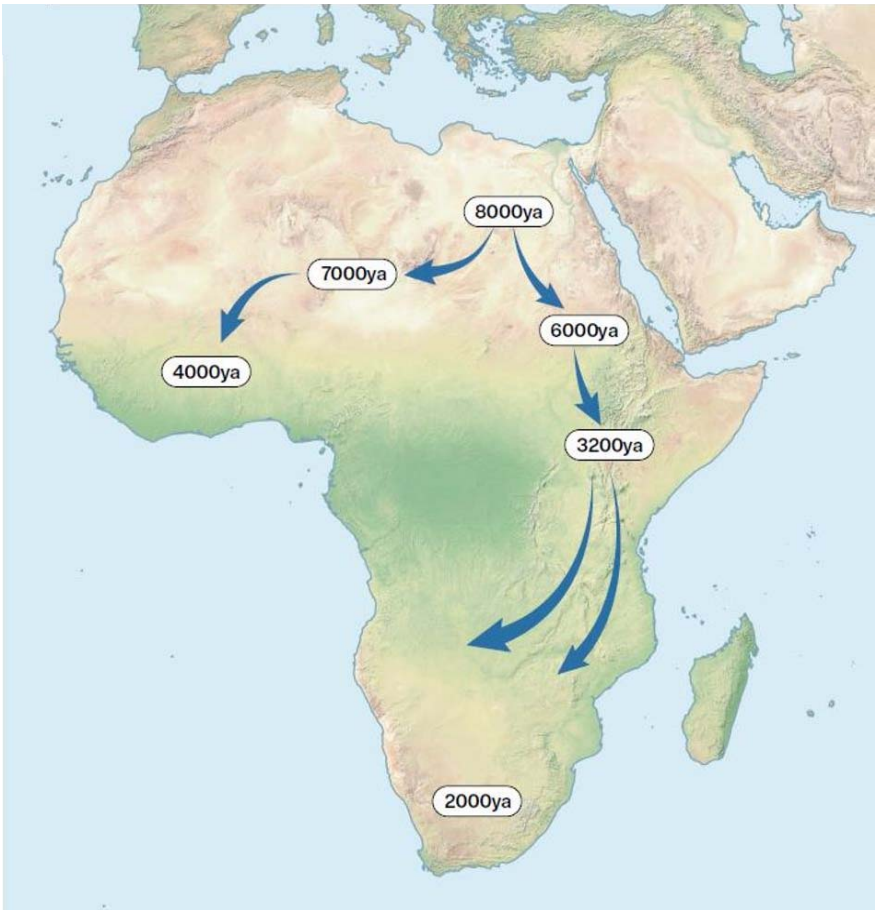

Figure 1: Map of Africa showing the spread of cattle-based pastoralism in Africa based on sites with a high prevalence of domestic cattle (after [5])

2.2 Milk consumption: nutritional and cultural implications

The transition from food procurement to food production marked a significant turning point in human history. The shift from hunting wild animals to raising domesticates for meat and secondary products, such as milk, as well as the domestication and cultivation of plants, fundamentally changed the economic, social and cultural landscape. Almost forty years have passed since Andrew Sherratt termed this transition the *"Secondary Products Revolution"* [36] which first occurred in the Near East during the Chalcolithic Period (~6000 to 5000 cal. BP) and subsequently spread to Europe and Asia. Sherratt argued that the exploitation of secondary animal products, such as milk, occurred long after initial domestication. Early attempts to test this model for dairying relied heavily on the ability to reconstruct kill-off patterns for herds based on well-preserved archaeological animal remains. Now a suite of methods, including lipid residue analysis of ceramics, peptide mass fingerprinting of unidentifiable faunal remains, identification of milk proteins in human dental calculus, can provide new evidence for when, and how dairying developed.

Humans are unique amongst other mammals in consuming the milk of a different species long after the age of weaning. For many communities across the globe, milk and dairy products are important dietary staples and dairy animals retain important ideological and social importance. Milk is a protein-rich, nutritious and renewable food source and the ability to regularly produce and consume animal milk would have been a crucial adaptation for managing climatic risk. Access to a sustainable food source could provide environmental buffering as herding communities inhabited new areas. Furthermore, livestock had significant ideological value, and, as demonstrated by ethnography, the consumption, processing and storage of milk was shaped by cultural practices and beliefs [37,38].

Through archaeological scientific research we can begin to unravel the complex narrative for the emergence of milk consumption and explore the biological and cultural context for this important dietary transition. Despite the importance of dairying in the human past, for many parts of Africa we lack precise, regional models for the emergence of milk-centred subsistence strategies. In Manuscript A, we synthesise all existing evidence for early dairying in Africa for the first time. By drawing together multidisciplinary datasets we place the emergence of dairying within regional climatic models and explore the heterogeneous patterns for dairying across Africa while also identifying key regions and avenues for future research.

2.3 Tracing the origins of milk consumption in Africa

In sub-Saharan Africa today an estimated 50 million people base their livelihood on the management of livestock [39] and, for many, dairy products are important dietary staples. The importance of milk in some regions of Africa is evidenced by modern human genetic studies of the genetic adaptation to fully digest milk, or lactase persistence (hereafter LP). African populations display five of the known LP-associated alleles and eastern African societies display more LP alleles than any other region of the continent (-13915*G, -14009*G, -14010*C and -13907*G) [40–42]. While modern LP data reflects the importance of dairying, such frequencies may not be representative of past populations due to recent population admixture. Data generated from ancient genome sequencing has therefore been important in investigating the potential relationship between LP and dairying practices. Current ancient DNA studies of prehistoric African populations suggest LP frequency was extremely low or absent in most pastoral populations [43–45]. This narrative complements the emerging picture globally that milk consumption preceded high frequencies of LP in both Bronze Age Mongolia [45,46] and Neolithic Britain [47]. However, direct evidence for when pastoralists were

first consuming milk in Africa remains patchy making it difficult to compare milk consumption and LP status. Manuscript B provides new, dated species-specific evidence for milk consumption through the proteomic analysis of dental calculus and isotope analysis of human and faunal remains. These new lines of evidence tentatively contribute to studies concerning the potential drivers for the selection of LP.

Investigating early instances of pastoralism and dairying remains challenging but current evidence suggests dairying originated in northern Africa. The identification of dairy fats from ceramics along with faunal evidence suggests that dairy consumption was part of pastoral economies in Libyan Sahara by 7200 cal.BP [26,48–50] the Nile Valley in Sudan by 6600 cal.BP [50,51], and Algeria by 6200 cal.BP [50,52]. The apparent delay in the emergence of dairying in mediterranean North Africa can, in part, be linked to the slower establishment of pastoralism in the region. In the eastern Maghreb communities practiced mixed subsistence systems until around 7000 cal.BP and pastoral activities were initially integrated into strategies that also included hunted fauna and wild plants [53].

It has previously been suggested that strategies of herd management specialized for milk production in eastern Africa would not have been viable until herders reached the bi-modal rainfall zones of southern Kenya [7]. Increased rainfall from the bi-modal ITCZ-driven monsoons in southern Kenya and northern Tanzania enabled livestock to have two birthing seasons a year increasing the amount of milk available for human consumption [7,54]. In other regions, such as West Africa, the occurrence of one rainy season per year may have constrained the emergence of intensive dairy-based economies due to lower milk production [55]. Furthermore, epizootic diseases, such as trypanosomiasis and Wildebeest-derived malignant catarrhal fever (WD-MCF), may have posed a significant barrier to the diffusion of cattle-based pastoralism [56,57]. For example, there was a significant delay in the appearance of domestic livestock in southern Africa compared to other regions and small-stock herding preceded the development of cattle-based herding [58,59]. It is possible that zoonotic stressors delayed ability to sustain large herd sizes would have limited the opportunity for surplus milk production [54]. In order to assess theories about climate and disease it is vital to determine the timing of pastoral expansions in Africa. In Manuscript B, we present new, dated evidence for milk consumption to help refine models for the emergence of dairying as pastoralists migrated into eastern Africa.

3. Agricultural reliance in Central Africa

3.1 Agricultural systems in the Tropics

Central Africa presents a quite different set of challenges for food production when compared to eastern Africa. Tropical rainforests are considered some of the most ecologically diverse places on the planet playing a crucial role in global weather systems and biodiversity. However, until relatively recently they have been overlooked when considering sustainable food systems due to their portrayal as barriers to cereal agriculture with the global demand for high-yielding agricultural crops resulting in devastating anthropogenic deforestation [60]. While there is recognition that monoculture systems can have detrimental environmental impacts and, particularly in Africa, diverse subsistence strategies can successfully mediate environmental stressors [2] relatively little is known about how the earliest communities adapted to tropical environments.

While more studies are investigating tropical subsistence strategies in equatorial Africa [61], including the importance of vegetative Asian crops [62], assessing such models remains difficult. The lack of domestic animals and plants recovered from archaeological contexts situated in tropical settings means many questions remain about the use of cultivated crops in present-day tropical environments. Manuscript C offers new information about direct dietary reliance in the Democratic Republic of the Congo during the Iron Age through the application of stable isotopes to faunal and human remains, as well as the identification of phytoliths in human dental calculus. It aims to provide new insights into how early food producing communities in the Inner Congo Basin relied on introduced domestic crops compared to local wild resources.

3.2 The Bantu Expansion and changing diets in Central Africa

In Africa, cereal crop domestication occurred several millennia after herding. Indigenous grains such as pearl millet (*Pennisetum glaucum*), cowpea (*Vigna unguiculata),* finger millet (*Eleusine coracana*) and sorghum *(Sorghum bicolor)* emerged from different centres of domestication across Africa **(Figure 2)** [63,64]). Models for how farming then spread throughout sub-Saharan Africa have been dominated by the “Bantu Expansion”, the dispersal of Bantu speaking people from present-day Cameroon [65–67]. While there is no universal “Bantu package”, other innovations appear to have occurred at a similar time to the diffusion of Bantu languages such as farming, pottery and metalworking [68–70].

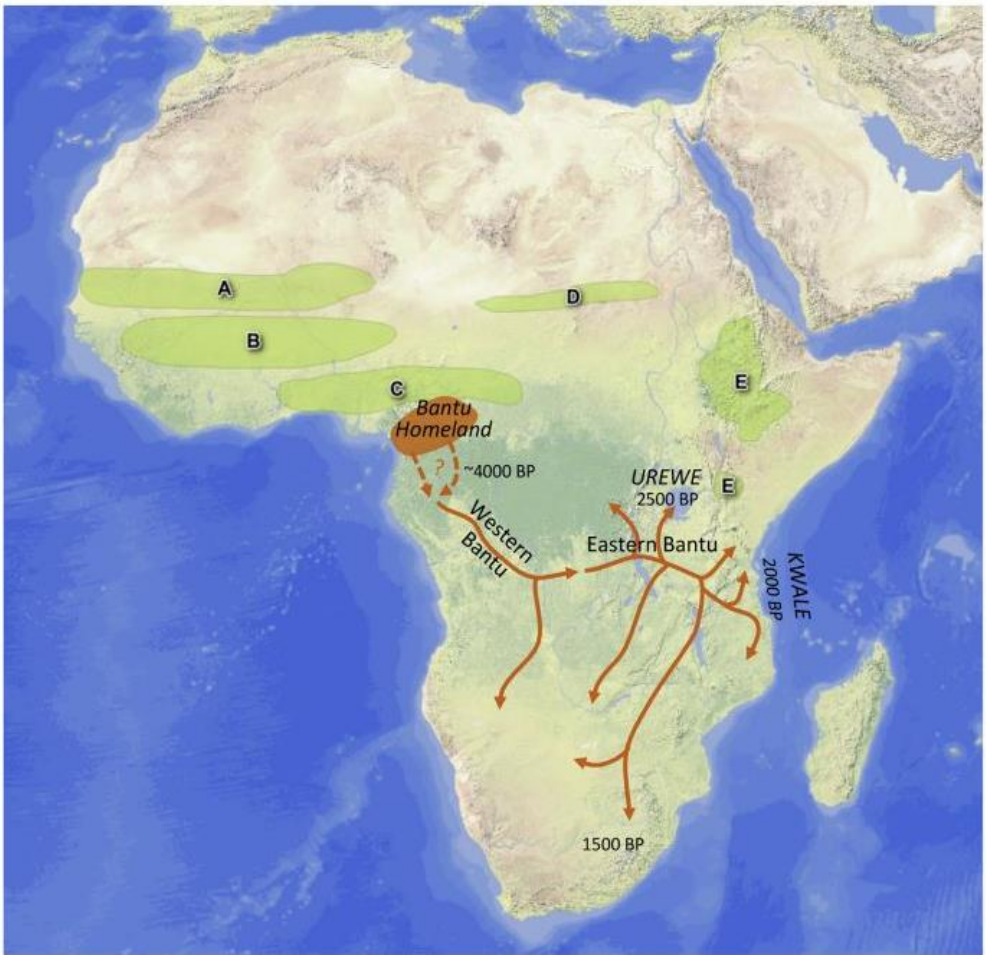

Figure 2: Map of Africa showing origins of key domesticated crops (pearl millet, sorghum, finger millet) and proposed routes in associated with the Bantu Expansion (map from [71])

The lack of domestic animals and plants recovered from sites in tropical contexts from Africa means they are often overlooked when examining the origins of agriculture. The equatorial rainforests of Africa have long been regarded as barriers for the expansion of pastoralism and agriculture. It has been hypothesised, based on linguistic evidence, that rainforests presented significant barriers to the migration of early food producing Bantu-speaking groups [9]. It has been proposed that, in contrast to western Africa where cereal-based subsistence systems intensified, in many regions of Central Africa small-scale food production, hunting, gathering, and fishing persisted [72,73]. The earliest evidence for domesticated pearl millet in the central African rainforest are charred grains recovered from Early Iron Age contexts in southwestern Cameroon [74]. Pearl millet is now a staple in the Sahel, flourishing in primarily arid or semiarid conditions, and so its discovery in Cameroon stimulated hypotheses that its successful cultivation in the tropics would have necessitated pronounced seasonality of rainfall and air humidity [64,75]. These theories have come under increased scrutiny due to the

successful cultivation of the crop today in the Inner Congo basin during high annual rainfall as well as late finds of pearl millet in the same region [76,77].

4. Methods of palaeodietary reconstruction used in this thesis

Over the past decade the number of studies applying genetic, isotopic and proteomic approaches to archaeological materials has increased significantly [78,79 a,80]. Multidisciplinary approaches not only offer the potential to examine many aspects of individual and population-level histories but can be particularly effective for examining diet when only fragmentary archaeobotanical or zooarchaeological datasets are available [81]. Since Africa is a vast and ecologically diverse continent multi-method approaches can help circumvent some, albeit not all, of the challenges caused by variable preservation [82–84]. The primary methods employed in this thesis are the proteomic analysis of dental calculus and the stable isotope analysis of bulk collagen and tooth enamel. The identification of proteins in calcified dental plaque is still an emerging discipline [85 b] whereas stable isotope methodologies have been used for dietary reconstructions in archaeology for over 50 years [86] . Therefore, both approaches have their own merits and limitations. The following sections summarise the history of the two main methodologies and provide necessary context for the discussion section of this thesis which explores some of these challenges in detail.

4.1 Palaeoproteomics

Proteins have been shown to survive into deep-time [87], surpassing the temporal limits for ancient DNA recovery. The study of ancient proteins therefore holds great potential for archaeological investigations. The origins of palaeoproteomics can be traced back to the 1950s when P. Abelson proposed that amino acids were preserved in fossilised bones, shells and teeth [88]. Subsequent developments in chromatography [89] accelerated the exploration of amino acid properties and led to the discovery that the rate of amino acid racemization (AAR), the measurement of the relative proportions of the L- and D-chiral forms, could be used to determine relative age [90,91]. In the years that followed the potential of using immunological methods to detect proteins in ancient materials was investigated [92–94]. While immunoassay-based methods became powerful tools in modern proteomics, applications to ancient materials were confounded by the higher levels of contamination and diagenesis exhibited by ancient samples [95]. Indeed, the issue of protein degradation in archaeological materials also meant that the available peptide sequencing methods at the time (i.e. Edman degradation [96]) were unsuitable for studies of ancient proteins.

A key turning point for the field was the use of mass spectrometry to detect proteins in ancient bones [97]. Mass spectrometry overcame some of the previous issues with damaged proteins because it identifies multiple short peptides and early studies focused on the most

abundant proteins in bones such as collagen and osteocalcin [97]. Following the emergence of high-sensitivity tandem mass spectrometry (MS/MS) complex mixtures of proteins can now be analysed including ceramic residues [98 c], preserved foodstuffs [99] and dental calculus [47,100]. While it is possible to analyse intact proteins, 'top-down' proteomics, for archaeological samples it is more common to analyse peptides, known as 'bottom-up' proteomics. The methods of extracting proteins from archaeological materials vary according to substrate and anticipated abundance of proteins, these include Gel-aided Sample Preparation (GASP) [101], Filter-aided Sample Preparation (FASP) [102] and Single-pot, Solid-phase-enhanced Sample Preparation (SP3) [103]. In this study, samples were extracted using FASP and SP3 and analysed using LC-MS/MS **(Figure 3)**.

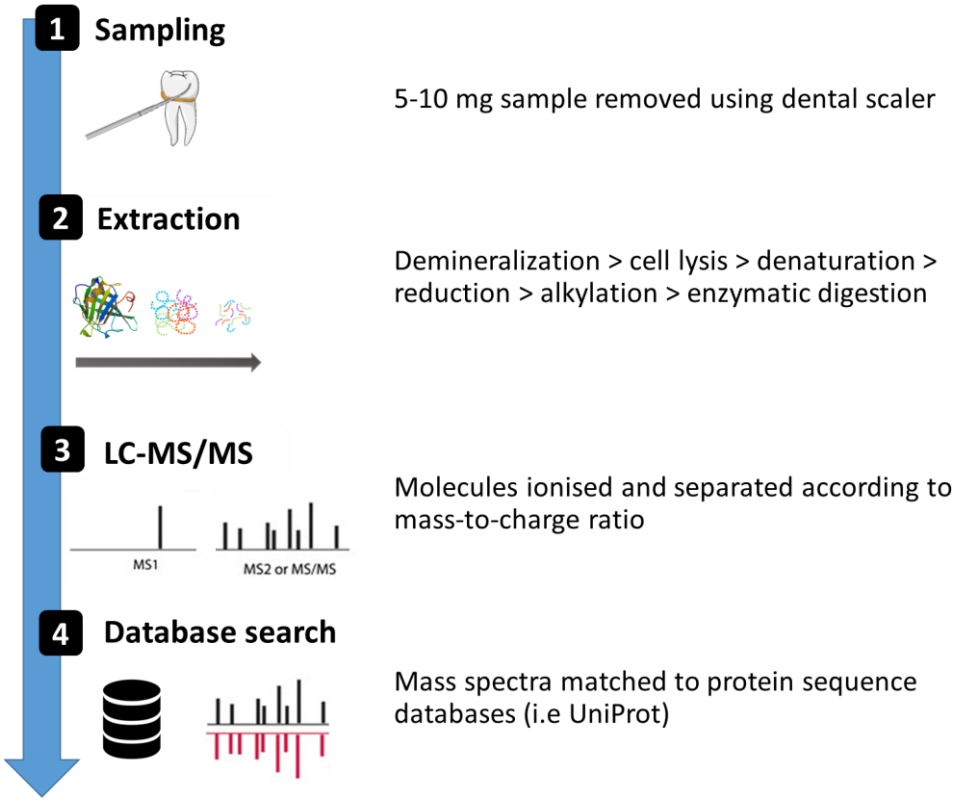

Figure 3: Shotgun proteomics workflow. 1) Dental calculus is removed from a tooth and demineralized, 2) Proteins are extracted, denatured and digested with trypsin, 3) Peptides are purified and injected into LC-MS/MS instrument. Precursor ions undergo one round of mass/charge (m/z) selection (MS1). These are further fragmented to generate product ions (MS2) for detection, 4) Experimental tandem mass spectra are matched against known protein sequences

4.2 Dietary proteins in dental calculus

Dental calculus or calcified dental plaque is a biofilm that traps and preserves biomolecules including DNA and proteins [104,105]. An increasing number of studies are using proteins identified in ancient dental calculus to explore dietary intake and from this growing body of

data some important trends have emerged. Firstly, dietary proteins appear to make up a very small proportion of the total proteins retrieved from dental calculus. The majority of proteins retrieved are microbial including those from the oral cavity [85,106]. Milk proteins are one of the most frequently reported dietary proteins retrieved from dental calculus [85] and, of these, β-lactoglobublin (hereafter BLG), the whey protein in milk, is the most commonly identified milk protein [46,47,107]. In contrast, the recovery of plant-derived proteins is far lower. To date only a small number of plants have been identified from proteins retrieved from ancient dental calculus these include peas (*Pisum sativum*), oats (*Avena sativa*) and Brassicaceae [85] in addition the cellular protein glyceraldehyde 3-phosphate dehydrogenase (GAPDH) has been identified and is believed to be of probable plant origin [107].

Lower retrieval of plant-derived proteins could be due to the way in which proteins are incorporated and preserved in calculus or due to biases in the reference databases. In the main protein databases a few, well studied, organisms have complete proteomes, such as mice, while the majority of animals and plants are not as well characterised. In recognition of such biases some studies focused on the identification of milk proteins are now using custom-made database that include dairy proteins from animals of interest [46], [108]. Fortunately, for the main three domesticates of interest in this study (cow, sheep and goat) there is already good coverage of the proteins of interest in the open-access Universal Protein Resource (UniProt) and SwissProt [109]. Although camels are milked today they were not introduced into Africa until far later and their milk does not contain one of the most commonly retrieved milk proteins (BLG) from ancient dental calculus [110].

Protein recovery for ancient calculus is also influenced by time and burial environment. The total number of proteins recovered from dental calculus decreases over time even when from a similar geographical region such as the United Kingdom [85]. Proteins and peptides are also known to break down via hydrolysis, a process requiring energy (heat), and therefore recovery is generally lower for archaeological materials retrieved from hotter environments. While proteins have been retrieved from ~4 million year old ostrich eggshells from Tanzania this is due to the specific mechanism in which peptides bind to the calcite surface and stabilization of associated water molecules [87] and the limits for protein preservation in dental calculus is still unknown. Through the successful identification of proteins from calculus from sites across Africa, Manuscript B aims to provide a new perspective on the utility of proteomics methodologies in African archaeology.

4.3 Species-specific evidence from milk proteins

A major advantage of using proteins identified in dental calculus to explore milk consumption over other methods, such as lipid residue analysis of ceramics, is the possibility of retrieving species-specific information about the animals from which the milk originated. Milk is primarily made of water, fat and proteins. In cow's milk, approximately 80% of the proteins are caseins and 20% are whey proteins **(Table 1)** recovered from the liquid fraction of milk after processing.

Protein	Concentration (g/litre)
α-S1 casein	10
α-S2 casein	2.6
β-casein	9.3
K-casein	3.3
ɣ-casein	0.8
β-lactoglobulin	3.2
α-lactalbumin	1.2
Bovine serum albumin	0.4
Immunoglobulins	0.8
Proteose peptone 8s & 8f	0.5
Proteose peptone component 3	0.3
Lactoferrin	0.1
Transferrin	0.1
Milk fat globule membrane	0.4
Total	33

Table 1: Protein content of bovine milk (adapted from [111]). β-lactoglobulin is the most commonly identified milk protein in ancient dental calculus but makes up approximately 10% of the total protein content of milk.

Although BLG is not the most abundant protein in milk (Table 2), it appears to be one of the most frequently retrieved dietary proteins from ancient dental calculus [85]. Due to differences in the amino acid sequence of proteins such as BLG, it can be possible to distinguish between different taxa **(Figure 4.a)**. However, amino acids can undergo changes over time and diagenesis-induced modifications such as deamidation can potentially reduce the resolution of taxonomic information derived from milk peptides. For example, in BLG peptide TPEVDDEALEK an unmodified aspartic acid (D) is indistinguishable, based on mass, from a deamidated asparagine (de.N) and therefore could be "bovinae or ovis" **(Figure 4.b).** While the number of specific foodstuffs that can be identified within dental calculus through proteomics has been expanding [47,85,107], one of the key questions has remained the degree to which proteomics can provide a quantitative estimate of relative consumption of these

foods. Therefore, in this thesis stable isotope analysis was used alongside proteomics to examine whether not only milk was consumed but also to what degree humans may have been relying on animal products.

b)

Sequence	Score	Mass
TPEVDDEALEK	67.1	1244.5772
TPEVDNEALEK	67.1	1244.5772

Figure 4. a) Variant site in beta-lactoglobulin peptide that can distinguish between different dairying species present in Africa. Aspartic acid (D) for Bos taurus, asparagine (N) for Ovis aries and lysine (K) for Capra hircus. **b)** The deamidation of asparagine results in its conversion to aspartic acid. In such cases, an unmodified (D) is indistinguishable from (de.N) and therefore only an assignment of "Bovinae/Ovis" could be made (adapted from [85])

4.4 Dietary reconstruction using stable isotope analyses

Stable light isotope ratio analyses are well-established tools for paleodietary reconstruction. The measurement of carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) ratios of human and faunal tissues are commonly applied to archaeological remains to investigate dietary reliance [86]. The first study of this nature, published in the 1970s, applied stable carbon isotopes to investigate the consumption of maize in North America [112,113]. This research built upon the principle that carbon fixation in plants differs according to photosynthetic pathways [114]. In terrestrial systems, the two dominant pathways are C_3 and C_4 which can be distinguished from one another based on their net discrimination against ^{13}C during the fixation of CO_2. C_3 plants such as barley, wheat and legumes have lower $\delta^{13}C$ values than C_4 plants such as maize and millets [115–117]. These observable differences in $\delta^{13}C$ are passed into the tissues of the consumers of these plants and the $\delta^{13}C$ of bone collagen is approximately 5‰ enriched in ^{13}C relative to the diet [118,119]. In the case of the North American study, increasingly positive human bone collagen $\delta^{13}C$ values showed the transition from diets composed of mainly C_3 sources to a strong reliance on C_4 maize [112,113].

Stable nitrogen isotope analysis of bone collagen can be used to investigate dietary protein intake by providing information about the trophic level. The majority of plants obtain nitrogen directly from soil with the exception of nitrogen-fixing plants, which obtain nitrogen through symbiotic relationships with mycorrhiza [120]. Many factors can influence the $\delta^{15}N$ values of soil and plants including temperature, precipitation, salinity and manuring [121,122]. In general,

$\delta^{15}N$ is enriched by *c.* 3-5‰ with each step in a food chain [123], although some studies suggest it could be up to ~6‰ [124]. As food chains in aquatic systems tend to be longer, aquatic resources tend to have higher $\delta^{15}N$ values, enabling their distinction from terrestrial resources [125,126].

Isotopic investigations of diet differ from the identification of animal or plant-specific proteins from dental calculus as they can assess dietary reliance over time rather providing a snapshot of the foods consumed. By determining the isotopic composition of different human tissues, it is possible to investigate different components of an individual's diet. For example, the $\delta^{13}C$ of bone collagen primarily reflects protein intake because dietary amino acids are preferentially used for collagen construction [127]. Whereas $\delta^{13}C$ of carbonate of tooth enamel is more reflective of the whole diet (proteins, fats, and carbohydrates) and therefore can provide more information about plant consumption.

There are also differences in the way bone collagen and enamel develop and consequently their isotopic composition reflects dietary intake at different stages of an individual's life. Bone collagen remodels over time and turnover varies according to overall bone composition (trabecular vs cortical) as well as skeletal element for example, femur bone collagen $\delta^{13}C$ represents approximately the last 10 year of protein intake in the diet whereas a rib, which has a faster turnover rate, represents an average over 2-5 years [128]. In contrast, tooth enamel grows progressively and is metabolically inert once formed and therefore reflects dietary intake at the time of tooth formation, for example, a third molar would reflect whole diet between 9-16 years of age [129]. In Manuscripts B and C, multi-tissue isotope analyses were performed in order to investigate dietary intake (protein vs “whole diet) as well as diet during different stages of an individual's life (childhood/adolescence vs adulthood).

5. Aims of the thesis

The thesis uses a multidisciplinary approach to investigate the diet of communities living in Africa during the Holocene. Two key subsistence practices; dairying and cereal agriculture, are assessed in the highly different ecological, temporal and cultural contexts of mid-holocene eastern Africa and Iron Age Central Africa. Proteomic and isotopic evidence from these two different case studies illuminate the diversity of human subsistence strategies across Africa over the past 6000 years and highlight the strength of a multi-method approach.

The thesis addresses the following specific questions:

Manuscript A:

- What is our current understanding of the emergence of dairying across Africa?
- How has climate and local ecology influenced the trajectory of pastoralism and dairying in different regions?
- How could novel scientific approaches, such as paleoproteomics, advance our understanding of dairy-based subsistence in Africa, and its relationship to climatic, pathogenic, and cultural factors?

Manuscript B:

- When were prehistoric communities in Sudan and Kenya consuming milk?
- Can the proteomic analysis of dental calculus provide species-specific information about animals raised for dairying?
- How does dairying in Africa fit within our global understanding of the origins of dairying and modern LP frequencies?
- How can we assess if proteins recovered from calculus are endogenous or contamination?

Manuscript C:

- To what degree were communities in the Congo Basin consuming (C_4) crops, such as pearl millet, compared to wild or forest C_3 sources?
- Which animals and other resources were humans reliant on during the Iron Age in Central Africa?
- How applicable are traditional “Bantu Expansion” models for the adoption and development of cereal crop use in Central Africa?

- How might human dietary variation relate to locally ecologies as well as representing cultural or close-contact exchange between foraging and farming communities?

6. Overview of manuscripts and author contributions

6.1 Manuscript A

"The Archaeology of Dairying in Holocene Africa"

M. Bleasdale, S.T. Goldstein, J. Hendy and N. Boivin

Accepted for publication in *Journal of World Prehistory*, 11th November 2019 (Submitted 3rd October 2018)

In Manuscript A, we present the first systematic review of multidisciplinary evidence for ancient dairying practices for the whole of the African continent. This work synthesises existing datasets from African archaeology including more traditional lines of evidence for dairying such as mortality-profiles of herds and rock art images of milking scenes. Additionally, we consider isotopic and biomolecular approaches including the identification of dairy lipids on ceramics, isotopic analysis of human and faunal tissues, and the identification of dairy proteins in ancient dental calculus. By reviewing the evidence over four broad regions (Northern Africa, Eastern and the Horn of Africa, Western and Central Africa and Southern Africa), we explore climatic and ecological factors that shaped the emergence of pastoralism and dairying. We also identify foci for future research such as the proteomic analysis of ancient dental calculus.

Author contributions: M. Bleasdale compiled and reviewed all existing evidence for dairying across Africa. M. Bleasdale wrote the manuscript with input from J. Hendy, S.T. Goldstein and N. Boivin.

In total, M. Bleasdale contributed 90% to the project, including data collection, synthesis and manuscript construction.

6.2 Manuscript B

"Ancient proteins provide evidence of dairy consumption in eastern Africa"

M. Bleasdale, K.K. Richter, A. Janzen, S. Brown, A. Scott, J. Zech, S. Wilkin, K. Wang, S. Schiffels, J. Desideri, M. Besse, J. Reinold, M. Saad, H. Babiker, R. C. Power, E. Ndiema, C. Ogola, F. K. Manthi, M. Zahir, M. Petraglia, C. Trachsel, P. Nanni, J. Grossmann, J. Hendy, A. Crowther, P. Roberts, S T. Goldstein, N. Boivin

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In Manuscript B, we present proteomic data from ancient dental calculus from 41 individuals from 13 sites across Africa. We found direct evidence for milk consumption (n=8) at five sites across Sudan and Kenya. Additionally, isotopic measurement of humans and fauna from the sites in Kenya showed a general reliance on domestic animals. As the study aims to identify dietary-derived proteins issues of authenticity were considered and an accompanying Oral Signature Screening Database for ancient dental calculus was constructed and published in an online public repository.

***Oral Signature Screening Database for Palaeoproteomic Analyses of Dental Calculus*:** full methods and database available on Zenodo [https://doi.org/10.5281/zenodo.3698271].

Author contributions:

M. Bleasdale, N. Boivin, A. Crowther and S.T Goldstein designed the study. M. Bleasdale coordinated the project. M. Bleasdale, S. Wilkin, M. Zahir, R.C Power and H. Babiker sampled material for the study. J. Desideri, M. Besse, J. Reinold, M. Saad, E. Ndiema, C. Ogola, F. K. Manthi, M. Petraglia and S T. Goldstein facilitated sampling and assisted with sample provenancing. M. Bleasdale conducted proteomic extractions and pretreatment of remains for isotopic analysis with supervision by P. Roberts and J. Hendy. Identification of zooarchaeological remains was conducted by A. Janzen and S. Brown. Mass spectrometry was conducted by J. Zech, C. Trachsel, P. Nanni and J. Grossmann. M. Bleasdale, K.K Richter and A. Scott conducted proteomic data analysis. M. Bleasdale conducted isotopic data analysis. M. Bleasdale, N. Boivin and S. T Goldstein composed the manuscript with input from all authors.

In total, M. Bleasdale contributed 80% to the project, including the majority of the laboratory work for the study, computational analysis and manuscript drafting.

6.3 Manuscript C

"Isotopic and Microbotanical Insights into Iron Age agricultural reliance in the Central African rainforest"

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In Manuscript C, we present the first multi-isotopic study of human dietary reliance in the Congo Basin during the African Iron Age. Stable isotope analyses ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) of

bone collagen and tooth enamel were performed on human and faunal remains from four sites across the Congo Basin. Through isotopic distinctions between local, wild C_3 plants and incoming domestic C_4 cereals, we investigate human dietary reliance on these different foodstuffs. In addition, charred food fragments and phytoliths and starches recovered from dental calculus are analysed to further investigate dietary intake.

Author contributions:

M. Bleasdale and P. Roberts designed the study. HP Wotzka, B.Eichhorn, J, Mercader and V. Linseele provided materials and provenancing information. A. Styring conducted isotopic analyses of charred food remains. M. Bleadale conducted isotope pretreatment of human and faunal tissue samples with assistance from J. Zech, S. Marzo and B. Fiedler. M. Bleasdale performed isotopic data analysis. J. Mercader, M. Soto, J. Inwood and S. Clarke conducted and facilitated analysis of dental calculus. M. Bleasdale and P. Roberts composed the manuscript with input from all authors.

In total, M. Bleasdale contributed 70% to the project, including the majority of analysis and manuscript composition.

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7. Manuscript A

The Archaeology of Dairying in Holocene Africa

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Abstract

Animal milk and dairy products have been an important food source for humans in various regions of Africa for millennia, and for many herder and farmer populations today access to milk also retains socially and ideological importance. Despite its critical role in modern Africa, there are gaps in our understanding on the origins and development of dairying practices in African contexts during the Holocene. This review is a comprehensive synthesis of the archaeological and biomolecular evidence for dairying across the entire African continent. We provide an evaluation both of proxy data for milking, and of the various methods for milk detection in terms of their respective strengths and weaknesses. Recognising that environmental conditions both constrain and support different milking strategies, the existing scholarship is reviewed with particular attention to known climatic shifts during the Holocene. Reviewing the data in this light allows us to generate several hypotheses for explaining the heterogeneous patterns of dairying observed across ancient Africa.

Keywords: Africa, Dairying, Pastoralism, Holocene, Archaeology

Introduction

Human domestication and management of herd animals like cattle, caprines, and camelids fundamentally altered the social and economic lifeways of societies around the world. While many herd species were likely first brought under human management for direct subsistence in terms of meat, ancient populations also came to use animals for secondary products, transport, and traction. The exploitation of animals for their milk and the development of dairy foods proved particularly important, offering renewable and highly nutritious food sources in a wide range of environments. Whilst the precise mechanisms and timing of the *"Secondary Products Revolution"* (Sherratt 1981) continue to be debated (Greenfield 2010; Marciniak 2011), animal milk has been an essential food source for millennia, with some

suggesting that it played an important role in animal domestication (Evershed et al. 2008; Helmer and Vigne 2007, Roffet-Salque et al. 2018). Moreover, dairying and lactase persistence is one of the best supported and most studied examples of gene-culture co-evolution, with the development of independent mutations linked to the ability to digest lactose emerging across the world (Gerbault et al. 2012; Ségurel and Bon 2017).

Africa presents a compelling case study for the inception of dairying practices as trajectories of food production on the continent differ from models for the transition to farming and herding in other parts of the world. Populations living in Africa demonstrate more identified lactase persistence-associated alleles than any other continent (Ingram et al. 2009a, 2009b; Jones et al. 2013; Priehodová et al. 2014; Tishkoff et al. 2007) and dairying practices remain socially and economically important to many pastoral populations today (Barnard 1992; Bollig et al. 2013; Dahl and Hjort 1976; Fratkin 2001). The ability to transform otherwise inedible plants into food-source for humans without having to sacrifice livestock is critical for the survival of modern herders in arid and unpredictable environments. In parts of Africa, mobile pastoralism developed before crop cultivation (Marshall and Hildebrand 2002), which could have made dairy products an even more important source of calories and nutrients for early food producers. Despite the importance of dairy products for recent African pastoralists, the possible role of dairying strategies and technologies in the spread and resilience of Africa's earliest pastoral communities' remains poorly understood.

Studying this important dietary transition in Africa is not without its challenges. Amongst these is the fact that not all regions of the continent have received the same intensity of archaeological investigation, resulting in a patchy dataset for the Holocene. Preservation of the traces of dairying is also complicated by harsh taphonomic processes, as well as high temperatures and, in some regions, tropical ecologies. The evidence that currently exists for the emergence and spread of dairying across Africa also comes from multiple disciplinary sources that have seen insufficient engagement and dialogue. Here we attempt to draw together some of this diverse and still fragmentary evidence, focusing on four broad areas of the African continent (northern, eastern and the Horn of Africa, western and central, and southern Africa) **(Fig.1)**. The role of climate in shaping early dairying practices has not been well explored for Africa and so the existing published evidence is reviewed with particular focus on palaeoenvironmental conditions throughout the Holocene. We hope that this focus will generate hypotheses for many regions that can be tested as new evidence emerges. We argue that understanding patterning in the development and intensification of milking strategies is crucial for addressing major questions regarding the spread of pastoralism across the continent (Gifford-Gonzalez 1998, 2000, 2017; Sadr 2015), thus linking dairying

to larger scale processes of population expansion and migration that have reshaped the genetic, linguistic, and economic landscape of Africa in recent millennia.

Figure 1: The four regions of Africa as referred to in this study

Identifying Ancient Dairying in Archaeological and Genetic Datasets

Identifying dairying in the archaeological record is notoriously difficult. An interdisciplinary approach synthesising multiple lines of evidence has potential to greatly advance our understanding of an otherwise elusive practice. Rock art and other images of milking scenes from various regions of the world demonstrate that animals have been raised for milk for thousands of years. The depiction of calves being present during milking (e.g. frieze from Tell al-'Ubaid, Temple of Ninhursag, Iraq, c.2500 BC, (Aruz and Wallenfels 2003, p.28), for example, is taken as evidence that, like modern herders, ancient pastoralists used the biological response of lactating mothers to their young to encourage milk let-down (Orihuela 1990; Ryan 2005). Other forms of early evidence for milking include Egyptian cylinder-seals

depicting cattle and later hieroglyphic texts listing animal products and their uses (Atici 2014; Green 1980; Sherratt 1981).

The detection of milking species and the emergence of large numbers of domesticates in comparison to wild fauna has also been used to trace the development of livestock-orientated economies; some of which would have been focused on milk production (Gifford et al. 1980; Kherbouche et al. 2016). There are however challenges with the identification of domesticates (Grigson 2000) as metric analyses rely on the assumption that domestic mammals can be recognized based on the reduced size of dental and skeletal elements (Meadow 1989; Payne and Bull 1988) but this is not a universal process (Meiri and Dayan 2003; Ozgul et al. 2009; Zeder et al. 2006).

The presence of domestic dairy species alone does not confirm dairying however it is sometimes possible to recognise mortality profiles that are consistent with animals being raised with an emphasis on milk. To ensure continued milk supply, large numbers of older lactating females are maintained in herds, while young males are culled. Demographic profiling has therefore been used to identify animals raised for milk in various time periods and parts of the world (Helmer et al. 2007; Marshall 1990; McCormick 1992; Merzoug et al. 2016; Payne 1973). However accurate age and sex determination may not always be possible as it requires the presence of several dental and skeletal markers (Grigson 1982; Halstead 1998; Legge 2005; McGrory et al. 2012; Telldahl et al. 2012). Further insights about herd management, mobility and seasonality might be gained through the stable isotope analysis (^{13}C, ^{15}N, ^{18}O and $^{87}Sr/^{86}Sr$) of human and faunal bone collagen and tooth enamel (Balasse et al. 2002; Mashkour et al. 2005). Alongside other lines of evidence, stable isotope analysis may go some way to identify animals raised for milk by detecting static herds fed on local sources (Gerling et al. 2017) and reconstructing weaning signals to determine if male animals were slaughtered at the end of the mother's lactation period (Gillis et al. 2013). Broader dietary shifts shown through the carbon and nitrogen stable isotope analysis of human bone collagen may also reflect the consumption of milk from ruminants eating specific plants (Sealy 2010) however the same variation could be attributed to eating meat of the same animals (Kornexl et al. 1997; O'Connell and Hedges 2001; Privat et al. 2005). The utility of calcium isotopes ($\delta^{44}/^{42}Ca$) for studying dairying has also been explored (Chu et al. 2006), but isotopic studies of bone collagen have demonstrated variations in both milk-drinking and non-milking populations and inconsistent differences between juveniles and adults (Reynard et al. 2011, 2013) thus warranting further investigation.

Modern genetic studies of the frequency of lactase persistence can provide broad insights into the history of milk consumption as the ability to digest milk into adulthood is determined largely by the presence of this genetic adaptation. However, the connection between the consumption of animal milk in adulthood and the evolution of lactase persistence is not well understood. In mammals, the intestinal enzyme lactase-phlorizin hydrolase (Ingram et al. 2009a; Troelsen 2005) breaks lactose down into absorbable glucose and galactose but this ability is usually lost after weaning. In humans, the *MCM6* gene contains two regulatory regions for the gene encoding for lactase (LCT) and different mutations in these regions (single nucleotide polymorphisms or SNPs) cause the lactase enzyme to continue to be produced even after weaning (Ingram et al. 2009a; Jones et al. 2015; Tishkoff et al. 2007). Therefore, the frequency and distribution of lactase persistence associated variants (Table 1) provide a framework for examining the variation in the adoption and intensification of dairying across the world, providing that population and migration histories in individual regions are considered. Ancient DNA can go some way to address the challenges of reconstructing long-term patterns of lactase persistence (Marciniak and Perry 2017). Despite the overall increase in the number of published ancient genome-wide studies, including those for the African Holocene (Schlebusch et al. 2017; Skoglund et al. 2017), for many regions and periods coverage remains relatively low, limiting the utility of this approach at present.

Global variations in lactase persistence frequency are, in part, explained by the variation in selective pressures throughout human history across different regions, as well as by historical population movements In Eurasia a single mutation, -13910*T underlies the lactase persistence phenotype and is found at very high frequency in European populations (Leonardi et al. 2012). In contrast, four lactase persistence-associated mutations have been identified in Eastern African populations and lactase persistence distribution is patchy (Swallow 2003). It is possible that lactase persistence was positively selected in populations where dairying was already taking place (Hassan et al. 2016; McCracken 1971; Simoons 1970). It appears to have evolved in areas with a long history of pastoralism (Ségurel and Bon 2017), and milk residues have been identified in the Early Neolithic in South-eastern Europe and the Near East despite presumably low frequencies of lactase persistence at the time (Evershed et al. 2008; Malmström et al. 2010). The nutritional value of milk was likely another important factor in driving the selection of lactase persistence. Milk is a good source of calcium and contains vitamin D, which is essential for calcium absorption (Pereira 2014). Therefore the Calcium Assimilation Hypothesis (Flatz and Rotthauwe 1973) proposes that lactase persistence was selected in northern Europe because populations would have not been able to synthesise enough vitamin D through exposure to sunlight. This hypothesis has

since come into question (Sverrisdóttir et al. 2014) and it may be that accessing the full calorific content of milk was a more significant selective pressure (Wijesinha-Bettoni and Burlingame 2013). It seems increasingly unlikely that there is single monocausal explanation for the selection of lactase persistence and it is clear that more research is needed to understand the benefits of dairy consumption to different populations throughout the human past.

LP-associated allele	**Geographic region**	**References**
-13907*G	Eastern Africa (Sudan, Ethiopia)	Hassan et al. 2016; Ingram et al. 2009b; Tishkoff et al. 2007
-13915*G	Middle East (Saudi Arabia, Palestine) Eastern Africa (Sudan, Ethiopia)	Hassan et al. 2016; Imtiaz et al. 2007; Ingram et al. 2007; Ranciaro et al. 2014
-14009*G	Eastern Africa (Sudan, Ethiopia)	Ingram et al. 2009b
-14010*C	Eastern Africa (Ethiopia, Tanzania, Kenya) South Africa	Jones et al. 2015; Ranciaro et al. 2014; Tishkoff et al. 2007
-13910*T	Eurasia, Eastern Africa (Sudan) Central Africa (Cameroon)	Bersaglieri et al. 2004; Enattah et al. 2002; Hassan et al. 2016; Itan et al. 2009; Jones et al. 2015

Table 1: Lactase persistence-associated alleles and their geographic region, after Ségurel and Bon (2017:Table 1)

Early herding communities may have circumvented the absence of a genetic adaptation to milk drinking by processing milk into yoghurt and cheese, since the fermentation process lowers the lactose content of dairy foods. Identification of the storage and processing of milk-derived foodstuffs in the archaeological record is therefore crucial in our understanding of early dairying practices. Biomolecular approaches have contributed significantly to our understanding of food production and consumption in the past through the detection of food residues on ceramic vessels and processing equipment **(Fig.2)** (Correa-Ascencio and Evershed 2014; Evershed et al. 1990). Through the identification of fatty acids consistent with ruminant adipose and dairy fats, milk has been detected on ceramics from Europe (Craig et al. 2005; Cramp et al. 2014; Copley et al. 2005; Evershed et al. 2008) the Near East (Evershed et al. 2008), Central Asia (Outram et al. 2009) and Africa (Dunne et al. 2012, 2018a). Biomolecular analyses have also been used to support the identification of ceramics used as “cheese strainers” during the European Neolithic (Bogucki 1984; Salque et al. 2012). Additionally, proteomic methods have identified dairy-derived proteins, verifying the presence of whole dairy products (Yang et al. 2014), as well as milk proteins trapped in the surface residues of pottery (Hong et al. 2012) and non-ceramic containers (Buckley et al. 2013; Xie et al. 2016).

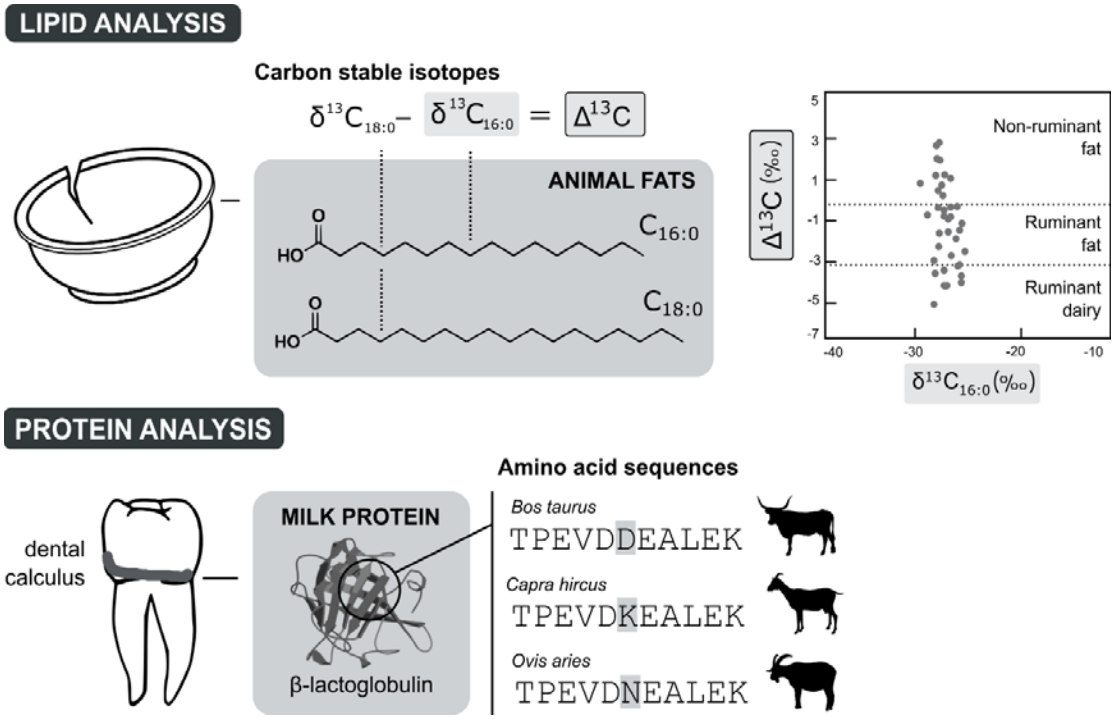

Figure 2: Visual summary of two methods used to identify ancient dairying and consumption; lipid analysis of archaeological ceramics and proteomic analysis of ancient dental calculus (mineralized tooth plaque)

The study of ceramic lipid residues continues to provide important insights into dairying, but caution must be exercised when using such evidence as a direct proxy for the first emergence of dairy-based economies because milk may not have been processed in ceramic vessels (Dunne et al. 2018b, Grillo 2014). Another method that has the potential to provide information about milk consumption at the individual-level is the proteomics analysis of ancient human dental calculus **(Fig.2).** As dental calculus (calcified plaque) forms, it traps pathogens, food debris and various dietary biomolecules (Adler et al. 2013; Hardy et al. 2009; Warinner et al. 2014a, 2014b; Weyrich et al. 2015); therefore, through the extraction and detection of proteins it is possible to learn more about the diet of an individual. In particular, the identification of the major whey protein in milk (β-Lactoglobulin or BLG) from dental calculus has provided the first direct archaeological evidence for milk consumption (Warinner et al. 2014a). Due to variations in the amino acid residues in BLG, it is often possible to establish which species archaeologically-identified milk derives from, with cattle, buffalo, sheep, goat and donkey-specific BLG all differentiable (Warinner et al. 2014a). This extraordinary degree of resolution holds revolutionary potential for reconstructing diet when faunal evidence is lacking, as well as for revealing regional variations in animal-based subsistence (Craig et al. 2005). However palaeoproteomics is still a developing field (Cappellini et al. 2014; Mackie et al. 2017) and requires favourable preservation conditions (Buckley and Wadsworth 2014; Collins et al. 2002). As of yet, more research is needed to

fully assess its potential as a tool for investigating dairying in tropical regions, where aDNA survival, for example, has been more limited (Pinhasi et al. 2015; Reed et al. 2003).

There are inherent challenges in identifying dairying in the archaeological record but an increasing suite of methods can now be applied to try to explore this practice. The combination of more traditional approaches alongside novel biomolecular methods has the potential to generate multiproxy datasets that can provide greater resolution, as well as extend the temporal and spatial exploration of dairying in the global archaeological record.

Pastoralism and Dairying in Holocene Africa

The narratives of pastoralism and dairying in Holocene Africa are intertwined, and the term 'pastoralism' is used here to broadly describe communities that relied heavily on domesticates for subsistence and often moved to new pasture to support their livestock (Gifford-Gonzalez 2005, p.188; Marshall and Hildebrand 2002, p.114; Salzman 2004, p.1). The term "nomadic" or "mobile" pastoralist is often applied to African herders following the criteria of Dyson-Hudson and Dyson-Hudson (1980), that these groups structure their mobility around the needs of their herds over other economic concerns. "Pastoralism" also encompasses the ideological and cultural aspects of human-animal interactions (Ingold 1980). This is especially true for many pastoralist populations in eastern Africa who see themselves as "people of cattle", and for whom livestock ownership is a critical dimension not just of their cultural identity, but of their status as human beings (Spencer, 1993). The consumption and use of animal products has important symbolic connotations and in many pastoral communities today milk is associated with life and growth (Århem 1989; Lombard and Parsons 2015). While the majority of this review is centred on the economic aspects of pastoralism and dairying, it is important to recognise that; as with modern pastoral communities, cultural practices may have shaped the way milk was consumed, used, and stored (Grillo 2014; Parsons and Lombard 2017).

Northern Africa and the Nile Valley

Northern Africa is the natural starting point for exploring the emergence of dairying in Africa, having yielded the earliest examples of domesticated ruminants on the continent. Domesticated sheep (*Ovis aries*) and goats (*Capra hircus*) are believed to have been introduced around 7800-7000 BP from Southwest Asia (Blench and MacDonald 2000; Bollig et al. 2013; di Lernia 2013; Smith 2005), entering via the Sinai Peninsula and/or the Isthmus

of Suez with Mediterranean maritime trade aiding their diffusion across the northern coastline (Muigai and Hanotte 2013; Oren 1979; Pereira et al. 2006). Whether African domestic cattle (*Bos taurus*) were derived from Southwest Asian cattle or native African aurochs (*Bos primigenius africanus*) or some combination thereof has been debated for nearly forty years (Brass 2018; Gautier 1984; Gonzalez 2013; Pitt et al. 2018; Smith et al. 1984; Stock and Gifford-Gonzalez 2013; Wendorf and Schild 1980, 1994). Many aspects of the precise timing and mechanism of cattle domestication in Africa remain unresolved but archaeological evidence shows that people were practicing cattle keeping by 8000 BP (for review of AMS dated sites see Riemer 2007).

The development of African pastoralism appears to relate to the extreme climatic changes in northern Africa during the early Holocene. From c.11-8k BP, a more northerly Inter Tropical Convergence Zone (ITCZ) brought increased rainfall across the Sahara, enabling the formation of wetlands and ephemeral lakes, river systems, and lush savannas (Kuper and Kröpelin 2006; Quade et al. 2018). After 7300 BP, a southward shift in the monsoon saw gradual drying out of the Sahara (Mayewski et al. 2004; Tierney and deMenocal 2013). Populations responded to the increased aridity by incorporating different degrees of mobile pastoralism (Cremaschi and Zerboni 2009, 2010; Manning and Timpson 2014). By the Middle Holocene, pastoral communities in the Libyan Sahara were using cattle, sheep and goats, and the presence of a mature animal management strategy is evident from abundant collections of livestock depicted in Saharan rock art (Muzzolini 2000).

Disparate archaeological evidence for dairying suggests it did not directly coincide with the arrival of livestock in northern Africa and was not adopted uniformly across the region **(Fig.3)**. Images found across the Tassili-n-Ajjer plateau in Algeria, depict cattle surrounded by people and vessels (Simoons 1971, p.436, p.438) and also include possible milking scenes, such as those at Wadi Aramat and Wadi Djerat (Le Quellec 2011). There are also reported depictions of cattle being milked in rock art in the Libyan Sahara (Le Quellec 2011) including part of an image from Wadi Teshuinat **(Fig. 4a)** which is believed to show a person milking a cow and collecting the milk in a vessel (Lutz and Lutz 1995; Simoons 1971). In contrast to the numerous depictions of herders with their livestock found in Central Saharan rock art, images from the Western Saharan are dominated by wild or isolated animals making the identification of pastoral scenes extremely challenging (Soler and Soler 2016). In general, definitive images of milking scenes remain scare in the archaeological record, and despite improved methods and approaches to establishing chronologies for pastoral rock art in Africa (Bonneau et al. 2017; Riemer et al. 2017), the majority of these images still lack precise dates. Rock art evidence offers tantalizing clues regarding ancient dairying, but

cannot yet provide information on the timing and inception of dairying practices in the Sahara.

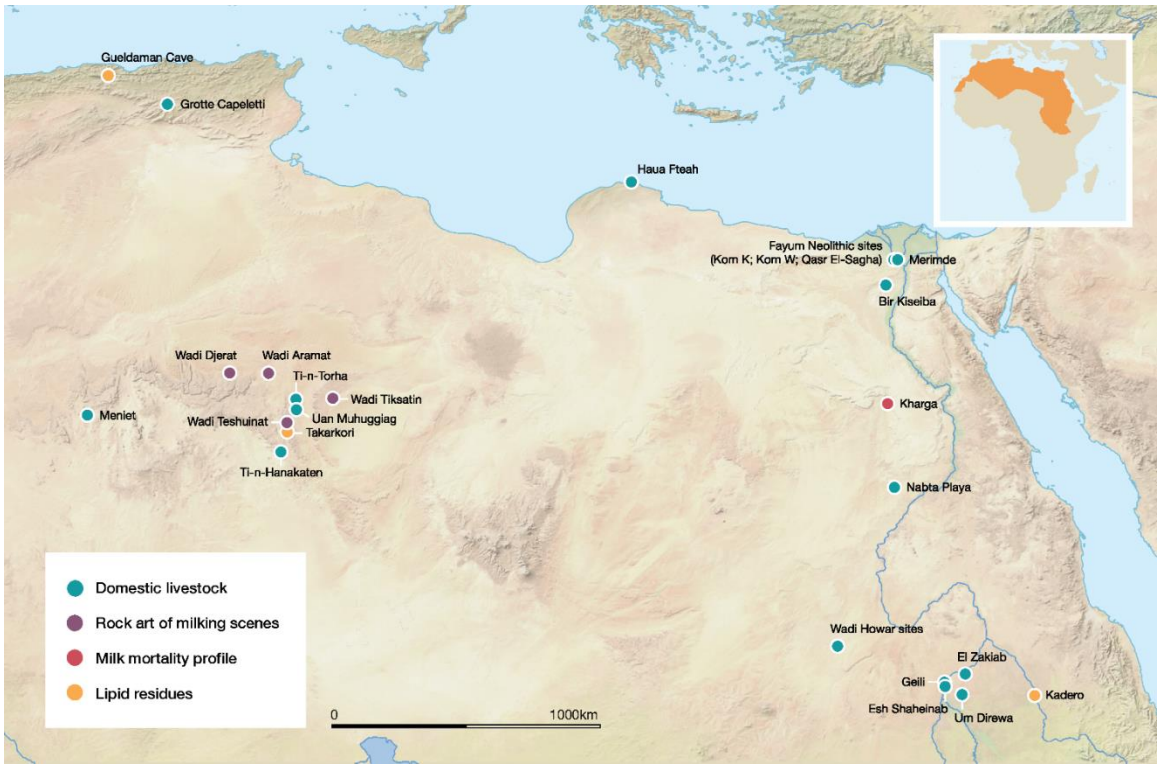

Figure 3: Location of archaeological sites across Northern Africa and the Nile Valley with early domestic livestock and evidence of dairying

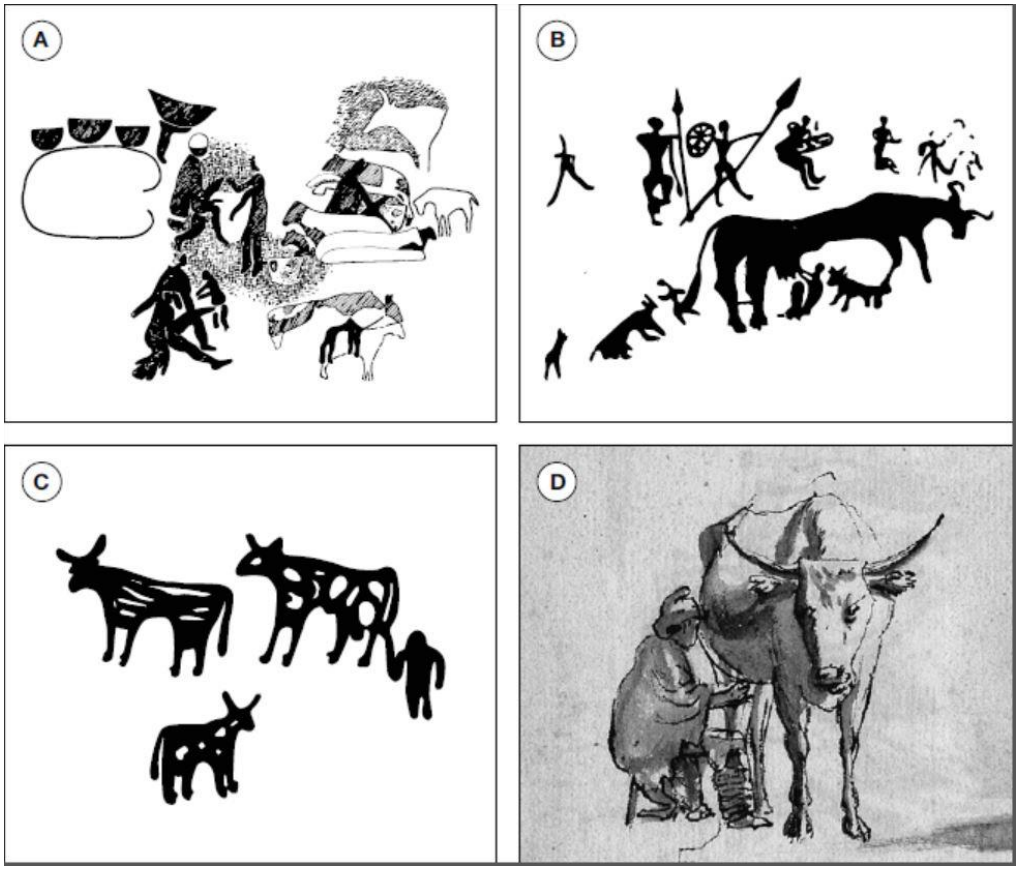

Figure 4: Archaeological and historic depictions of people with livestock and milking scenes **a)** Rock carving, Wadi Teshuinat, Libya. After Simoons (1971); **b)** Rock painting, Zeban Ona Libanos, Eritrea. After Graziosi (1964); **c)** Rock engraving, Dhar Tichitt, Mauritania. After Amblard and Vernet (1984); **d)** Drawing of Khoekhoe herder milking a cow, pre-dating 1713 (National Library of South Africa, INIL 6256)

More precise chronological information for milking has been gained by analysing residues from ceramics from Takarkori rock shelter in Libya (Dunne et al. 2012, 2018a). These findings combined with faunal data, demonstrate that milking was part of a pastoral economy by 7200 BP (Biagetti and di Lernia, 2013; di Lernia and Tafuri 2013). This is further supported by pollen and sediment records that indicate the increased pastoral occupation of the site (c.8180-5610 cal BP) coincided with new increased water availability (Cremaschi et al. 2014) which would have supported larger numbers of dairy animals. During the Late Pastoral Neolithic phase of the site (c.5700-4650 cal BP) increased aridity and environmental instability prompted the adoption of a more mobile form of pastoralism with an emphasis on goat herding (Biagetti and di Lernia, 2013; Cremaschi et al. 2014).

Climatic variations and access to reliable water sources also played a fundamental role in shaping pastoralism in the Nile Valley. The increasing desiccation of the Sahara during the Middle Holocene prompted populations to withdraw to the banks of River Nile and oases, which offered predictable supplies of water even as rainfall patterns were becoming more

erratic (Kuper and Kröpelin 2006; Kuper and Riemer 2013). In the Sudanese Nile Valley pastoralists used semi-permanent camps such as at Esh Shaheinab and Geili (Arkell 1953; Caneva 1988). Domestic animals were also dominant at many sites, including Kadero which has provided some of the earliest evidence for pastoralism in the region with the presence of domesticated sheep, goat and cattle (Krzyżaniak, 1991; Tigani El Mahi 1988). Faunal kill-off patterns and residue analysis of ceramics from the site suggest that dairying was practiced by approximately 6600 BP (Dunne et al. 2018a; Gautier and Van Neer 2011; Krzyzaniak 1991). It is possible that dairying was established far earlier in the region given the favourable conditions for raising herds, however the complex stratigraphy at Kadero means more direct evidence is required to develop a refined chronology. Moreover, there remain many gaps in our understanding of small-scale, local changes in water availability and aridity across the Nile Valley. Occupational hiatuses in the Holocene sequence at Kerma before and after the presence of pastoral societies have been attributed to changes in aridity (Honegger and Williams 2015), but more research is needed to investigate the relationship between local water availability, herding and the potential viability of dairy-based economies across other areas.

The timing of the emergence of herding and dairying across northern Africa shows great regional variation due to the diverse scope of human adaptation to local environments. In Mediterranean North Africa, there was a more gradual transition to pastoralism and dairying, with some communities practicing mixed subsistence systems including hunting, fishing and gathering until around 7000 BP (di Lernia 2013). At coastal sites, populations were able to utilise a large range of wild food resources, including marine animals, in addition to keeping domestic livestock (Mercuri 2011, Ramos et al 2011). At Gueldaman Cave, Algeria, faunal evidence and lipid residue analysis suggest initial low-levels of dairying followed by the development of a specialized pastoral economy by around 6200 BP (Dunne et al. 2018a) and a more sedentary lifestyle (Kherbouche et al. 2016; Tierney et al. 2017).

While a growing number of studies are beginning to specifically explore aspects of the development of dairying in northern Africa, the overall archaeological evidence for the region as a whole remains fragmentary (Mulazzani et al. 2016). The less intensive archaeological investigations of Western Sahara, Algeria and Tunisia have resulted in a patchy dataset, limiting the exploration of different regional trends surrounding the exploitation of domesticates for milk.

The combination of early faunal remains of domestic cattle, sheep and goat, and biomolecular evidence for milk residues suggests a very early history of dairying in northern

Africa. However there is not a clear understanding of how lactase persistence fits into the origins of dairy consumption in the region. The main lactase persistence-associated mutation in modern Northern African populations is -13910*T (Bersaglieri et al. 2004; Myles et al. 2005; Ranciaro et al. 2014) with suggestions that it was introduced by populations from the Middle East or Europe (Myles et al. 2005). This is supported by a recent genetic study of modern populations in Tunisia, with the caveat that the current lactase persistence mutations were introduced by relatively recent admixture with populations of European ancestry (Halima et al. 2017), this pattern in not surprising given historic population migrations in northern Africa and the high degree of mobility and interaction across the Mediterranean.

Patterns of lactase persistence in prehistoric northern African populations cannot be reconstructed without genomic data from individuals who lived in the Early and Middle Holocene. In comparison to other regions of the continent such as eastern Africa, the northern region remains phenotypically understudied and further work is needed to fully understand the spread of lactase persistence. There is also the possibility that early populations may have consumed milk in the absence of a lactase persistence allele by fermenting and processing milk to reduce its lactose content. For example in Morocco today, raw cow's milk is left to sour at room temperature to make *'lben'* and then used to make fermented butter *'smen'* and cheese *'jben'* (Benkerroum and Tamime 2004; Jans et al. 2017; Ouadghiri et al. 2009).

Despite northern Africa being the likely starting point for the emergence of dairying in Africa, archaeological research in the region is far more limited than on the European side of the western Mediterranean, and there has been an overemphasis on particular areas such as coastal Morocco, Egypt and Libya (e.g. Dunne et al. 20012; Cremaschi and Zerboni 2009; di Lernia 2013; Linseele et al. 2014; Martinez-Sanchez et al. 2017; Wengrow et al. 2006). As a result, the picture for the emergence of food production in northern Africa remains fragmentary, and the expansion of these datasets (Brooks et al. 2009) would significantly deepen our understanding of the development of dairying and complex interaction of populations from the Sahara, Mediterranean and Near East (Linstädter 2008; Mulazzani et al. 2016).

Eastern Africa and the Horn of Africa

Eastern Africa has a long history of pastoralism, and likely dairying given that its populations possess more identified lactase persistence-associated alleles than any other region of the African continent (Ingram et al. 2007; Priehodová et al. 2014; Tishkoff et al. 2007). As

herding and later farming economies spread into the region, they became enmeshed in a complex process of population dispersal, environmental modification, and technological transition. Although this process has fundamentally shaped the environmental, ethno-linguistic, and economic diversity of modern eastern Africa, its dynamics remain poorly understood. This section examines the archaeological and genetic data for dairying from eastern Africa here defined as the region covering the present-day nation states of Kenya, Tanzania, Uganda, Rwanda and Burundi, and the Horn of Africa comprising of Ethiopia, Somalia, Djibouti and Eritrea **(Fig. 5)**.

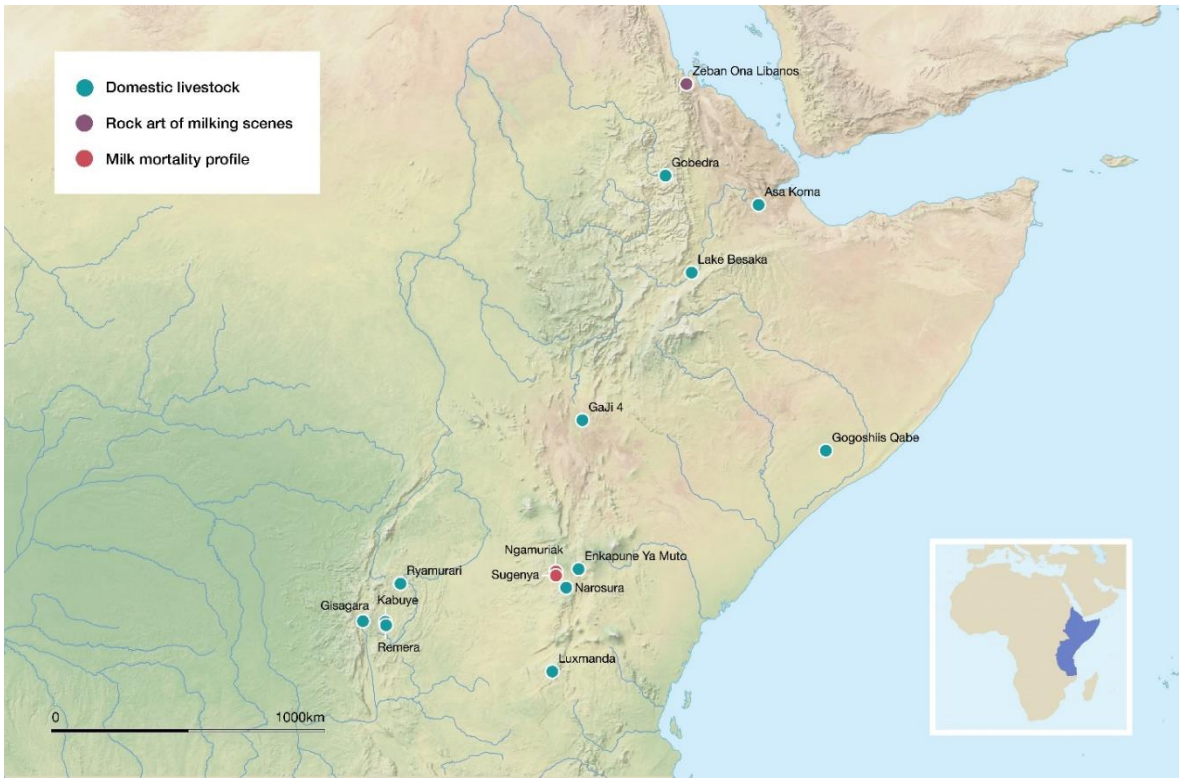

Figure 5: Location of archaeological sites across Eastern Africa and the Horn of Africa with early domestic livestock and evidence of dairying

The transition to herding in eastern Africa was a fragmentary process in which different populations across space and through time practiced a broad range of diverse subsistence economies, incorporating herding to varying degrees. In northern Kenya, the initial transition to generalised mixed pastoralism is believed to have occurred by 4800 BP (Marshall et al. 1984), when domestic cattle (*Bos taurus*) and caprines (*Ovis aries/Capra hircus*) were introduced by mobile pastoralists migrating south of the Sahara (Kuper and Kröpelin 2006; Marshall and Hildebrand 2002). Faunal evidence suggests that there was a delay in the emergence of herding in the Horn of Africa and early pastoral sites seem to cluster in the northern and eastern regions of the Horn of Africa (Lesur et al. 2014). Domesticates appear at 3800 BP at Asa Koma in western Djibouti (Guerin and Faure 1996; Gutherz et al. 1996;

Newton et al. 2008) and 4250-2950 BP at Gogoshiis Qabe rockshelter in Somalia (Brandt and Carder 1987). Tracing the emergence of dairying in the Horn of Africa is challenging due to the limited available datasets, but it is possible that the wooded, humid areas of western and southern Ethiopia, which offer abundant plant food opportunities, would have been less conducive to early arid-adapting pastoralism.

In Ethiopia today, cattle-based pastoralism is well established, and in other regions such as Somalia and northern Kenya, the one-humped camel, *Camelus dromedaries,* is an important dairy animal. Although dated camel remains are relatively recent (1300-1600 AD), it is possible that camel was present earlier (Clark and Williams 1978), with communities able to benefit from the species' long lactation period and ability to produce milk throughout the dry seasons (Bekele et al. 2002; Dahl and Hjort 1976;). Another potential option is donkey, which was domesticated in Africa from wild *Equus africanus,* and is occasionally present in early herding sites in east Africa (Kimura et al. 2013; Rossel et al. 2008).

Palaeoclimatic conditions across eastern Africa shaped the trajectory of pastoralism as these strategies spread further through eastern Africa. While, as noted, broad shifts such as the increasing acidification of the Sahara drove pastoralists southwards (Kuper and Kröpelin 2006), the degree to which environment influenced the adoption of livestock, and later milk-centred economies, can also be observed at the site and regional level. The extreme aridity in Kenya from c.5000-3000 BP would have constrained calving episodes, making it difficult to generate excess milk from animal herds in a large enough quantity for milk to be a dietary staple. As a result, patterns of mixed-herding continue to dominate the archaeological record as herding moves into southern Kenya after c.3200 BP. Early pastoralist sites like Crescent Island at Lake Naivasha and Prolonged Drift in the Lake Nakuru basin of the southern Rift Valley has a high percentage of wild fauna, suggesting that some communities continued to rely on both wild and domestic fauna, possibly as a way of mediating environmental uncertainty (Gifford et al. 1980; Onyango-Abuje 1977). These sites belong to an assemblage-group termed the "Savanna Pastoral Neolithic" (SPN), which are found across a wide area occupying mainly savanna environments in the Central Rift Valley, southwestern highland, and Athi-Kapiti Plains of Kenya. They encompass several different ceramic traditions such as the Nderit, Ileret, Maringishu, Akira, and Narosura (Ambrose 2001; Lane 2013), with suggestions that populations included migrants as well as local adopters of herding (Marshall et al. 2011).

A more consistently specialized form of pastoralism appears in southern Kenya after c.3000 BP largely constrained to highland ecologies and with its own distinctive mortuary traditions,

lithic technologies, and ceramics, which are given the designation “Elmenteitan” (Ambrose 2001; Goldstein 2018). The appearance and expansion of Elmenteitan patterns is coincident with the stabilization of the ITCZ, which fuelled a long rainy season from June to October and short rains in late November. Increased rainfall, in turn, resulted in lake-recharge and expansion of savanna grassland environments following the previous hyper-arid phase (Ambrose and Sikes 1991).

Domesticates account for a high percentage (up to 99%) of the identifiable fauna recovered at many Elmenteitan sites (Marshall 1990; Robertshaw 1988; Simons 2004). It is possible that this degree of reliance on domesticates was made possible by the emergence of the bimodal rainfall regime present in southern Kenya which would have permitted two calving seasons a year (Marshall 1990b). Ethnographic research for the region shows a clear link between rainfall and cattle productivity, with reports that lactating cattle can produce 2-5 kg of milk a day during the rainy season, but only 1-2 kg to none in the dry season (Dahl and Hjort 1976; Meyn 1970). Increased production and availability of milk may have facilitated the development of dairying from 2500 BP at Elmenteitan sites like Ngamuriak, Sugenya, and Oldurotua, where cattle mortality profiles tentatively reflect strategies wherein livestock were kept alive beyond prime-age, which is consistent with an emphasis on milk production (Marshall 1990a, 1990b).

With direct evidence of plant or other wild resource consumption lacking at Pastoral Neolithic (PN) sites, it is possible that milk (along with blood) would have supplied critical micro-nutrients that humans need to survive (but see Ambrose and DeNiro 1986). The ability to collect milk for a longer period of the year would offer more economic security in increasingly unpredictable eastern African environments, possibly supporting the emergence of the larger herder habitation sites that develop in the southwestern Highlands at this time. In turn, any increase in population would have accelerated expansion into frontier zones. Milk-derived calories may have supported the rapid spread of herders into north-central Tanzania (Prendergast et al. 2011), for example. On the other hand, without some milk consumption, it is questionable whether such specialized herding signatures would be feasible. Iron Age sites from Rwanda (Gisagara, Kabuye and Remera) have also produced faunal assemblages dominated by domestic stock (Van Neer 2000) but small sample sizes mean specific herd management strategies cannot be identified.

Spatial variation in the adoption of livestock-centred economies and dairying may also be reflected in lactase persistence data for eastern Africa. The presence of four identified lactase persistence-associated alleles (-13915*G, -14009*G, -14010*C and -13907*G)

appears to echo the long tradition of pastoralism and milk consumption in the region (Ingram et al. 2007; Jones et al. 2015; Priehodová et al. 2014; Tishkoff et al. 2007) as well as broader population movements. However not all of these mutations emerged locally – for example, -13915*G was likely introduced from Arabia – and at least one, -14009*G, has been observed at low frequency with a marginal association with lactase persistence (Al-Abri et al. 2012; Ingram et al. 2009b; Jones et al. 2015; Priehodová et al. 2014). The relationship between milk drinking and lactase persistence is further blurred by the presence of communities that drink significant quantities of milk but have a low lactase persistence-frequency, as is seen amongst Somali camel herders (Ingram et al. 2009b), for example. It is likely that many factors have shaped patterns of lactase persistence in eastern Africa, including ecological variation across space, population admixture, and the length of time over which selection operated.

Along with environmental factors, social and cultural beliefs may have also shaped the emergence of livestock-centred economies and dairying practices. Rich collections of paintings and engravings of herders and their animals such as those at Kakapelin Kenya (Brandt and Carder 1987; Červíček and Braukämper 1975; Odak 1977), including rare depictions of milking at Zeban Ona Libanos, Eritrea **(Fig. 4b)** could indicate the increasing economic and symbolic importance of domesticates in eastern Africa, at least by the early Iron Age. Early herders, like many modern pastoral communities, are likely to have seen their dairy animals as more than just “walking larders” (Clutton-brook 1989). Some have gone as far to say that early milk consumption may have been restricted to elite individuals (Durham 1991; Simoons 1970), but this is difficult to test from archaeological evidence. Turning to ethnography, it has been shown in many African pastoral communities that children always take priority in terms of the distribution of milk, especially in times of resource stress (Gulliver 1966; Wienpahl 1984). Dairying practices are, however, highly gendered in some pastoral groups (Parsons and Lombard 2017); for example, Maasai and Turkana women largely take control of the milking of cattle and the distribution of milk within the household (Talle 1987; Wienpahl 1984).

Ethnographic research has furthermore revealed that cultural values also influence the way in which milk is stored and cooked, which could have important implications for the visibility of milk storage and consumption in the archaeological record. For instance, the Samburu, pastoralists in north-central Kenya, do not collect, store or cook milk in ceramic pots, and even bringing pots near cattle is viewed as disrespectful (Grillo 2014). It is especially important for archaeological researchers to acknowledge that, as with many modern pastoral populations, communities likely used degradable storage containers such as gourds, wood

and/or animal skin bags to store, transport and process milk (Grillo 2014; Lore et al. 2005). Such practices reduce the overall visibility of material culture associated with dairying in the archaeological record (Dunne et al. 2018b). Furthermore, when ceramics are used, it is possible that milk could have been used in the manufacture of ceramics, for example, Ethiopian potters have been observed pouring cold milk into newly fired pots, swirling it around the vessel until cool, which helps to seal the vessel (Messing 1957, p.134). While ethnographic and archaeological records cannot necessarily be equated, ethnography emphasises the complex cultural component to many aspects of dairying practice.

Western and Central Africa

The western and central regions of Africa demonstrate tremendous ecological diversity, ranging from the semi-arid southern Sahel, to grasslands, deciduous forest and tropical rainforest (Seo et al. 2009). These are key locations for exploring the link between past food production and human adaptations to diverse environments. However, due to the relatively small number of well-dated early pastoral sites it is challenging to investigate when dairying may have first occurred, and it is likely that early domestic dairy animals are underrepresented in the archaeological record for many forested regions.

Domesticated ruminants were introduced to sub-saharan West Africa by mixed-economy herders migrating southwards from the Sahara (Kuper and Kröpelin 2006; Linseele 2013) with communities settling in the wetlands of the Inner Niger Delta (Breunig 2013). Highly controversial dated domestic cattle remains have been discovered from 6000 BP in Niger at Adrar Bous and Takene Bawat in the Azawagh (Paris 1996; Roset 1987). By 3000 BP cattle and ovicaprines were present at Chin Tafidet, Niger (Paris 1992) and Winde Koroji Ouest, Mali (MacDonald 1996). The Middle Senegal Valley would have also offered favourable conditions for pastoralism (MacDonald and MacDonald 2000) and there is evidence of agropastoralists occupying the site of Walaldé in Senegal around 2800-2500 BP along with domestic sheep and goats (Dème and McIntosh 2006). Returning eastwards, the increased aridity after the mid Holocene caused Megalake Chad to recede opening up new ground suitable for grazing (Armitage et al. 2015) with pastoralists raising domestic cattle, sheep and goats at sites such as Bukarkurari (Linseele 2007).

Investigations of Holocene sites in forested zones and tropical areas are more limited, with the exception of Kintampo Tradition sites in Ghana. At Kintampo rock shelter (K6) domestic caprines; and possibly cattle, have been identified (Carter and Flight 1972; Gautier 1987; Stahl 1985). However, the high diversity of wild species (Stahl 1985) and plants

demonstrates that these populations practiced mixed subsistence strategies incorporating both herding and hunting. The oldest evidence of ovicaprines in Central Africa comes from Nkang in Cameroon, dating to around 2760 - 2400 BP (Mbida et al. 2000; Van Neer 2000). Preservation biases and limited sample sizes have reduced the visibility of domestic dairy animals in many tropical and forested areas; nevertheless, unstable climatic conditions, dense forest and the occurrence of endemic diseases such as trypanosomiasis may have made larger-scale, sedentary cattle herding unfeasible (Brun et al. 2010).

Furthermore, the occurrence of only one rainy season a year; unlike Eastern Africa's bimodal rainfall system, means milk production is more limited and West African pastoralists cannot rely on milk to the same degree as other communities across Africa (Linseele 2010). Small-stock herding is far more viable in such conditions and it appears dwarf species of goat, such as the West African Dwarf and Nigerian Dwarf, evolved locally as a consequence of the unfavourable environment and may have been trypanotolerant (Chiejina and Behnke 2011; Gifford-Gonzalez and Hanotte 2011). Although goats are less efficient milk producers than cattle, their suitability for dairying in the tropics has been noted (Knights and Garcia 1997) with studies of modern West African Dwarf goats demonstrating their reasonable lactation periods, milk yields and ability to produce milk in the absence of their young (Jaitner et al. 2006).

Direct evidence for early dairying in the archaeological record for western and central Africa is sparse **(Fig. 6)**. For many of the early sites, such as Winde Koroji Ouest, it is not possible to reconstruct herd management strategies based on mortality profiles due to small sample numbers of animal bones and poor preservation (Linseele 2007; MacDonald and MacDonald 2000). Rock art of the late third-early second millennium BC from Dhar Tichittin Mauritania depicts bovids with large udders and herders with their cattle **(Fig. 4c)**, but there are no definitive images of dairying practices (Amblard and Vernet 1984; Holl 1998).

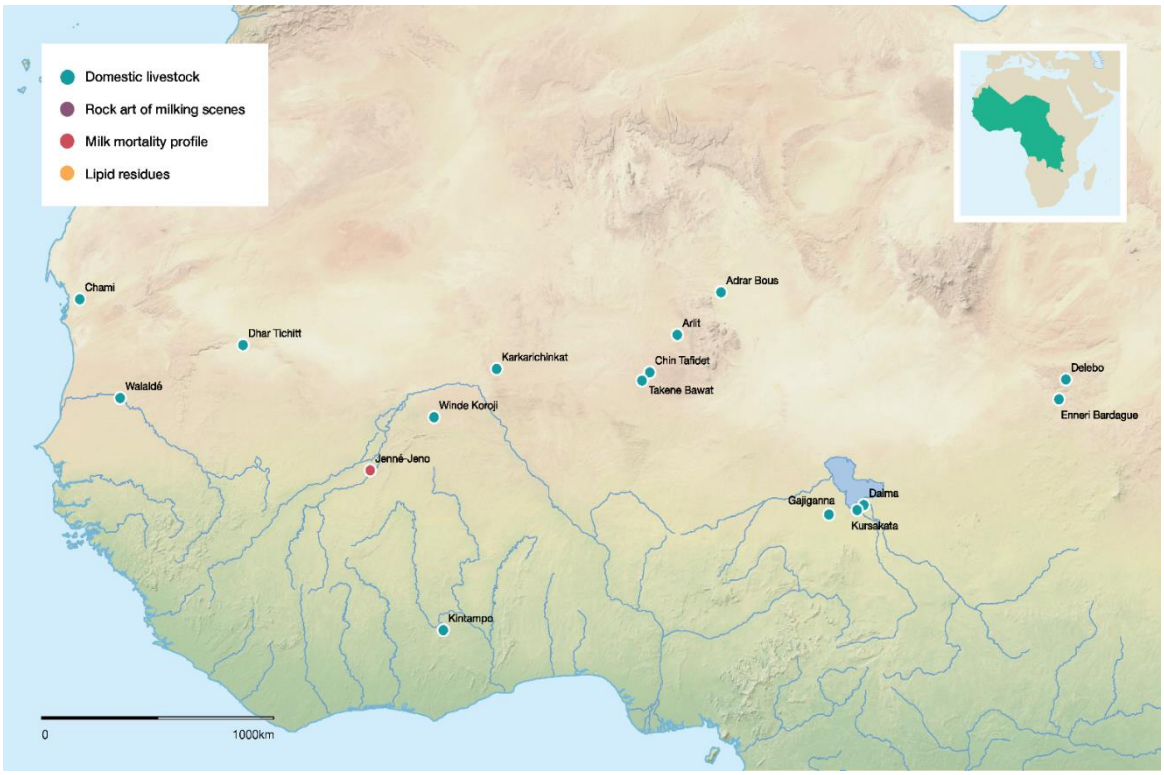

Figure 6: Location of archaeological sites across Western and Central Africa with early domestic livestock and evidence of dairying

Specialised pastoralism likely originated in West Africa during the Iron Age and may have been accompanied by a degree of dairying but identifying early nomadic forms of herding remains challenging. It may be that it was not until the second half of the first millennium AD, with the introduction of zebu-type cattle from Asia that dairying became more established (Linseele 2013). In general, zebu cattle (*Bos indicus*) are more resilient to fluctuations in food and water availability and under specific conditions may have been more reliable milk-producers than taurine or humpless-cattle (Lampkin and Lampkin 1960). In contrast, indigenous taurine cattle (*Bos Taurus*) are more resistant to trypanosomiasis (Murray et al. 1984) and are more widely used by farmers (FAO 2001). By 300 AD, previously small agricultural settlements such a Jenné-Jeno, Mali, had developed into large urban centers with evidence for herding, crop cultivation, hunting and fishing (McIntosh and McIntosh 1980; Stone 2017). Mortality profiles confirm the establishment of a mixed meat and milk economy by 600 AD (MacDonald and MacDonald 2000, p.135) and the presence of smaller and larger cattle types could be evidence of exchange with nomadic herders who introduced lager breeds to pre-existing smaller stock herds (MacDonald 1995).

Based on the current archaeological evidence it is difficult to ascertain how and when dairying fits into the narrative of the emergence of herding in the western and central regions

of Africa. However we do know that by the later medieval period dairy consumption was well-established, with written accounts from Arab travellers Ibn Battuta and Al-Bakri documenting the popular consumption of soured milk products in communities in western Africa (Translations: Monteil 1968; Ibn Battuta 1983, see also: Levtzion and Hopkins 1981). Modern genetic studies have identified -13910*T as the main lactase persistence-associated mutation in Fulani pastoralists from Cameroon, Mali and Sudan (Kulichová et al. 2017; Lokki et al. 2011; Ranciaro et al. 2014). This variant is most commonly present in European populations and could indicate relatively recent admixture with populations from outside of Africa (Myles et al. 2005). However, as discussed previously, milk consumption does not always correlate with lactase persistence and some pastoral groups from neighbouring areas such as South Sudan have low lactase persistence frequencies despite regularly consuming milk without discomfort (Bayoumi et al. 1982). More ancient genetic and archaeological investigation is needed to fill gaps in our knowledge of the development of pastoralist lifeways, dairying and milk consumption in western and central Africa.

Southern Africa

Recent southern African populations display rich genetic, linguistic and cultural diversity (Güldemann 2008; Mitchell 2010; Pickrell et al. 2012; Tishkoff et al. 2009) reflective of several waves of migration and interaction over several millennia. Early pastoralists brought domesticated sheep, cattle and goat to southern Africa, though how and when this happened is contentious. Long-standing debates centre on whether they were brought by migrating Khoe-speaking pastoralists (Güldemann 2008; Smith 1983, 2006) or were locally integrated into the lifeways of indigenous populations (Sadr 1998, 2003).

Archaeological investigations have centered on the distribution of ceramics in relation to subsistence economies (Bollong et al. 1997). The “Situmpa” ceramics in southern Zambia and “Bambata” ceramics of Botswana are both associated with early domesticated animals (particularly sheep), but remain poorly dated and minimally investigated (Huffman 2005; Plug 1981; Smith 2008). Nothing is known about the potential for dairying or extent of milk consumption during this first hypothesized phase of herding. For coastal populations the exploitation of marine mammals for meat, skin and subcutaneous tissues may have may have provided an alternative reliable source of calories. The analysis of residues from ceramics at Kasteelberg site D East (8th-11th century AD) indicates that inhabitants were utilising marine mammal products despite high quantities of goats in some contexts (Copley et al. 2004). It is possible that milking practices were introduced to these regions during later expansions of Bantu-speaking agro-pastoralists. Given the course resolution of the

archaeological record, it is only possible to say that early livestock appears associated with a period of grassland expansion around (Robinson and Rowan 2017). In southern Africa this appears associated with dryer-cooler conditions that reduced bushy environments where disease vectors flourished, providing possible corridors for herders but likely limiting the viability of intensive dairying (Castañeda et al. 2007; Schefuß et al. 2011; Woltering et al. 2011).

More recent archaeological and genetic evidence supports the idea that pastoralism in southern Africa involved at least an element of population displacement from eastern Africa (Pickrell et al. 2014; Sadr 2015; Skoglund et al. 2017). This model is also supported by lactase persistence data demonstrating the presence of the eastern African lactase persistence variant -14010*C at high frequency in the Khoe-speaking Nama of Namibia, (Breton et al. 2014; Macholdt et al. 2014), suggesting that dairying may have spread with migrating populations into southern Africa together with the introduction of domesticated animals. Again, the chronology is problematic and it is unclear when these alleles were introduced, by which dispersal routes, and in relation to which economic strategies or environmental conditions.

Questions of *how* domesticates reached southern Africa continue to be debated but *when* they were introduced is also key for exploring the development of dairying. However, the overall reliability and security of faunal identifications of early domestic species in southern Africa has come under increased scrutiny (Horsburgh et al. 2016). The earliest dated domestic sheep appear in the record around 2100-200 BP at Blombos Cave, southern Cape and have also been reported at Spoegrivier, Namibia **(Fig. 7)** (Henshilwood 1996; Pleurdeau et al. 2012; Sadr 2015; Sealy and Yates 1994). Potentially earlier caprine remains were identified at Blydefontein shelter, South Africa, but the chronology, DNA evidence and morphology have been critiqued (Bousman et al. 2016; Horsburgh and Moreno-Mayar 2015; Scott and Plug 2016). The earliest directly dated cattle bones, from Toteng 1 in Botswana, gave a date of 2070 BP (Robbins et al. 2005), but the level of preservation of the bones has created doubt over the security of the morphological based identification (Horsburgh et al. 2016). Problematic faunal identifications may be verified using ZooMS or aDNA and in some cases, such as the maxilla from Reception Shelter, this could result in revised identifications (Orton et al. 2013). There also is a need for more dated remains to strengthen our understanding of the introduction and spread of herding in southern Africa.

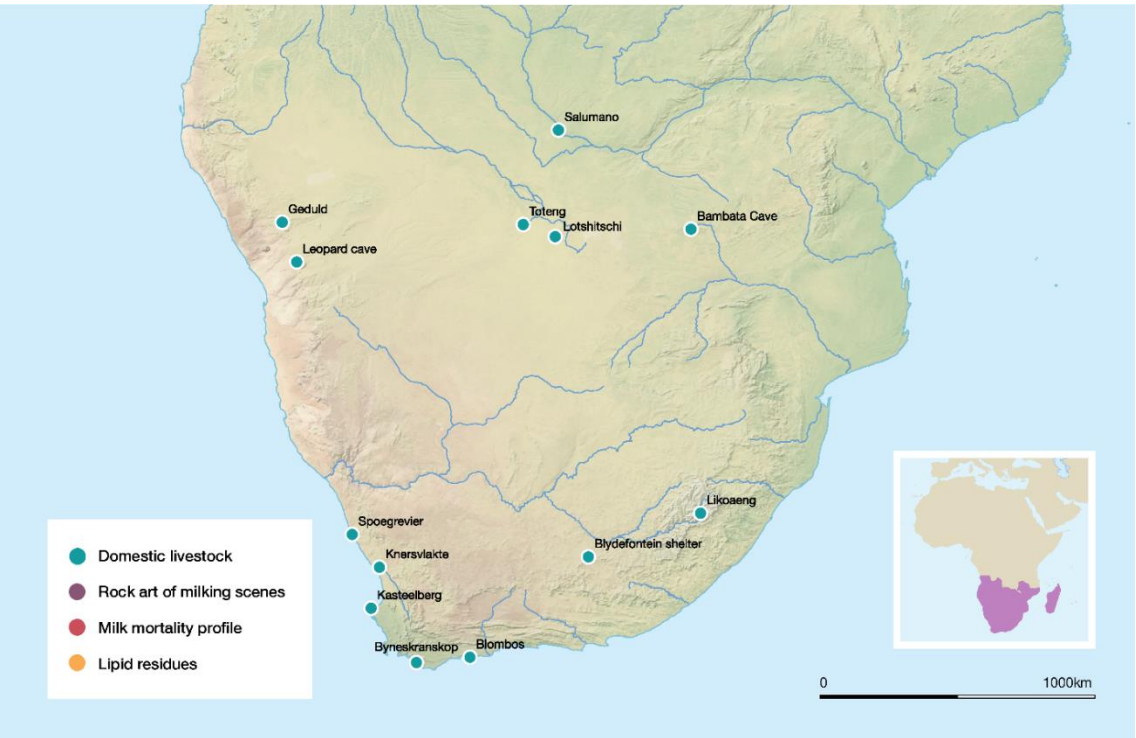

Figure 7: Location of archaeological sites across Southern Africa with early domestic livestock and evidence of dairying

Archaeological evidence indicates cattle diffused into southern Africa several hundred years after sheep and goats (Plug 1996). Rock art, for example, seems to support the later adoption of cattle, with depictions of sheep appearing widespread at sites inhabited by herders and hunter-gatherers, and cattle appearing only later and over a smaller geographical range (Hollmann 1993; Manhire et al. 1986). The $\delta^{13}C$ and $\delta^{15}N$ isotope values of bone collagen from pastoralists show no significant shift in values relative to those of hunter-gatherers upon the introduction of domesticates (Sealy 2010). Therefore suggesting a more limited reliance on domesticates for food, including dairy products, at this time.

It is possible that epizootic disease may been a considerable barrier for rapid diffusion of cattle-based pastoralism, and subsequent adoption of dairying, across the region with communities needing time to implement strategies that would ensure the survival of larger livestock (Gifford-Gonzalez 2000, 2017; Mitchell 2017). Early pastoralism in the southernmost regions of Africa may thus have centred on small stock, as seen with goat herding in the Kalahari (Ikeya 1993). Modern indigenous breeds, such as the Kalahari Red goat, have demonstrated the suitability of goat herding in humid areas as well as the potential of goats as dairy animals due to their long lactation period and high milk yield (Adewumi et al. 2017; Dhal and Hjort 1979). It is therefore possible that sheep/goat pastoralism and the use and consumption of these species' milk was enough to drive the

selection of the eastern African lactase persistence allele despite the late introduction of cattle into the region.

Accounts from the 17th and 18th century document pastoralist Khoekhoe people in southern Africa keeping large herds and regularly milking livestock (Fig. 4d) (Barrow 1801; Sparrman 1785). Milk was a major component of their diet, fresh milk was sometimes drunk straight from the udder (Kolb 1719/1745 in Jopp 1979) and fermented or curdled milk was also popular (Schultze 1907). Generally, livestock were not slaughtered for meat (Alexander 1838; Barrow 1801) due to their importance in providing milk. However, the relatively low milk yields of South African cattle were also noted in European historical accounts (Dornan 1925). The importance of milk in the diet of historically recorded Khoekhoe pastoralists is evident but tracing this back into the archaeological record for across southern Africa remains difficult. Herding groups practicing more mobile forms of pastoralism will be more difficult to identify archaeologically and in general there are relatively low proportions of dairy animal remains at South African LSA sites and a greater focus on rockshelter sites (Sadr 2008, 2013; Smith 2006).

There have been few studies that have retrieved information about dairying in southern Africa. Isotope evidence from sites across southern Africa show a dietary shift after 1000 AD which could reflect an increased intake of dairy products from cattle feeding on C_4 plants (Sealy 2010). However, it is not possible; differentiate whether dairy or other cattle products caused the observed trend. Organic residue analyses were carried out on vessels from Kasteelberg to investigate if sprouted-ware pottery recovered from contexts containing high proportions of sheep bones were used for dairying, however only marine mammal fats were detected (Copley et al. 2004). It is possible that early pastoralists used leather bags and wooden pails for milk, as observed for the Khoekhoe people in the 18th century (Barrow 1801; Sparrman 1785) and therefore the presence of dairying-related material culture in the archaeological record is hampered by the poor preservation of organic remains.

Discussion

Food production in Africa during the Holocene involved a complex mosaic of subsistence economies, with dairying forming just one, albeit important, component. The adoption of dairying itself was a multifaceted process, with populations practicing nomadic and sedentary pastoralism, large-stock and small-stock herding, and relying on milk to varying degrees. By reviewing different lines of archaeological evidence, however, some broader themes and trends emerge, including the complexities of the relationship between lactase

persistence and milk consumption, the link between changing environments in Africa and spread of pastoralism, and issues of visibility of the data in the archaeological record.

The evolutionary history of lactase persistence and dairying in Africa differs greatly from other parts of the globe. In Africa, lactase persistence distribution is fragmented, with some alleles (-13910*T and -13915*G) ascribed to gene flow from populations outside of the continent and others (-14009*G) seeming to emerge independently (Hassan et al. 2016; Ségurel and Bon 2017). In Tanzania, lactase persistence seems to show a hard selective sweep (Tishkoff et al. 2007), but for other populations it is more difficult to infer the strength of selection due to the coexistence of multiple alleles. Overall, lactase persistence diversity is highest in eastern Africa and declines further south and west into areas where pastoralism emerged later. While geneticists have proposed dates for the emergence of different alleles, modern African populations have been observed consuming milk in the absence of any lactase persistence-associate allele, thus we cannot use these time estimates as a proxy for the beginnings of milk consumption. Lactase persistence allele data does, however, demonstrate the complexity of population admixture in Africa, suggesting that dairying histories may be diverse and multi-faceted.

In Africa, the long history of dairying and complex picture for lactase persistence may in part be explained by the complexity of Holocene climatic and environmental change. The selection pressures for lactase persistence would have differed across the continent and varied due the periodicity of opportunities for significant dairy consumptions by humans in the archaeological record. All else being equal, the increased rainfall of the bimodal ITCZ pattern in southwestern Kenya and northern Tanzania enables livestock to have two birthing seasons a year, resulting in the possibility of more surplus milk for human consumption than more arid regions (Dahl and Hjort 1976). It is perhaps not coincidental that large pastoralist settlements dominated by domesticates with late-age culling patterns became more common in this region with the advent of a bimodal rainfall system (Bower 1991; Marshall 1990).

There may have been strong selective pressures for humans to be able to digest milk, whether through its regular consumption or use as an important fall back food source. Major drought periods have been common since the establishment of the bimodal rainfall pattern and in such periods accessing the full calorific content of milk and being able to comfortably digest large quantities of dairy foods would have posed a significant advantage. However, extreme or prolonged droughts would have resulted in substantial (up to 90%) herd loss, periodically limiting or eliminating the potential for milk in the diet of early herders. In such periods of severe drought, modern pastoralists recognize that limited milk production must

go to new born animals or they risk losing the herds altogether, although milk will still be given to human infants in emergencies (Dahl and Hjort 1976; Gulliver 1966; Wienpahl 1984). As diverse herder populations migrated into Eastern Africa, and climates ameliorated, reinvigorated selective pressure for lactase persistence may have contributed to the diversity in lactase persistence alleles seen in modern populations inhabiting this region.

In other regions, such as Western Africa, milk production was constrained by the occurrence of one rainy season a year so communities could not rely on milk to the same degree. An absence of wide-spread, intensive milk-centred economies would be one possible explanation for the identification of only one lactase persistence-associated allele (-13910*T) in modern populations and that it is likely a product of recent population migrations and admixture (Kulichová et al. 2017; Lokki et al. 2011; Myles et al. 2005). Similarly, if limited milk surplus were preferentially given to infants and children, rather than adults, it would not create selective pressures for lactase persistence mutations.

Environments where diseases were prevalent could have also influenced the spread of pastoralism. Diseases such as such as trypanosomiasis acted as barriers for pastoral expansion across Africa, and limited herd size, reducing opportunities for surplus milk production (Gifford-Gonzalez 2000, 2017). The record for early pastoralism in southern Africa is dominated by a narrative of ephemeral small stock herders preceding focused cattle-based economies (Fauvelle-Aymar et al. 2006; Sealy and Yates, 1994). Zoonotic stresses could have kept herd sizes small in these regions, and mirroring the case of hyper-aridity in the mid-Holocene, this would have limited the potential for surplus milk. Whilst many questions surrounding lactase persistence remain unanswered, renewed archaeological investigation of pastoral sites and ancient DNA studies of Holocene populations should shed more light on the development of dairying and emergence of lactase persistence across Africa.

Concluding Remarks

One of the main challenges for exploring the origins and development of dairying across Africa is the unequal coverage of archaeological investigations of Holocene sites across the continent and subsequent fragmentary datasets. Large regions of the African continent harbour tropical and heavily forested zones. While dairying is not commonly associated with such environments, these landscapes should not be excluded from our explorations of early dairying. Humans have successfully adapted to these landscapes since the Pleistocene (Roberts et al. 2017; Roberts and Petraglia 2015) and although archaeological evidence is

scare, domestic species have been found at early sites in the tropical areas of central and western Africa.

There are also preservational and taphonomic challenges that may reduce the visibility of dairying in the record across Africa. Milk and milk products may also have been stored in organic vessels, which are unlikely to preserve in archaeological contexts (see Grillo 2014). A more multidisciplinary approach may yet extend the temporal and spatial explorations of dairying. In particular, the incorporation of biomolecular methods can bring greater resolution to more traditional approaches, for example, the application of Zooarchaeology by Mass Spectrometry (ZooMS) to distinguish between sheep and goat, providing insight into small stock herd-keeping (Buckley et al. 2010).

The variation in the intensity of dairying and its visibility in the record have implications for uncovering evidence of milk consumption or processing. While the absence of evidence is not necessarily evidence of absence, we must also consider the possibility that there are periods and regions in African prehistory in which peoples relying on domesticated livestock did not consume dairy. A hypothesis for climate-driven dairy use and intensification has the potential to drive new, more focused research looking for biomolecular traces of dairy. Large scale studies of assemblages from sites across diverse range of time periods and regions is, of course, essential to begin grappling with questions of when and why dairying becomes an economic staple. In such studies, both positive and negative results are potentially informative and useful in establishing large-scale as well as regional patterns. Pursuing expanded research has added obstacles. Very few sites have been thoroughly excavated, precisely dated, and well published in Africa relative to many other parts of the world where the pattern for dairying is clearer. Africa's archaeological, ethnographic and genetic record suggests a rich history of dairying, which was shaped by the diversity of environments, strategies and population histories that Africa contains. Renewed research on early dairying through multi-disciplinary analyses has the potential to enhance our exploration of the interaction between culture, biology, and environment over the long-term.

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8. Manuscript B

Ancient proteins provide evidence of dairy consumption in eastern Africa

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Abstract

Consuming the milk of other species is a unique adaptation of *Homo sapiens*, with implications for health, birth spacing and evolution. Key questions nonetheless remain regarding the origins of dairying and its relationship to the genetically-determined ability to drink milk into adulthood through lactase persistence (LP). As a major centre of LP diversity, Africa is of significant interest to the evolution of dairying. Here we report proteomic evidence for milk consumption in ancient Africa. Using liquid chromatography tandem mass spectrometry (LC-MS/MS) we identify dairy proteins in human dental calculus from northeastern Africa, directly demonstrating milk consumption at least six millennia ago. Our findings indicate that pastoralist groups were drinking milk as soon as herding spread into eastern Africa, at a time when the genetic adaptation for milk digestion was absent or rare. Our study links LP status in specific ancient individuals with direct evidence for their consumption of dairy products.

Introduction

Human experimentation with collecting, processing, and consuming milk from other animals enabled one of the most profound revolutions in human diet since the initial emergence of agriculture. Animal milk is rich in proteins, fat, and micronutrients and, particularly in arid environments, provides an important way of converting scarce natural resources into a portable, renewable food source[1]. Animal milk also offers the opportunity for early weaning, and can play a role in reducing birth spacing, with significant demographic implications[2,3]. The transition to consuming animal milk is thought to have been so important in human history that it drove intensive selection for lactase persistence (LP), a genetic adaptation among certain populations across Asia, Europe and Africa that allows people to digest lactose into adulthood[4–6]. The strong selection of LP-conferring alleles in the context of milk consumption represents one of the most widely-cited examples of gene-culture coevolution[5,7,8].

In spite of its status as a textbook example of gene-culture coevolution[7], however, many questions remain about the emergence of LP. In particular, the question of whether milk drinking drove LP, or if low frequencies of LP encouraged milk drinking, has not been adequately answered[5,9]. It is still unclear what selective pressures drove LP to such high frequencies, since numerous populations manage to consume milk in the absence of this genetic adaptation, possibly through external fermentation (yoghurt, cheeses) and/or microbiome adaptations[10,11,12]. Additionally, the early spread of livestock milking practices, and the global timing and pattern for LP emergence are poorly understood. Initial

hypotheses that LP emerged rapidly among early farmers have been challenged by ancient DNA (aDNA) studies that demonstrate low frequencies of the LP-allele during the Neolithic period (~8000–4000 cal. BP) in Europe[13,14], and reports that present-day LP allele frequency levels in Germany were only attained in the last 850 years or so[15]. Palaeoproteomic studies have demonstrated that, in Europe, milk drinking was present thousands of years prior to the emergence of high LP frequencies[16], while in Mongolia prehistoric populations lacking any known LP alleles were regularly consuming milk products[17].

Forms of mobile animal pastoralism have supported large populations in Africa's arid and semi-arid grassland for several thousand years, with milk from cattle, sheep, and goat herds continuing to be vital food sources for millions of people in Africa today[18,19]. The importance of animal milk on the continent is probably key to understanding why Africa today hosts the greatest diversity of LP variants in the world[4,20,21]. All five of the known LP-associated variants are present in contemporary African populations, and three of these are believed to have independently emerged in northeastern or eastern parts of the continent, where hotspots of high LP frequencies are found today[4,5,21,22]. Despite this, the historical relationship between LP selection and milking in Africa is vastly under-studied. Counterintuitive patterns, such as the persistence of high LP frequencies amongst foraging populations in eastern Africa that are not assumed to have deep histories of animal management or milk consumption (e.g. Hadza), and low LP frequencies amongst pastoralist populations that regularly consume dairy products (e.g. Dinka), are not fully understood[4,5]. Furthermore, repeated ancient and historical population movements in Africa problematise attempts to assess the selective relationship between LP and environmental context (e.g., aridity, UV radiation[23]).

The growing corpus of aDNA research for Africa, which has generated genetic data for over 100 individuals[24-33] has so far identified only one LP-associated allele in a single individual from northern Tanzania (~2000 cal. BP)[32]. Establishing the true frequency of LP in ancient Africa is constrained by the relatively small ancient genomic dataset for the continent. In addition, the absence of a LP-associated allele in some individuals can be attributed to poor coverage on LP-associated genomic regions rather than a true absence of the LP-related genetic signature. Nevertheless, current available evidence places LP in Africa ~6000 years after the introduction of livestock to the continent, and ~1000 years after the development of specialized cattle, sheep and goat herding economies in eastern Africa[34,35]. As in Europe, higher frequencies of LP may have emerged relatively late and well after the beginnings of dairy consumption in Africa, though more chronological information for early milk drinking and high-quality ancient LP data is needed to test this.

While researchers assume that milk consumption must have existed before LP-conferring alleles could be subjected to positive selection, there is uncertainty surrounding when and where economic strategies emphasising milk production first emerged in Africa. The remains of domesticated cattle, sheep, and goats[35] and images of milking scenes in rock art[36] attest to early herding in Africa, but neither provide strong evidence for the extent or antiquity of dairying practices. In northern Africa, the identification of milk fats in ceramics together with the presence of domestic fauna suggest that early pastoralists were consuming milk by c. 7200 cal. BP in Libya[37,38] and by c. 6600 cal. BP in Sudan[38,39]. A recent lipid residue study of ceramics (n = 40) from early herder sites in the Lake Turkana Basin identified one sample with a $\Delta^{13}C$ value consistent with ruminant milk fats, suggesting some use of dairy products by 5000 BP in eastern Africa[40]. The lipid analysis also returned three other positive results (and one possible positive result) for milk fats from c. 3000-year-old ceramics (n = 85) from southern Kenya and northern Tanzania[40].

Lipid residue findings thus suggest early pastoral use of milk, but lipid analyses for milk residues suffer a number of constraints. For example, lipid evidence sometimes encounters issues of equifinality, particularly when local isotopic baselines are lacking, as mixing different food sources can mask dairy signatures[41]. A more important concern in the present case is that the identification of dairy fats in ceramic vessels does not necessarily mean milk was regularly consumed as food, as it is known to have medicinal[42] and ritual uses[43]. This concern is heightened when recovery rates are low. While the recent eastern African lipid study[40] appears to confirm early pastoralist use of milk, it remains unclear what the very low recovery rates of lipids with $\Delta^{13}C$ values within the range of milk fats mean in terms of consistency or frequency of milk consumption in the past. Ceramic lipid residue analysis is also unable to link milk consumption to particular individuals in a population that may have had mixed LP frequencies.

Palaeoproteomics provides a more direct method for detecting the consumption of milk by ancient humans. The extraction and identification of proteins from ancient dental calculus[16,17,44–46] not only enables the identification of milk drinking in specific individuals, but also in some cases the animal from which the milk was derived, since some dairy peptides are genus- or species-specific due to single amino-acid polymorphisms in different taxa. Typically, these studies have identified the milk whey protein β-lactoglobulin as opposed to other proteins more abundant in whole milk, such as caseins, although the reason for this detection bias is not yet well-understood[45]. Proteomic analysis of dental calculus can be combined with mortuary or aDNA information for specific individuals, allowing dietary

patterns to be linked to circumstances like age, sex, status and, especially, the existence of LP alleles.

Here, we draw on this application to examine the origins of milk consumption relative to existing genetic data on LP for ancient individuals in eastern Africa. We use liquid chromatography tandem mass spectrometry (LC-MS/MS) to analyse proteins extracted from the calculus of 41 human individuals from 13 sites across Sudan and Kenya. The sites from Sudan span the Neolithic (~8000–5500 cal. BP) to Meroitic (~2300–1600 cal. BP) periods and the sites from Kenya all date to the Pastoral Neolithic (~3500-1200 cal. BP) (Supplementary Note 1, Supplementary Tables 1-2, Supplementary Data 1).Today, these regions of eastern Africa display high diversity of LP-associated alleles **(Fig. 1)** and have yielded some of the earliest evidence for pastoralism in Africa, making them key geographical loci for exploring the spread of herding from northeastern into sub-Saharan Africa. To further assess whether these communities were reliant on animal-derived products (milk and/or meat), a total of 17 humans (including six whose dental calculus yielded milk proteins) and associated fauna from three Kenyan sites were also analysed using stable carbon ($\delta^{13}C$) nitrogen ($\delta^{15}N$) analysis of bone collagen and $\delta^{13}C$ analysis of tooth enamel (Supplementary Note 4, Supplementary Data 8, Supplementary Data 9). Stable oxygen ($\delta^{18}O$) isotope data for the tooth enamel samples is also presented. Ancient genetic information[31,32] was obtained previously from several of the individuals tested here, as well as from other individuals from the sites sampled in the present study, enabling comparison of dairying status, ancestry and LP allele-related data for a number of individuals and sites.

Results

We identified milk peptides, in some cases genus- or species-specific, in the dental calculus of eight individuals, deriving from two sites in Sudan and three sites in Kenya (**Fig. 2**, Supplementary Tables 1-2, Supplementary Data 6). The earliest milk peptides recovered are from Sudan, from a c. 6th millennium BP individual; milk was also identified as early as the fourth millennium BP in Kenya. These findings provide direct evidence of milk consumption in prehistoric Africa, and demonstrate the importance of animal milk for early pastoralists south of the Sahara.

The total number of proteins per calculus sample was variable (Supplementary Data 2) and lower than those previously reported in studies of archaeological calculus from Europe and Central Asia [16,44-47], likely reflecting poorer biomolecular preservation in the warmer environments of the African continent. Research has shown that dental calculus traps

molecular signatures of the human oral proteome and oral microbiome[47,48], therefore well-preserved ancient dental calculus proteomes should contain an oral signature. Since some samples derived from extensively handled museum collections, each sample was searched against a custom-made, oral signature screening database (hereafter OSSD) to avoid conducting analysis for dietary proteins on samples for which oral signatures did not support authenticity (for full details of the OSSD see Supplementary Note 3). Dental calculus samples (n = 21) from 19 individuals across eight sites (46% of the total individuals analysed) passed the OSSD threshold (Supplementary Data 3).

Of the 21 samples (19 individuals) with a characteristic oral proteomic signature, 11 samples (10 individuals) had at least one milk peptide. Nine of these samples (8 individuals) were considered to have authentic dairy proteins after screening **(Fig. 2, Methods)**. All nine samples had peptides from the milk whey protein β-lactoglobulin (BLG) (Supplementary Data 6, Supplementary Figs. 1-8). The most common peptide recovered was at position 143 (Supplementary Data 6) accounting for 57% (30/52) of the total BLG peptides identified, consistent with other studies reporting milk proteins in ancient calculus[16,17,45,46]. BLG proteins from one individual from Sudan produced species-specific information, providing evidence of goat milk consumption. For all other individuals (n = 6) only genus, subfamily, family or infraorder-level identifications could be made for the type of milk consumed (Supplementary Table 4, Supplementary Table 5, Supplementary Data 6). In addition, casein proteins, which constitute c. 80% of the proteins in cow, sheep and goat's milk, were recovered in the calculus of one individual (Supplementary Fig.8). No evidence consistent with camel milk was recovered, which was not unexpected since camels were introduced from Arabia in the last c. 2000 years and only reached large numbers by ~1600 BP.

Dairying evidence in prehistoric northeastern Africa (Sudan)

We analysed dental calculus from individuals from seven sites across Sudan, and detected milk peptides (BLG and caseins) at the sites of Kadruka and Berber Meroitic Cemetery. Dairy proteins were identified in the calculus of two individuals from Kadruka, a series of cemeteries dating from the Neolithic (~8000-5500 cal. BP) to Kerma period (~4450-3450 cal. BP) located in the Northern Dongola Reach, south of the 3rd Cataract of the Nile[49] (Supplementary Note 1). One individual from Kadruka 21 (Z452, DA351), a Neolithic cemetery dating to ~6000 cal. BP[49] possessed milk proteins assigned to Bovinae (domestic cow/zebu) or *Ovis* (sheep). We detected BLG peptides associated with *Capra* (goat) in the calculus of an individual from Kadruka 1 (Z708) **(Fig. 3)**, directly radiocarbon dated to 4140-3930 cal. BP (Supplementary Table 2, Supplementary Fig. 14), providing the earliest direct evidence of goat milk consumption in Africa. Faunal remains recovered from Kadruka 1 and

21 include cattle, sheep, and goat[50,51], in line with the proteomic results. BLG peptides derived from Bovinae or *Ovis* were also present in the calculus of an individual (DA156) from the Berber Meroitic Cemetery site (~2300–1600 cal. BP) in northern Sudan. In the calculus of this individual, Bovinae Alpha-S2-casein and Beta-casein were also detected, including one peptide sequence for Kappa-casein derived from *Bos*. The individual was recovered from inside a mudbrick substructure, likely the tomb within a pyramid[52].

New evidence for milk consumption in eastern Africa (Kenya)

Individuals were analysed from six sites in Kenya and milk proteins were identified from three, spanning different time periods and ecological zones. The earliest evidence of milk consumption in Kenya was identified at Lukenya Hill, an inselberg in the Athi-Kapiti plains of southern Kenya. Lukenya Hill is associated with several archaeological sites with early evidence of pastoralist material culture and domesticated animal remains[53]. Two samples that yielded results for this study come from locality GvJm202, which is a rockshelter containing human burials attributed to the Pastoral Neolithic phase (~3500-1200 cal. BP)[54] (Supplementary Note 1). Another individual from this locality was directly radiocarbon dated to 3610–3460 cal. BP[31] (Supplementary Note 1, Supplementary Table 2) confirming early pastoral occupation at GvJm202. Genetic analyses of two individuals from GvJm202 have not identified any known LP-alleles[31].

A second early pastoralist site where BLG proteins were recovered from human dental calculus was Cole's Burial Site (GrJj5a) near Lake Elmenteita within the Central Rift Valley, approximately 150 km northwest of Lukenya Hill[55] (Supplementary Note 1). Dental calculus recovered from one complete human burial yielded BLG peptide sequences belonging to Bovinae/*Ovis* (DA325). This individual is directly radiocarbon dated to 3350–3180 cal. BP (Supplementary Note 1, Supplementary Table 2)[32], with genetic data demonstrating shared ancestry with other early pastoralist communities, including those at Lukenya Hill[31]. Despite the likely pastoralist diet and lifestyle, and pastoralist ancestry, this individual lacked a genetic signature of derived alleles associated with LP in their genome[32]. Dental calculus was also analysed from an isolated incisor (DA346) from another individual from which BLG peptides (Bovinae/*Ovis*) were identified.

A single individual (DA144) (Skeleton 1) from Molo Cave (GoJi3), also in the Central Rift Valley (Supplementary Note 1), had BLG peptides matching to Bovinae/*Ovis* in their dental calculus. A fragment from the petrous portion of the skull from this individual was directly radiocarbon dated to 1415–1320 cal. BP[31] (Supplementary Note 1, Supplementary Table 2). The Molo Cave individual dates to very near the transition from the Pastoral Neolithic to the

Pastoral Iron Age period in the region and their genetic ancestry[31] reflects relatively late admixture between indigenous eastern Africa foragers and early herders. Despite the direct evidence for milk consumption, this individual also lacks LP-related alleles[31].

Results of stable isotope analysis for all three sites (Lukenya Hill, Cole's Burial, Molo Cave) **(Fig. 4A**, Supplementary Note 4, Supplementary Figs. 9-13, Supplementary Data 8, Supplementary Data 9) support the proteomic findings. Human bone collagen and tooth enamel $\delta^{13}C$ results for the three sites indicate a dietary signal that is aligned with the local environment, mainly C_4 grasses, consistent with the consumption of herd animal products (meat or milk). For Lukenya Hill, the average $\delta^{15}N$ for human bone collagen is 12.7‰ compared to 8.0‰ for *Bos* (identified using peptide mass sequencing) (Supplementary Note 4, Supplementary Data 8) which could indicate the consumption of milk or meat from domesticates. Similarly, the difference between the average $\delta^{15}N$ for humans from Molo Cave (11.6‰) and that of the associated *Bos* and *Capra* specimens (7.0‰) is within the expected range for trophic enrichment (Supplementary Note 4, Supplementary Data 8) indicating humans were heavily reliant on animal food sources for their protein intake. Overall, $\delta^{13}C$ results for the three Kenyan sites are consistent with people reliant on associated herd animals for meat or milk products[55,56].

Oxygen ($\delta^{18}O$) values for humans (n = 12) and faunal (n = 9) tooth enamel for all three sites are variable, ranging from -3.7‰ to 0.9‰ and -7.8‰ to 2.2‰ respectively (**Fig. 4B**, Supplementary Data 9). Of the sites sampled, Molo Cave has the largest faunal isotopic dataset (n = 6) including wild taxa (i.e. *Dendrohyrax*, *Heterohyrax*) and domesticates (*Capra* and *Bos*). Caprine $\delta^{18}O$ (-1.5‰ and 1.7‰) are higher than that for the single *Bos* (-4.0‰) sampled from the site. A similar distinction between caprines and cattle has been observed for Pastoral Neolithic sites in Kenya[57] and likely reflects differences in drinking behaviours, the proportions of ingested surface water and plant water, and/or taxonomic differences in fractionation. Given the known multifarious dietary and environmental influences on $\delta^{18}O$ and small number of enamel samples in this study, we cannot further assess these values, nor those obtained for Lukenya Hill and Cole's Burial, with great confidence. We present them for completeness and future use by other scholars undertaking more detailed $\delta^{18}O$ analysis of the assemblages, or compiling regional datasets.

Discussion

The identification of milk proteins in the calculus of eight individuals from five sites across northeastern and eastern Africa provides direct, palaeoproteomic evidence of milk consumption in Africa. Despite the importance of the dietary transition to dairying, and the

complex emergence and spread alluded to by current LP allele demography in Africa, understanding of dairying origins in the continent has been impeded by poor preservation of early pastoralist settlements and ancient organic remains. Our provision of direct evidence for the consumption of milk products provides critical insight into the human cultural practices that may have driven selection for diverse LP alleles in Africa.

In this study, milk proteins were detected in calculus from individuals at archaeological sites in Sudan dating to the Neolithic, Kerma, and Meroitic periods. Our results from Kadruka 21 and 1, dating to ~6000 cal. BP and ~4000 cal. BP, respectively, provide direct evidence that milk products were being consumed in the Northern Dongola Reach by at least the sixth millennium BP. This supports previous lipid residue and zooarchaeological evidence from the Neolithic site of Kadero (c. 400 km south of Kadruka) that suggest human use of animal milk by ~6600 cal. BP[38,58]. While much attention has been given to the economic, social, and symbolic significance of cattle in early North Africa due to their predominance in faunal records across the region from the Neolithic onwards[58], our results directly demonstrate, that goats and possibly sheep were also sources of milk products in these early dairying cultures. Obtaining milk from arid-adapted species may have been critical during times of drought in the past as it is amongst northern African pastoralists today[59,60]. Later in time, the identification of Bovidae and *Bos* milk proteins in calculus from one individual from the Meroitic Period is consistent with textual evidence and rock art images emphasising the importance of cow's milk at this time in Sudan[61].

The identification of milk proteins in the calculus of individuals from Lukenya Hill and Cole's Burial (~3600-3200 cal. BP) constitutes some of the earliest direct evidence for the arrival of herders in southern Kenya and demonstrates that these groups were already consuming animal milk during the very earliest expansions of herding into the region. Combined with proteomic data from Sudan, and milk lipid residues from one sherd at Dongodien (GaJi4) in northern Kenya by 5000 cal. BP and three sherds at Ngamuriak (GuJf6) and Luxmanda in SW Kenya/northern Tanzania after c. 3000 cal. BP[40], these data point to milk consumption having been a widespread and persistent component of early herder lifeways. Together, these studies provide strong, multi-proxy evidence that people were regularly relying on access to animal milk throughout the expansion of pastoralism from the Sahara through eastern Africa with no detectable regional or temporal gaps. It is possible that, as has been argued for Mongolia[17,46], caloric and nutritional contributions from animal milk were in fact necessary for the survival of early African herders during expansion across arid regions before widespread plant agriculture (see [62,63]). Increased aridification and drought frequency after ~4500 cal. BP across eastern Africa[64] may have provided the bottleneck effects

necessary for rapid selection of LP genes among small populations of herders such that they become evident in ancient genomes by 2100 cal. BP[32]. In such a scenario, an enhanced ability to digest milk products through and after adolescence would have significantly increased an individual's chances of reaching reproductive age.

Our study provides a rare opportunity to link the LP status of specific ancient individuals with direct evidence for their consumption of milk, drawing on combined palaeoproteomic and archaeogenetic investigations. This research clearly demonstrates that ancient African individuals who do not appear to have had a genetic adaptation enabling lactose digestion were nonetheless drinking milk. As noted, not all individuals or populations that drink milk have LP, and ancient dairy consumption may have been enabled by fermentation practices[10,11] or gut microflora[12], as is suggested for populations today. Our study points to the early consumption of dairy having had a role to play in driving selection for LP in eastern Africa. Whether this related to the increased nutritional benefits provided by milk in a particular dietary context, its benefits as a fluid source in arid environments; and/or its utility as a mechanism for withstanding drought or food shortages, for example, remains to be investigated. But it may be that exceptional periods of stress, such as drought, were necessary for driving strong selection for LP. In Africa, drinking milk may have enabled expansion of pastoralists into new regions, and persistence of populations through periods of climatic aridity.

While the proteomic data reported here cannot yet provide a clear explanation for modern LP patterns in Africa, which undoubtedly reflect palimpsests of migrations across the continent and a potential range of selection pressures, they nonetheless add to a growing body of literature that shows milk consumption began millennia before LP became widespread in several regions of the world. These results show an emerging picture, on a global-scale, of dairying and milk consumption as cultural adaptations that preceded widespread LP, in numerous cases by several millennia. Our results further demonstrate that proteomic evidence for milk consumption has the potential to refine existing narratives for the spread of dairying even in hot environments. Dairy proteins survive in dental calculus from at least 6000 years ago in spite of challenging preservation conditions. Nonetheless, the limited application to date of palaeoproteomic analysis to dental calculus from tropical and arid regions of the world means that extraction methods and data analysis pipelines still need to be optimised for low abundance samples. In order to validate our findings, we created a custom-made oral signature screening database (See: Supplementary Note 3 for full details) to investigate if calculus samples matched expected oral protein signatures.

However, larger global datasets for ancient dental calculus are needed to fully assess temporal and geographic trends relating to overall preservation[65,66].

Lipid residue analysis has been applied to investigate the inception and development of pastoral economies in northern and eastern Africa, finding that dairy products were used or stored in ceramic vessels[37,38,40,67]. Models for interpreting lipid residue results in European studies[68] may suggest that the very low detection rate for milk lipids from African ceramics indicates minimal contributions of milk to African herder diets. However, proteomic evidence for milk consumption in several individuals supports the idea that the African lipid recovery rates instead reflect preservation biases in arid and tropical climates or that milk was processed in organic containers[70, 71]. This reinforces the need for multi-proxy studies that, together, strengthen arguments for widespread use of animal milk among ancient eastern African herders.

Protein-based research opens up the possibility of exploring variation in the relative reliance on the milk of different animal species, and patterns of livestock management, across different regions and populations. The milking of species better suited to arid environments, such as goats, provides potential insight into the adaptations pastoralists developed to survive droughts as well as longer-term climatic aridification. Goats and sheep reproduce much faster following drought events[63] and their role in rapid recovery from climatic stress in the past merits further exploration. Palaeoproteomic analysis also enables dairying to be identified directly, and in specific individuals, with the possibility, as we have demonstrated here, of linking milk consumption to specific genetic ancestries and LP status. In Africa and other parts of the world, preference for storing milk in organic vessels like gourds rather than ceramics[69-71] challenges the use of lipid residue analysis of ceramics as an approach to the study of early milking. Nonetheless, lipid studies remain useful, particularly when skeletal material is unavailable, and in investigations of how fresh milk may have been converted into low lactose products like cheese and yoghurt.

Our data demonstrate that the identification of milk proteins in dental calculus has significant potential to expand our understanding of the global origins and emergence of dairy-based economies, including in warmer and tropical regions of the world. Its ability to identify milk drinking in specific individuals, who can also be assessed using archaeogenetic methods to understand their specific lactase persistence status and mutations, offers the opportunity to develop fine-scale proteomic-genomic studies in Africa and elsewhere in future. This study also highlights the strength of combining proteomic and isotopic evidence to provide direct, species-specific information about milk consumption as well as assess human dietary

reliance on animal-derived products. Moving forward, the integration of proteomic evidence from dental calculus with the analysis of lipid and protein residues in ceramics, and application of peptide-mass fingerprinting to aid the identification of domesticates, will undoubtedly refine and even reshape our understanding of the emergence and intensification of herding and dairy consumption in Africa and beyond.

Methods

Experimental Design

Individuals were analysed from 13 sites across Sudan and Kenya from a range of periods and locations (Supplementary Data 1) All material from Kenya was sampled and exported under permits issued by the National Museums of Kenya. Material from Kadruka 1 and Kadruka 21 was sampled and exported from the Laboratory of Prehistoric Archaeology and Anthropology, University of Geneva under the terms of an agreement with the Section française de la direction des antiquités au Soudan (SFDAS). All other archaeological remains from sites in Sudan were sampled and exported in accordance with section (31A) of the Sudan Antiquities Ordinance 1999. Permission "Ref. NCAM/4/B" was issued by the National Corporation for Antiquities and Museums (NCAM), Khartoum, Sudan.

Fifty-one human dental calculus samples, representing 41 individuals were analysed for proteins (Supplementary Data 1-2), 13 human bones and 21 teeth were analysed using stable isotope analyses ($\delta^{15}N$, $\delta^{13}C$, $\delta^{18}O$) (Supplementary Figs. 9-13, Supplementary Data 8, Supplementary Data 9), bones from 8 animals were analysed using peptide-mass-fingerprinting to confirm taxonomic identification (Supplementary Table 6, Supplementary Data 7) and a radiocarbon date was obtained for one human individual (Supplementary Fig. 14).

Dental calculus sampling

Dental calculus samples were collected from a number of archaeological remains curated by the National Museums of Kenya, Sudan National Museum and Laboratory of Prehistoric Archaeology and Anthropology Geneva (Supplementary Table 1). Dental calculus was removed from the teeth using a sterile dental scaler and transferred to 2 ml microcentrifuge tubes. Protein extractions were conducted in a clean room facility at the Max Planck Institute for the Science of Human History, Jena.

Proteomic Extraction Methods

Dental calculus samples were extracted using two methods (for discussion of method selection see Supplementary Note 2). Ten samples were extracted using a modified FASP (Filter-Aided Sample Preparation) protocol[17,72,73]. 10-20mg of each sample was crushed and briefly predigested in 0.5 M EDTA for 5 minutes. After the supernatant was removed, the samples were demineralised in 1.0 mL of 0.5 M EDTA for 3-5 days. After demineralization the samples were centrifuged to create a pellet. 10 kDa Millipore Microcon filter units were prepared by adding 50 µL of 8 M Urea in 100 mM Tris-HCl (UA). Then 200 µL of EDTA supernatant was transferred to the filter unit and mixed with the UA. The filter unit was centrifuged at 14,000xg for 10 minutes. The remaining supernatant was removed from the pellet and stored. Then 30 µL of sodium dodecyl sulfate (SDS)-lysis buffer (4% w/v SDS, 100 mM Tris/HCL pH 8.2, 0.1 M DTT) was added to the pellet and incubated at 95°C for 5 minutes followed by centrifugation. After lysis, 200 µL of UA and the supernatant from the lysis were added to the filter unit and mixed. The filter units were centrifuged at 14,000xg for 20 minutes and the flow through discarded. The samples were washed with 200 µL of UA followed by centrifugation at 14,000xg for 20 minutes.

To alkylate the samples, 100 µL of iodoacetamide (IAA) solution (0.5 M IAA in 8 M UA) was added to the filter units and mixed at 600 rpm for one minute in the dark followed by incubation in the dark without shaking for 5 minutes, followed by 15 minutes centrifugation at 14,000xg. The samples were washed twice with 200 µL of UA, then washed twice with 200 µL of 0.5 M NaCl with centrifugation at 14,000xg for 20 minutes at each wash step. 120 µL of 50mM triethylammoniumbicarbonate was added to the filters. The samples were digested with 0.4 µg of trypsin overnight at 37°C then centrifuged at 14,000xg for 20 minutes into a new tube. The flow though was acidified with 5% TFA to a final pH<2. Stage tips (Thermo Scientific StageTips 200µL C18 tips) were cleaned with 150 µL 100% methanol, followed by 150 µL 60% Acetonitrile (ACN) solution (60% ACN, 0.1% TFA, 39.9% ddH2O). Stage tips were then equilibrated with two washes of 150 µL of 3% ACN solution (3% ACN, 0.1% TFA, 96.9% ddH2O). Acidified samples were loaded onto the tips. Tips were then washed twice with 150 µL of 3% ACN solution and the flow through discarded. Peptides were eluted from the stage tip with 150 µL 60% ACN solution into a fresh collection tube, dried in centrifugal evaporator and stored at -80°C ready for LC-MS/MS.

41 samples were extracted using modified Single-pot, solid-phase-enhance sample preparation (SP3) protocol[74]. A 2-10mg sample of calculus, 3mg of powered archaeological sheep bone (positive control) and an extraction blank were demineralized in 500 µL of 0.5 M

EDTA on a rotator for 3-5 days. After demineralization, samples were centrifuged at 20,000xg for 10 minutes and 400 µL of EDTA supernatant was transferred to a new tube and stored in a -20ºC freezer as a potential back-up sample. Next, 200 µL 2 M GuHCl was added to each sample and mixed through resuspension. To each sample, 30 µL of 100 mM CAA/100 mM TCEP solution was added to a final concentration of 10 mM CAA/TCEP. Samples were vortexed briefly and then placed in a ThermoMixer for 10 minutes at 99°C. After 10 minutes, samples were removed from the ThermoMixer and left to cool on the bench for 5 minutes.

Next, 20 µL of prepared Sera-Mag Speedbeads (GE Healthcare) solution (20 µg/µL of 1:1 mixture of hydrophilic:hydrophobic mixture) was added to each sample and mixed through pipetting. A volume of 100% ethanol equal to the total volume (350 µL) was added and each sample briefly shaken to mix. Samples were incubated in a ThermoMixer for 5 minutes at 1000 rpm at 24°C. After removal from the ThermoMixer samples were placed in a magnetic rack and left for 1-2 minutes to allow the beads to migrate to the magnetic wall. The supernatant was removed and stored a -20 freezer. The beads were then washed three times with 200 µL 80% ethanol, incubated on the magnetic rack for 1-2 minutes and the supernatant discarded. The beads were resuspended in 75 µL of 100Mm ammonium bicarbonate and digested with 0.4 µg of trypsin overnight at 37°C in the ThermoMixer at 750 rpm. Samples were centrifuged at 20,000xg for 1 minute and placed in a magnetic rack. Once the beads had migrated to the wall the supernatant was transferred to a new tube. Samples were acidified with 5% TFA to pH<2. Samples were vortexed briefly to mix and centrifuged. Stage tips (Thermo Scientific StageTips 200µL C18 tips or three 3 M Empore C18 disks placed in 200 µL tips) were cleaned with 150 µL 100% methanol, followed by 150 µL 60% Acetonitrile (ACN) solution (60% ACN, 0.1% TFA, 39.9% ddH_2O). Stage tips were then equilibrated with two washes of 150 µL of 3% ACN solution (3% ACN, 0.1% TFA, 96.9% ddH_2O). Acidified samples were loaded onto the tips. Tips were then washed twice with 150 µL of 3% ACN solution and the flow through discarded. Tips were then sent for LC-MS/MS analysis to the Functional Genomics Centre Zurich where they were eluted with 150 µL 60% ACN solution. The SP3 protocol can be found on protocols.io [dx.doi.org/10.17504/protocols.io.bfgrjjv6].

LC-MS/MS Analysis

LC-MS/MS was conducted at the Functional Genomics Center Zurich using either a Q-Exactive or a Q-Exactive HF mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a Digital PicoView source (New Objective) and coupled to a nanoACQUITY or

an ACQUITY UPLC M-Class system (Waters AG, Baden-Dättwil, Switzerland), respectively. Solvent composition at the two channels was 0.1% formic acid for channel A and 0.1% formic acid, 99.9% acetonitrile for channel B. Column temperature was 50°C. For each sample 4 µL of peptides were loaded on a commercial MZ Symmetry C18 Trap Column (100Å, 5 µm, 180 µm x 20 mm, Waters) followed by nanoEase MZ C18 HSS T3 Column (100Å, 1.8 µm, 75 µm x 250 mm, Waters). The peptides were eluted at a flow rate of 300 nL/min by a gradient from 8 to 22% B in 49 min, 32% B in 11 min and 95% B in 1 min (Q-Exactive) or from 5 to 40% B in 120 min and 98% B in 5 min (Q-Exactive HF). The column was cleaned after each run with 98 % solvent B for 5 min and holding 98 % B for 8 min prior to re-establishing loading condition.

The mass spectrometers were operated in data-dependent mode performing HCD (higher-energy collision dissociation) fragmentation on the twelve most intense signals per cycle. The settings were slightly adapted for each instrument. For Q-Exactive analyses, full-scan MS spectra (300−1700 m/z) were acquired at a resolution of 70'000 at 200 m/z after accumulation to a target value (AGC) of 3E6, while HCD spectra were acquired at a resolution of 35'000 using a normalized collision energy of 25 (maximum injection time: 110 ms; AGC 50'000 ions). For Q-Exactive HF analyses, full-scan MS spectra (300−1500 m/z) were acquired at a resolution of 120'000 at 200 m/z after accumulation to a target value (AGC) of 3'000'000, while HCD spectra were acquired at a resolution of 30'000 using a normalized collision energy of 28 (maximum injection time: 50 ms; AGC 10'000 ions). Unassigned singly charged ions and ions were excluded. Precursor masses previously selected for MS/MS measurement were excluded from further selection for 30 s, and the exclusion window was set at 10 ppm. The samples were acquired using internal lock mass calibration on m/z 371.1012 and 445.1200. The mass spectrometry proteomics data were handled using the local laboratory information management system (LIMS)[75].

Proteomic Data Analysis

Authentication of dietary proteins is a major challenge for palaeoproteomic studies of dental calculus. In other studies, covering different regions and time periods, estimation of the deamidation rates of glutamate and asparagine[76,77] has been used to evaluate proteins identified from ancient dental calculus[16,46,78]. These studies compare the deamidation rates of the dietary peptides against those of the oral signature proteins (which are assumed to be authentic[47]). We explored the potential of using deamiDATE[78] to assess deamidation rates of detected milk peptides. However, in our samples the number of deamidation sites in the dairy peptides are too small for statistical comparison (Supplementary Table 3). Noting that the low numbers of detected peptides also hampered using existing strategies focusing on

post-translational modifications, we created the Oral Signature Screening Database (OSSD) to assess whether our samples contain ancient proteins representing the oral microbiome. The OSSD cannot verify if food-related proteins are ancient but relies on the assumption that if an oral signature is present, food-related proteins identified from ancient dental calculus are more likely to be endogenous rather than contamination. The OSSD contains a subset of oral microbes, human inflammatory response proteins and common contaminants previously reported in ancient dental calculus samples (for full details see Supplementary Note 3). All calculus samples were first processed with the OSSD using Byonic v.3.2.0 (Protein Metrics Inc.)[79] to identify samples that had an oral protein signature in line with previous reports of ancient dental calculus composition (Supplementary Note 3).

The OSSD does not assess the authenticity of a dietary signature. However, it does provide a standardised way of assessing if a calculus sample possesses an oral signature. In this respect, the OSSD is most effective when used as a screening tool for studies focusing on the identification of dietary proteins, rather than those reconstructing the full oral microbiome[47]. For poorly preserved samples, for which other methods of authentication (i.e. deamiDATE[78]) may not be suitable, the OSSD ensures problematic samples are easily identifiable. If dietary proteins are identified in poorly preserved calculus that lacks an oral signature, careful consideration is needed as to whether food-derived proteins are endogenous or modern contamination. In this study, all samples with milk proteins passed the OSSD.

In order to assess other types of damage, for the samples that passed OSSD, the degree to which tryptic vs. non-tryptic cleavage occurred was assessed as a fraction of the total peptides as an additional indication of quality (Supplementary Data 3). While we acknowledge that signatures of age-related damage in ancient dental calculus proteomes are not fully understood, we observe that non-specific cleavage occurs in all of the samples that passed the OSSD with rates of non-tryptic or semi-tryptic cleavage ranging 2.5-24.7% of the peptides in the OSSD searches and 3.4-39.8% of the peptides in the ByonicPreview error tolerant searches (Supplementary Data 3, Supplementary Data 5). Error-tolerant searches were also performed in Mascot and ByonicPreview against the SwissProt database in order to assess the presence of PTMs across all identified proteins (Supplementary Data 4, Supplementary Data 5).

Samples with oral signatures were run against SwissProt Release 2019_08 database (560,782 entries) using 1) Byonic (Protein Metrics Inc., Cupertino, California, United States, version 3.2.0) and 2) Mascot (Matrix Science, London, UK, version 2.6.0) and Scaffold

(Proteome Software Inc., Portland, Oregon, United States, version 4.9.0) (Supplementary Data 3) using a 95% protein probability cut off.

Byonic

Parameters for Byonic were set as follows: tryptic-specific digestion, a precursor mass tolerance of 5ppm, a fragment mass tolerance of 0.05Da, 2 missed cleavages allowed, carbamidomethyl of cysteine as a fixed modification, variable modifications (2 common, 1 rare) as deamidation of asparagine and glutamate (2 common), oxidation of lysine and methionine (2 common), phosphorylation of serine and threonine (1 common), glutamate or glutamic acid to pryo-glutamate (1 rare), and acetyl at the N-terminus (1 rare). Byonic automatically filters the results to show only proteins with less than 1% FDR or up to the 20th decoy, whichever allows more proteins. Protein identifications were filtered to remove any proteins with a log protein p-value less than 1 and peptides were filtered to remove any with a score of less than 200. Samples with a high value of non-tryptic cleavage indicated by the OSSD were run using the same parameters with non-specific digestion.

Mascot and Scaffold

Tandem mass spectra were extracted by MSConvert version 3.0.11781 using the 100 most intense peaks in each spectrum. Charge state deconvolution and deisotoping were not performed. Parameters for Mascot were set as follows: digestion enzyme trypsin, parent ion tolerance of 10ppm, fragment ion mass tolerance of 0.01 Da, carbamidomethyl of cysteine as a fixed modification, variable modifications of deamidation of asparagine and glutamate, oxidation of lysine and methionine, phosphorylation of serine and threonine, and acetyl at the N-terminus.

Scaffold was used to validate MS/MS based peptide and protein identifications. Peptide identifications were filtered to achieve an FDR less than 1.0% by the Peptide Prophet algorithm[80] with Scaffold delta-mass correction. Protein identifications were accepted if they could be established at an FDR of less than 5.0% and contained at least 2 unique identified peptides. Final protein and peptide FDR for each sample is listed (Supplementary Data 2). Protein probabilities were assigned by the Protein Prophet algorithm[80]. Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Proteins sharing significant peptide evidence were grouped into clusters. All raw data files and processed files are available through MassIVE repository with accession code MSV000085058 [doi:10.25345/C55M4S].

Milk Peptide Identifications

Milk peptides were individually assessed. A protein-protein alignment search of all peptide spectral matches (PSMs) identified in any sample as from milk proteins (lactoglobulins and caseins) was performed against the entire translated nucleotide database at NCBI using BLAST. Positive peptide identification required 100% homology and 100% coverage of the peptide to the desired dairy protein (Supplementary Data 6). For individuals that passed the OSSD, milk peptides were filtered to achieve a peptide FDR of 0.5% or less in Byonic or a peptide FDR of 1% or less in Mascot/Scaffold (Supplementary Data 3). The differences in FDR scores are due to how each program assigns and reports FDR. Individuals were considered to have dairy proteins in their dental calculus if at least four milk PSMs (two unique sequences or all four PSMs starting at position 143) at least two of which were identified in both Mascot and Byonic (see Supplementary for more details, Supplementary Figs.1-7). Non-tryptic peptides were only assessed via the Byonic searches (Supplementary Data 3).

Stable isotope analysis of bone collagen

Bone collagen was extracted using a modified Longin[81] method. Approximately 1g of bone was cleaned using abrasion and demineralised in 0.5M HCl for 14 days. After demineralization, each sample was rinsed three times with ultra-pure H_2O. Samples were gelatinized at 70°C in pH3 HCl for 48hrs and the collagen solution Ezee-filtered. Following 48hrs in a freeze dryer, approximately 1.0 mg of collagen was weighed in duplicate into tin capsules for analysis. The $\delta^{13}C$ and $\delta^{15}N$ ratios of the bone collagen were determined using a Thermo Scientific Flash 2000 Elemental Analyser coupled to a Thermo Delta V Advantage mass spectrometer at the Isotope Laboratory, MPI-SHH, Jena. Isotopic values are reported as the ratio of the heavier isotope to the lighter isotope ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) as δ values in parts per mille (‰) relative to international standards, VPDB for $\delta^{13}C$ and atmospheric N2 (AIR) for $\delta^{15}N$ using the following the equation [$\delta = (R_{sample} – R_{standard})/R_{standard}$][82]. Results were calibrated against international standards of (IAEA-CH-6: $\delta^{13}C$ = -10.80 ± 0.47 ‰, IAEA-N-2: $\delta^{15}N$ = 20.3 ± 0.2 ‰, and USGS40: $\delta^{13}C$ = -26.38 ± 0.042 ‰, $\delta^{15}N$ = 4.5 ± 0.1 ‰) and a laboratory standard (fish gelatin: $\delta^{13}C$ = ~ -15.1 ‰, $\delta^{15}N$ = ~14.3 ‰). Based on replicate analyses long-term machine error over a year is ± 0.2‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{15}N$.

Stable isotope analysis of tooth enamel

Teeth were cleaned using air-abrasion and approximately 7 mg enamel was removed from each tooth using diamond-tipped drill bit. For each individual, a bulk sample was taken by sampling enamel from along the full length of the buccal surface. Enamel samples were pretreated with 1mL 1% NaClO for 1hr and then rinsed with ultra-pure H_2O for a total of three washes, centrifuging each time. Next, 1 mL 0.1M acetic acid was added for 10 minutes followed by three more washes with ultra-pure H_2O. After the final rinse, each sample was placed in a freeze dryer for 4 hrs. Alongside the samples of this study, an in-house standard of equid tooth enamel was processed. Approximately 2 mg of each sample was weighed out into a 12ml borosilicate glass vial. Following reaction with 100% phosphoric acid, gases evolved from the samples were analysed to stable carbon and oxygen isotopic composition using a Thermo Gas Bench 2 connected to a Thermo Delta V Advantage Mass Spectrometer. Carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) stable isotope values were calibrated against international standards IAEA NBS 18 (δ13C -5.014 ± 0.032 ‰, $\delta^{18}O$ -23.2±0.1 ‰), IAEA 603 (δ13C +2.46±0.01 ‰, $\delta^{18}O$ -2.37±0.04 ‰), IAEA CO8 (δ13C -5.764±0.032 ‰, $\delta^{18}O$ -22.7±0.2 ‰), and USGS44 (δ13C = ~ -42.1 ‰) Precision was assessed by repeat measurements of a laboratory standard (MERCK CaCO3) (n= 20, ± 0.2‰ for $\delta^{13}C$ and ± 0.2‰ for $\delta^{18}O$, $\delta^{13}C$ = ~ -40.6 ‰, $\delta^{18}O$= ~ -13.3 ‰) Measurement error was ± 0.3‰ or less for $\delta^{13}C$ and ± 0.2‰ or less for $\delta^{18}O$.

Morphological identification of faunal remains

Faunal samples from Lukenya Hill and Molo Cave were identified to element, and when possible, taxon (Supplementary Table 6). Identifications of teeth were made using photographs, drawings, and metric data of eastern African fauna from AJ, as well as published criteria on differentiating caprine teeth[83,84]. Less identifiable fragments were identified to the narrowest taxonomic category possible and size class[85]. Given the fragmentary nature of the postcranial remains, specimens from some sites (Lukenya Hill sites, and Molo Cave) could not be identified beyond mammal and size class. These faunal specimens underwent ZooMS analysis to refine identifications.

Zooarchaeology by Mass Spectrometry (ZooMS)

Highly fragmented faunal bones were analysed by Zooarchaeology by Mass Spectrometry (ZooMS) to confirm taxonomic identifications (Supplementary Data 7). Samples were analysed following established protocols[86] in which bone samples were demineralized in 0.6M hydrochloric acid (HCl) for 18 hours. The HCl was removed and the sample was rinsed

three times in pH 8 solution of 50mM ammonium bicarbonate (AmBic). After rinsing, the sample was incubated at 70°C for an hour in 100 µL of 50mM AmBic. 50 µL of the resulting supernatant was treated with trypsin (Pierce™ Trypsin Protease, Thermo Scientific) at 37°C for 18 hours. Following digestion, the samples were subjected to C18 cleanup (Pierce™ C18 Tips, Thermo Scientific), mixed with a matrix solution of α-cyano-4-hydroxycinnamic of 10mg/mL in 50% acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) and allowed to co-crystallize. Analysis was carried out on an Autoflex MALDI-TOF Bruker Ultraflex II (Bruker Daltonics, Bremen). The resulting mass spectra were screened for diagnostic markers using the FlexAnalysis software and compared against a reference library[87-89] and analysed using mMass. Samples were analysed alongside multiple blanks which all returned negative results and were determined to be empty.

Radiocarbon dating

A total of 5 samples (1 bone fragment from petrous portion, 3 teeth and 1 hair sample) were sent for radiocarbon dating and were split between the Centre for Isotope Research Groningen (CIO, Lab ID: GrM) and Scottish Universities Environmental Research Centre Radiocarbon Laboratory Glasgow (SUERC, Lab ID: GU). The hair sample from Kadruka 1 SK68 was pretreated with 4% HCl for a short period, rinsed with decarbonized water and dried before combustion. Four teeth were pretreated for dating at SUERC using published methods[90]. A cranial fragment from individual Kadruka 21 Skeleton 129 was pretreated at CIO following established protocols[91] Unfortunately none of the teeth or the cranial bone fragment yielded sufficient collagen for dating. A radiocarbon date was successfully obtained from the hair sample from Kadruka 1 SK68. ^{14}C ages were calibrated to calendar years with software program: OxCal, version 4.3[92], using calibration curve: IntCal13[93] (Supplementary Fig.14).

Data Availability

Raw and processed MS/MS files are available to download via MassIVE repository with accession code MSV000085058 [doi:10.25345/C55M4S]. The full Oral Signature Screening Database (OSSD) and associated results are available via MassIVE with accession code MSV000086557 [doi:10.25345/C5PR4T] and on the open-access repository Zenodo [https://doi.org/10.5281/zenodo.3698271]. The SP3 protocol is published open-access on protocols.io [dx.doi.org/10.17504/protocols.io.bfgrjjv6]. All other supporting data are available within the paper and supplementary information files.

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Author Contributions

M.Bleasdale., N.B., A.C and S.T.G. designed the study. M.Bleasdale., A.J., S.B., J.H., J.Z., and P.R. performed research. M.Bleasdale., K.K.R., A.J., S.B., A.S., J.Z., K.W., S.S., C.T., P.N., J.G., and P.R. analysed data. M.Bleasdale., S.W., J.D., M. Besse., J.R., M.S., H.B., R.C.P., E.N., C.O., F.K.M., M.Z., M.P. and S.T.G contributed material resources. M.Bleasdale., K.K.R., S.T.G., and N.B. wrote the paper.

Competing Interests

The authors declare no competing interests.

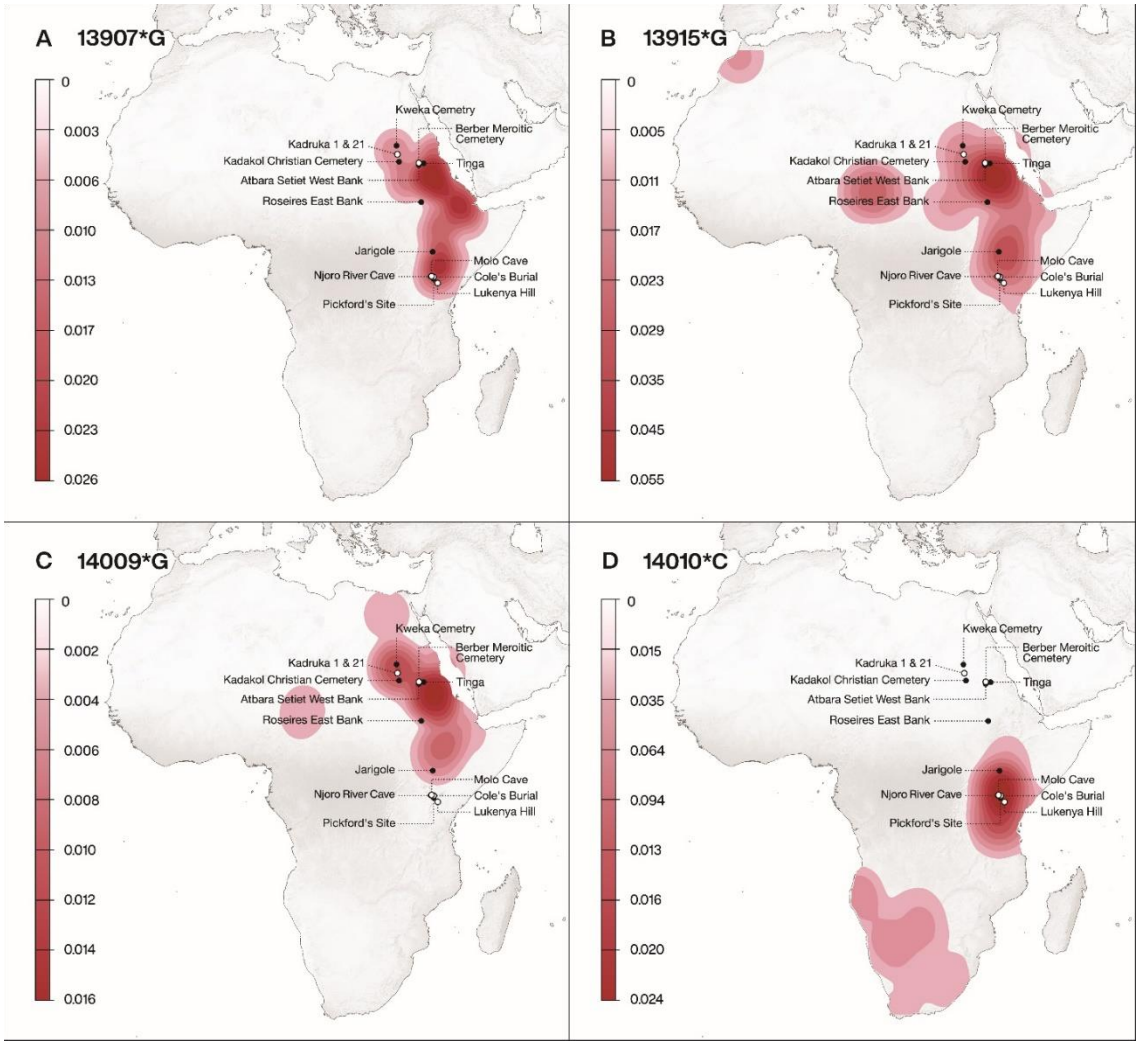

Figure 1: Locations of sampled archaeological sites in Kenya and Sudan in relation to the distribution of main lactase persistence alleles found in modern populations in Africa: (A) 13907*G, (B) 13915*G, (C) 14009*G and (D) 14010*C. Filled circles represent sites where milk proteins were identified in dental calculus samples, empty circles are sites where samples did not yield milk proteins. The map was created for this study by Michelle O'Reilly (Graphic Designer for the Max Planck Institute for the Science of Human History, Jena, Germany) using QGIS 3.12 [https://qgis.org/en/site/] and the Natural Earth Database from [https://www.naturalearthdata.com/downloads/] and Adobe Illustrator CC. Heat maps were generated using published LP distribution frequencies[6].

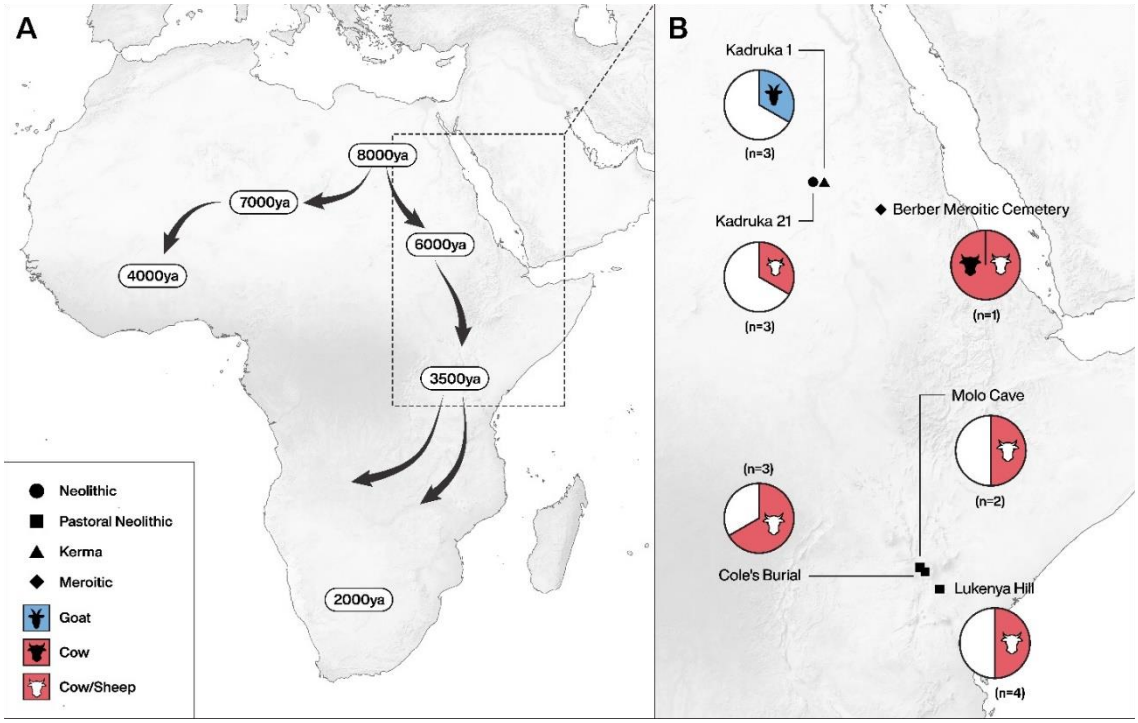

Figure 2: Map of sites with calculus containing milk proteins. *(A)* Area of study in relation to the spread of cattle-based pastoralism across Africa (after [35]). *(B)* Pie charts showing the number of individuals per site with milk proteins (shaded) proportionate to the total number of individuals that passed screening with Oral Signature Screening Database (see Methods and Supplementary Note 3 for full details). Neolithic: ~8000-5500 cal. BP; Kerma: ~4450-3450 cal. BP; Pastoral Neolithic: ~3500-1200 cal. BP; Meroitic: ~2300–1600 cal. BP. The maps were created for this study by Michelle O'Reilly (Graphic Designer for the Max Planck Institute for the Science of Human History, Jena, Germany) using QGIS 3.12 [https://qgis.org/en/site/] and the Natural Earth Database from [https://www.naturalearthdata.com/downloads/]. Additional edits were made using Adobe Illustrator CC.

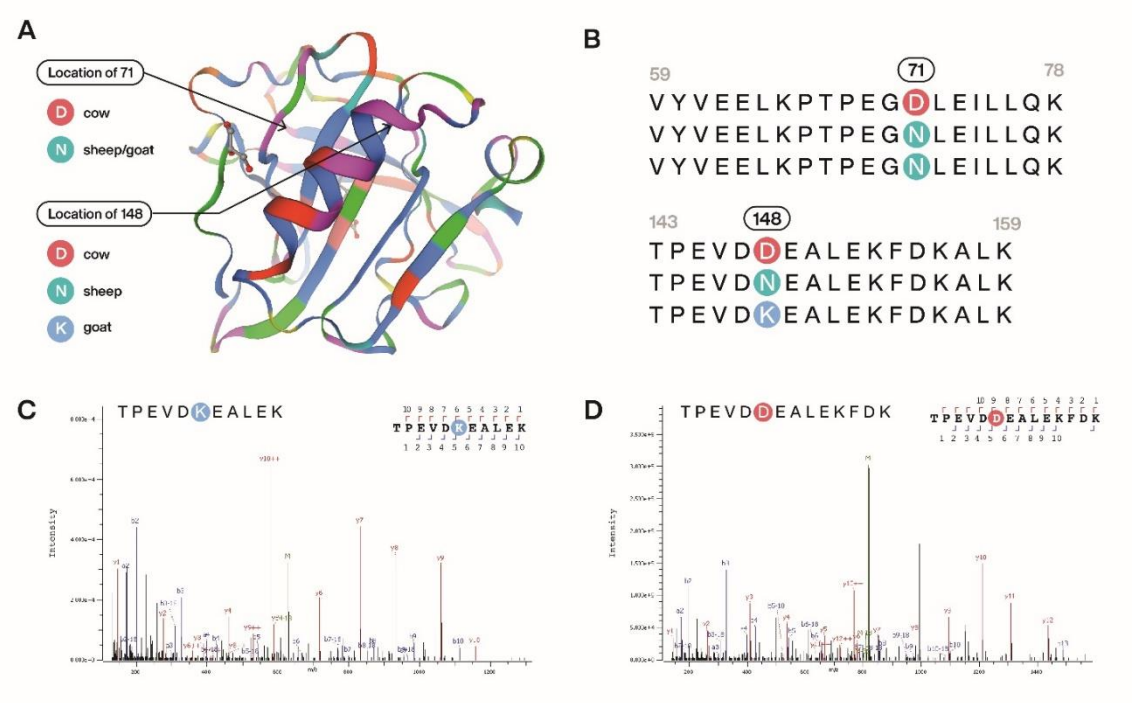

Figure 3. Milk β-lactoglobulin proteins identified from ancient dental calculus of individuals from Sudan and Kenya: *(A)* 3D model of β-lactoglobulin showing location of species variant sites. *(B)* Variations in the amino acid sequence at position 71 and 148 can be used to distinguish between *Bos*, *Ovis* and *Capra*. *(C)* Spectrum for TPEVDKEALEK specific to *Capra* from individual Z708. *(D)* Spectrum for TPEVDDEALEKFDK from individual DA323. The deamidation of asparagine results in its conversion to aspartic acid so an unmodified (D) is indistinguishable from (de.N) therefore this milk peptide is identified as Bovinae*/Ovis.* The 3D image of β-lactoglobulin is from SwissModel [https://swissmodel.expasy.org/repository/uniprot/P02754].

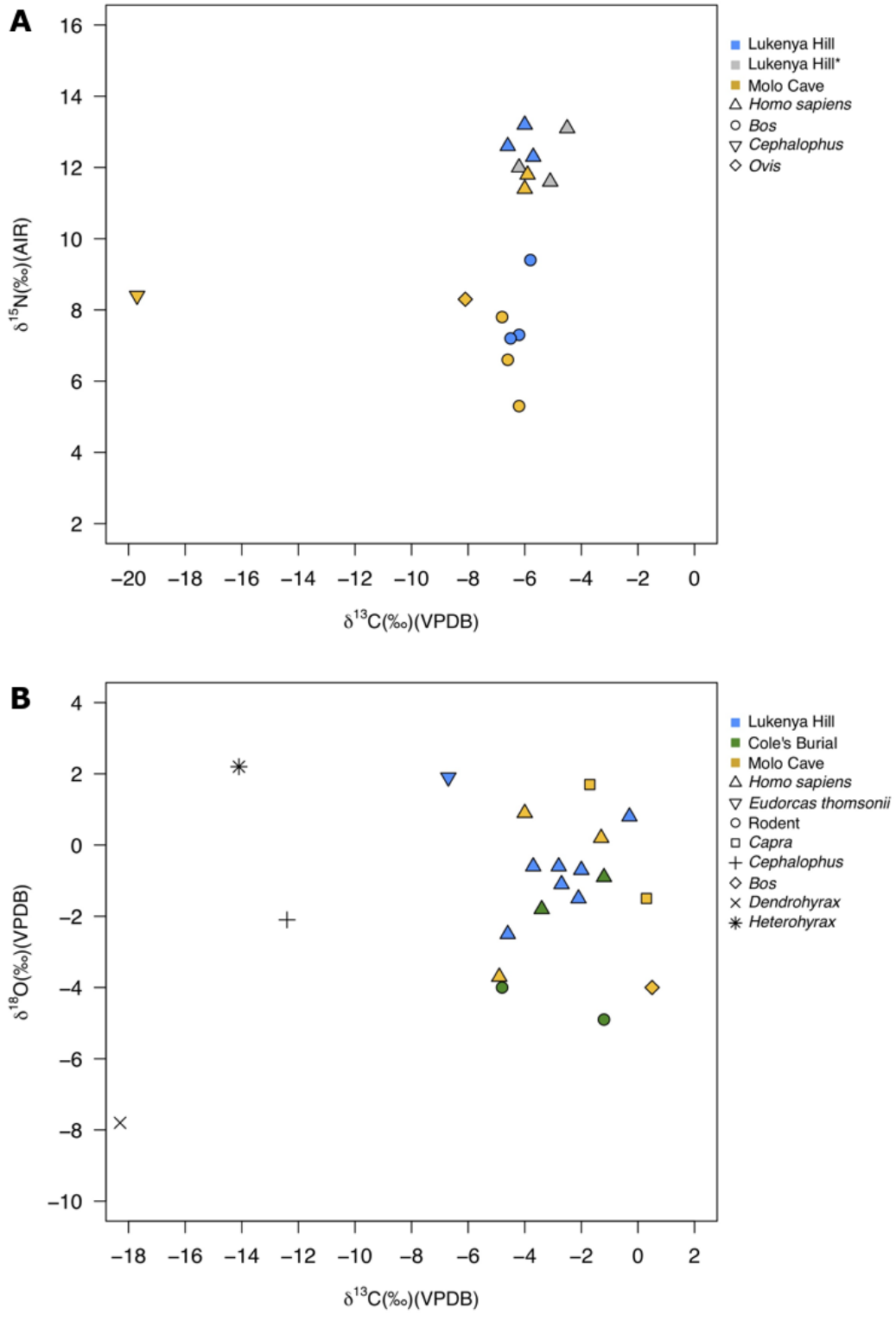

Figure 4: Stable isotope results for humans and fauna *(A)* Bone collagen stable carbon and nitrogen isotope values for Lukenya Hill and Molo Cave. *values previously published for Lukenya Hill [53]. *(B)* Tooth enamel stable carbon and oxygen isotope values for Lukenya Hill, Cole's Burial and Molo Cave. The *Cephalophus*, *Dendrohyrax* and *Heterohyrax* were sampled from Lukenya Hill.

Supplementary Information

Ancient proteins provide evidence of dairy consumption in eastern Africa

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Supplementary Figures:

Supplementary Tables:

Other Supplementary Materials for this manuscript include:

Supplementary Data 1-9 (provided separately as excel sheets).

Oral Signature Screening Database for Palaeoproteomic Analyses of Dental Calculus: Zenodo, [doi.org/10.5281/zenodo.3698271].

SP3 extraction protocol: protocols.io, [doi.org/10.17504/protocols.io.bfgrjjv6].

Supplementary Note 1: Materials

Kadruka 1 and Kadruka 21, Sudan

Kadruka is a Neolithic (~8000-5500 cal. BP), and Kerma period (~4450-3450 cal. BP) site located upstream of the 3rd Cataract of the Nile within the northern Dongola Reach. Fieldwork in the Kadruka District led by Jacques Reinold of the *Section française de la direction des antiquités du Soudan* (SFDAS) from 1985-2002 resulted in the discovery of 17 Neolithic Burial mounds. Of these, six mounds and over 700 individuals were excavated. At Kadruka 1, 124 individuals were excavated with approximately 70% (n = 96) from the Neolithic period and 30% (n = 46) form the Kerma period[1]. A total of 228 individuals were excavated at the cemetery site of Kadruka 21. For this study, a selection of skeletal remains (n = 10) were sampled: five each from Kadruka 1 (KDK 1) and Kadruka 21 (KDK 21), representing both the Neolithic and Kerma periods.

Preservation conditions at Kadruka have enabled the recovery of a large number of human remains in addition to a rich collection of material goods including cosmetic cases shaped from the canines of hippos, ivory handle tools, and painted vases[2]. The hot, dry climate of the site facilitated preservation of skin and hair, including hair on skeletal remains and preserved sheepskin[3,4]. At Kadruka 1, variations in burial arrangement and distribution of grave goods could indicate differences in the social stratigraphy[1]. One burial (KDK1/131), which was unavailable for sampling for this study, was located in the centre of the mound and contained a large quantity of grave goods including ivory bracelets, axe heads, ceramics, grindstones, and an Anthropoid sandstone figurine[1].

Collectively, the human remains from Kadruka were recovered from a funerary context, but zooarchaeological and botanical evidence has offered some important insights into the broad subsistence practices of the buried population. At Kadruka 1, sacks made from animal skin were discovered containing barley[2], and the remains of domestic sheep, goat, and cattle were reported[4]. Another potential line of evidence for the establishment of a dairy economy at the site is the presence of small perforated bowl (KDK1/120/3) from the Neolithic period[2] that bears similarities to Neolithic *"cheese strainers"* from Europe[5,6]. The Kadruka bowl was discovered filled with chaff and further analysis is needed to firmly establish its function.

Berber Meroitic Cemetery, Sudan

Berber Meroitic Cemetery is located on the east bank of the Nile River to the east of the centre of Berber City in northeastern Sudan. Archaeological excavations at the site started as a rescue project in 2009 in response to the construction of a plastics production factory. The subsequent discovery of a large, well-preserved Meroitic period cemetery instigated further excavations under the direction of Mahmoud Suliman Bashir of the *National Corporation for Antiquities and Museums* (NCAM) in Sudan. A number of substructures were uncovered during excavations including tombs and three mud brick pyramids. The total number of individuals buried at the site is still unknown as excavations are ongoing. Five individuals were analysed in this study but only one (BMC 38b) gave positive results for milk proteins. BMC 38 is the substructure of a mud brick structure, probably a pyramid, consisting of three courses with a funerary chapel located on the eastern side. Individual BMC 38b is an adult male buried in extended position east-west and was excavated close to another tomb directly dated to around 2160 cal. BP[7].

Lukenya Hill (GvJm 202), Kenya

Lukenya Hill is located in the Athi-Kapiti Plains east of the Central Rift Valley in southern Kenya. Lukenya Hill has several archaeological sites including rockshelters with Middle and Later Stone Age archaeology[8] and open-air Pastoral Neolithic (PN) sites[9,10]. For this study, dental calculus from five human teeth were analysed from the PN site of GvJm202. GvJm202 is a rockshelter containing the remains of at least six individuals consisting of five adults and one sub-adult[11,12]. Dental calculus was analysed from five teeth, including two discrete burials (Skeleton A and Skeleton C). The petrous portion of Skeleton C has been analysed for aDNA[13], and is dated to ~3635-3475 cal. BP[13]. Additionally, human bones from three individuals and seven teeth (including two teeth where milk proteins were identified in associated dental calculus) were analysed for stable isotope analysis. Bone collagen from three *Bos* specimens (identified using morphology and ZooMS: see methods) from Lukenya Hill was also analysed for stable carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope composition: two *Bos* specimens are from GvJm202 and one is from the neighbouring PN site of GvJm184. GvJm184 is dated to ~2715-1735 cal. BP[10,14] and has produced Savanna Pastoral Neolithic (SPN) pottery and the remains of domesticated cattle, sheep, and goat[14].

Cole's Burial (GrJj5a), Kenya

Cole's Burial site is located on the eastern side of Lake Elmenteita in central Kenya[15]. Charles Nelson and Stanley Ambrose documented the site in 1976 when fragmentary

human remains were observed in the cliffs above the lake. A minimum of three individuals were excavated. A tibia from one individual (CB1) was previously directly radiocarbon dated to 2750-2015 cal. BP on apatite (2355 ± 150 BP; GX4714-A) and 2854-2185 BP on gelatin (2500 ± 130 BP; GX4714-G)[15]. Genetic analysis and radiocarbon dating (3350-3180 cal. BP; 3070 ± 20 BP; PSUAMS4723) was carried out previously on CB1.01[16]. Bone collagen from all three individuals was analysed previously using stable isotopes of carbon and nitrogen[14]. For this study, dental calculus was sampled for proteomic analysis from two burials (CB1.01 hereafter Individual 1, and Individual 2) and from a loose incisor not associated directly with any of the three burials. To further investigate dietary intake, tooth enamel from Individual 1 and the isolated tooth were was analysed using stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope analysis. Additionally, enamel was sampled from a rodent and mole rat (*Tachyoryctes*).

Molo Cave (GoJi 3), Kenya

Molo Cave is located approximately 50 km west of Lake Nakuru in the Central Rift Valley of southern Kenya. The remains of three individuals were salvaged by Mary D. Leakey and are presently curated by the National Museums of Kenya. The individuals are believed to be associated with the Pastoral Neolithic period[11]. For this study, dental calculus was sampled from one discrete burial (Skeleton 1) and one loose tooth. Skeleton 1 has been analysed previously for aDNA[13] and directly radiocarbon dated to 1415-1320 cal. BP (OxA-37, 359) (Supplementary Table 2)[13] supporting the likely Pastoral Neolithic attribution. Tooth enamel from this individual was analysed as part of this study using stable carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope analyses along with enamel and bone collagen from two other individuals and a range of wild and domesticated fauna (*Bos, Cephalophus*, *Capra hircus*, *Dendrohyrax,* and *Heterohyrax)* (Supplementary Information Section 4, Supplementary Figs.12-13).

Supplementary Note 2: Proteomic Extraction Methods

Two proteomic extraction methods were used in this study: FASP and SP3. Modified FASP (Filter-Aided Sample Preparation)[17] protocols have successfully been applied in several ancient dental calculus studies[18-20], including the first study to report milk peptides[21]. However, filter-based protocols are less suited to small amounts of starting material[22]. Recently, Single-pot, solid-phase-enhance sample preparation (SP3) has been developed to overcome sample size limitations of FASP[23,24]. However, this method is based on protein precipitation and aggregation[25], which could be limited or absent in cases where proteins are heavily fragmented. For a full description of SP3, see Methods and protocol published on protocols.io [doi.org/10.17504/protocols.io.bfgrjjv6].

In this study, dental calculus samples from Kadruka (n = 10) were extracted using both methods. For other sites, sample were extracted only with SP3 because start weights were too low for FASP. While this is the first study using SP3 on archaeological dental calculus and differences were observed between the methods, statistical comparisons were not conducted due to the small number of samples prepared with both methods.

Supplementary Note 3: Oral Signature Screening Database (OSSD) for the Palaeoproteomic Analysis of Dental Calculus

Full methods, database, and results of pilot test available at open-access repository Zenodo [doi.org/10.5281/zenodo.3698271].

As the proteomic analysis of dietary peptides retrieved from ancient dental calculus becomes more common[19-21,26], new methods of authentication need to be considered. Here we present an Oral Signature Screening Database (hereafter OSSD) developed as a screening tool for ancient dental calculus.

A major challenge in palaeoproteomics of dental calculus is to show that the dietary proteins reported are endogenous (i.e. they became entrapped in the calculus during formation) as opposed to modern contamination. One method to investigate whether such proteins are truly "ancient" is through the estimation of deamidation rates of glutamate and asparagine[27,28]. This method has been used to assess proteins identified from ancient dental calculus samples from the United Kingdom and Mongolia[19,20,29]. However, it is more challenging to apply similar methods to assess poorly preserved samples due to a number of limitations. Firstly, it requires a large number of endogenous peptides in order to have adequate deamidation sites for statistical models. Secondly, it cannot authenticate individual peptides, only the entire identified sample or a sufficiently large subset of an identified sample. Finally, due to variations in deamidation rates between different peptides and different sites within a peptide[30], it is best suited to samples that have a high coverage of a small number of proteins.

The dental calculus samples in this study had a lower total number of proteins recovered when compared to published results for other geographical and archaeological contexts[19,20,26]. Consequently, there is a low percentage of endogenous peptides overall meaning a small number of deamidation sites. We therefore considered an alternative way to screen calculus samples in order to quickly identify potentially problematic samples as well as those which are more likely to yield proteins of endogenous origin. As reported in the literature, well-preserved dental calculus samples include human oral proteins and oral microbiome proteins[31]. However, this “oral signature” is not routinely reported in a standardised format in palaeoproteomic studies. In part, this is because the oral microbiome is diverse with reported differences between the oral microbiomes of modern plaque and ancient calculus[32]. Therefore, we selected a restricted list of the most abundant oral

signature proteins and microbial proteomes seen in ancient dental calculus. This enabled us to produce a screening database requiring minimal computational time.

The OSSD includes proteomes from a subset of the most common oral microbes, human inflammatory response proteins commonly found in archaeological samples and contaminants introduced during laboratory preparation (trypsin) or handling (keratins). The protein list for the database was created by finding commonalities amongst published datasets for dental calculus[18,21,26], as well as unpublished results generated by the Palaeoproteomics Lab Group in Jena (MPI-SHH).The full list of proteins in the database are available on Zenodo [doi.org/10.5281/zenodo.3698271]. Proteins were divided into four categories: lab contaminants, common contaminants, oral microbiome, and immune response. Common lab contaminants include trypsin, the enzyme used during the extraction process, and serum albumin which is often a contaminant in modern proteomics facilities. The common contaminants list includes collagens and keratins which are introduced through sample handling and proteins associated with the burial environment.

The primary aim of the OSSD is to provide a quick screening method to authenticate the oral signature in archaeological dental calculus samples. While we acknowledge oral biomes can contain numerous bacterial species and be highly variable, it is not the purpose of the OSSD to fully capture this diversity. Therefore, we only selected a subset of common oral bacteria identified in ancient dental calculus samples. This included the three members of the “red complex” (*Porphyromonas gingivalis, Treponema denticola*, and *Tannerella forsythia*) which are associated with periodontal disease[33,34] and other commonly identified microbes (*Actinomyces naeslundii, Treponema maltophilum, Streptococcus mutans, Streptococcus gordonii*, and *Methanobrevibacter oralis*). In order to ensure a short run time (<30 mins) for the OSSD when used with common MS/MS data analysis tools, we selected 11 bacteria proteomes in total. We recognise the list of oral bacteria is not extensive and that the OSSD is not a substitute for the comprehensive oral database eHOMD (expanded Human Oral Microbiome Database) which contains over 700 microbial species[35]. The OSSD is a screening tool and we would therefore recommend the use of other databases, such as eHOMD, for in-depth assessment of bacterial proteomes. In addition to oral bacteria proteomes, we also included two human proteins (lysozyme C and lactotransferrin) and 10 human immune proteins (immunoglobulin kappa constant, neutrophil elastase, cathepsin G, antithrombin-III, alpha-1-antitrypsin, myeloperoxidase, neutrophil defensin, S100-A9, S100-A8, and complement C3) commonly identified in palaeoproteoimc calculus samples[31].

The OSSD was tested on a number of published dental calculus samples and associated blanks: 11 samples from PXD009603[26], 15 samples from PXD012893[19], and 14 samples

from PXD008217[18]. In addition, we tested it against published results of archaeological and modern bones and sediments: 16 from PXD014657[36] and 12 from [37] MassIVE MSV000083687 [doi:10.25345/C5G04C], as well two internal bone extractions (unpublished). Samples were extracted with different methods (gelatinisation, GASP, FASP, SP3) in different laboratories, and are from modern and archaeological contexts from across the world.

The database was tested on Byonic Protein Metrics Inc.[38] with the following settings: non-specific digestion; a precursor mass tolerance of 5ppm; a fragment mass tolerance of 0.05Da, carbamidomethyl of cysteine as a fixed modification; variable modifications (2 common, 1 rare) as deamidation of asparagine and glutamate (2 common); oxidation of lysine and methionine (2 common); phosphorylation of serine and threonine (1 common); glutamate or glutamic acid to pryo-glutamate (1 rare); and acetyl at the n-terminus (1 rare). Proteins were manually assigned to each of the four categories and totals calculated. Proteins were considered authentic if they had at least four peptides assigned and had a log probability of greater than one, or greater than the highest scoring decoy, whichever was higher.

Proteins were considered authentic if they had at least four peptide spectral matches (PSMs) assigned and had a log probability of greater than one, or greater than the highest scoring decoy, whichever was higher. In the blank samples, there were no oral signature proteins and the total number of proteins was between 0 and 25. Over all of the samples, the average number of contaminant proteins was 5.5. Therefore, in order to pass OSSD cut-off (demonstrating that there was a “real” oral signature), samples required at least 10 total proteins with 45% of proteins assigned to the oral microbiome or immune response protein subcategories. In addition, samples that passed this threshold were assigned an OSSD score consisting of three levels, low, medium and high quality (Supplementary Data 3). The levels were as follows: low (10-19 proteins in total with <75% assigned to oral microbiome or immune response protein categories); medium (20-49 proteins in total with >75% assigned to oral microbiome or immune response protein categories or 50+ proteins in total with 75-90% assigned to oral microbiome or immune response protein categories); and high (50 or more total proteins with >90% assigned to oral microbiome or immune response protein categories).

The results of the pilot test were concordant with expectations; all bones and blank samples failed to meet the OSSD threshold (for full results see Zenodo [doi.org/10.5281/zenodo.3698271]). Thirty-one out of thirty-two calculus samples passed our

OSSD threshold, including all calculus samples that were reported in the published literature to have milk peptides.

At this time, the OSSD is not comprehensive and requires further development. As more ancient dental calculus results are published, the database will be tested, refined, and new versions will be made available. Additionally, more testing is needed to identify cut-off values for authenticity of the oral signature for different methods and regions of the world. Finally, this method does not overcome the problem of needing to authenticate individual peptides. Samples which have an endogenous oral signature could still be contaminated with peptides from modern food sources. Additionally, using the whole proteome of oral microbes likely allows for overlap between proteins found in both oral microbes and soil microbes. Nevertheless, for this study, the OSSD provided a quick method to screen and quantify any possible oral signature in the calculus samples, enabling the elimination of the most poorly preserved samples.

Additional Information on Milk Peptide Identifications

In previous studies of archaeological dental calculus, positive identifications of dietary proteins in an individual have required at least two unique peptide sequences[18–20,26]. However, these studies are largely based on well-preserved samples from Europe or Asia. Our study is the first to analyse ancient dental calculus from Africa with samples exhibiting a far lower total number of preserved proteins than those from other geographical contexts; we therefore modified this criterion in our study.

Across previously published calculus studies, the milk peptide beginning at position 143 (TPEVDDEALEK) is most frequently observed (both in ancient and modern samples) while other peptides are observed at a lower frequency[26,39]. As our samples had a lower overall level of preservation, we predicted to see a smaller subset of the possible recoverable peptides[40,41]. We therefore relaxed previously used parameters for a positive indication by allowing one unique sequence if, and only if, that sequence was the peptide starting at position 143. For all other protein identifications, two unique sequences were still required. We considered individuals to have authentic dairy proteins if the individual passed the OSSD, had at least four milk peptides (two unique sequences or all four PSMs starting at position 143) at least two of which were identified in both Mascot and Byonic.

In some cases, we are able to confirm the likely presence of cow, sheep, and goat's milk (Supplementary Table 4, Supplementary Table 5, Supplementary Data 6). Species-specific peptides were recovered from one sample. Other identifications could only be associated

with broader taxonomic categories. BLG is the most frequently identified milk protein in archaeological dental calculus, but many of the commonly recovered peptides such as TPEVDDEALEK[18-20] are shared among a variety of taxa. Additionally, post-translational modifications such as deamidation of asparagine can occur through normal sample processing, which make it difficult to determine species with these peptides[26]. However, when these peptides are present with sufficient b and y-ion series coverage, s identifications can be made confidently.

Supplementary Note 4: Stable carbon, nitrogen, and oxygen isotope analysis

Stable carbon and nitrogen isotope analysis of human and faunal bone collagen

The stable isotope ($\delta^{13}C$ and $\delta^{15}N$) analysis of bulk bone collagen from humans and animals from the archaeological record has been widely applied to investigate dietary variation in the past. In terrestrial ecosystems there is an isotopic distinction between the two major photosynthetic pathways, C_3 and C_4, which differ in their net discrimination against ^{13}C during the fixation of CO_2. C_3 plants have highly negative and variable $\delta^{13}C$ values (ranging from around -35‰ to -19‰). Plants using the C_4 photosynthetic pathway have higher values ranging from -8‰ to -13‰[42–45]. The differences between these two groups of plants is passed into the tissues of their consumers with the $\delta^{13}C$ of bone collagen being around 5‰ more positive than the diet[46,47].

The $\delta^{15}N$ of bone collagen reflects differences relating to the trophic level of consumers, increasing by approximately 3-5‰ with each trophic level[48] and therefore $\delta^{15}N$ measurements can be also used to separate consumers feeding exclusively on marine sources compared to terrestrial sources[49]. Furthermore, there is also a slight enrichment of 0–2‰ in $\delta^{13}C$ as trophic levels increase[48]. Determining freshwater fish consumption is more challenging due to middling $\delta^{15}N$ and $\delta^{13}C$ values. Generally, the consumption of freshwater fish will result in higher bone collagen $\delta^{15}N$ values, but the complex cycling of carbon in freshwater systems means there can be significant variation in $\delta^{13}C$ [50].

Stable carbon and oxygen isotope analysis of human and faunal tooth enamel

While $\delta^{13}C$ of bone collagen largely reflects the protein portion of an individual's diet, the $\delta^{13}C$ of carbonate in the bioapatite of tooth enamel is more representative of overall dietary intake including carbohydrates, proteins, and lipids, meaning that low protein foodstuffs such as crops may be more represented in human bioapatite than collagen[51]. Additionally, it is possible to measure the $\delta^{18}O$ of enamel that is reflective of ingested water (as water or food sources), as the water consumed is closely related to the isotopic composition of local precipitation, $\delta^{18}O$ measurements can therefore provide information about the environment and mobility[52–54].

Stable isotope approaches to investigate pastoralism in Africa

Stable isotopes have been used to explore the emergence and development of pastoral lifeways in Africa through the analysis of archaeological remains. The isotopic analysis ($^{87}Sr/^{86}Sr$, $\delta^{13}C$, $\delta^{18}O$) of human and faunal tooth enamel has been used to investigate pastoral mobility in the central Sahara[55], South Africa[56,57], and Kenya[58], and has revealed a more complex picture for the emergence of early herding in relation to ecological change[59]. Bulk bone collagen isotope analyses have been used to investigate the emergence of cattle-based pastoralism in southern Africa[60] and tease apart different subsistence strategies by analysing prehistoric and historic communities in Africa practicing herding, fishing, and farming[14,61]. Herders, who rely heavily on animals and animal products, generally have higher $\delta^{15}N$ than communities relying heavily on plant products. Furthermore, higher $\delta^{13}C$ in human collagen could reflect a reliance on grazers consuming local C_4 grasses[14,61].

Here we analysed humans from the three sites in Kenya that produced proteomic evidence for milk consumption: Lukenya Hill, Cole's Burial, and Molo Cave. While dietary proteins from dental calculus provide "snapshots" of dietary intake, isotope measurements of different tissues can be used to look at reliance on different food sources. In addition, bone collagen and tooth enamel were sampled from a range of local fauna to provide baselines against which human dietary signals could be examined. As some faunal remains were highly fragmented, we used Zooarchaeology by Mass Spectrometry (ZooMS) to improve taxonomic identifications. Of particular interest in this study were domestic dairy animals such as cattle, goats, and sheep, as well as any available wild fauna as a reference. $\delta^{13}C$ isotope analyses can be used to distinguish between different domesticates based on feeding behaviours. For example, modern sheep at low elevations in eastern Africa display higher $\delta^{13}C$, while goats have more variable values due to consuming mixed C_3/C_4 diet[62]. Furthermore, isotopic studies of faunal remains from sites across Kenya suggest that cattle (C_4-grazers) display minimal variability in diet, in contrast to other grazers (sheep and goats) which could have greater diversity[58,63]. Integrated approaches (such as those combining ZooMS and isotopes) appear most effective in archaeological contexts for confidently distinguishing species[64].

In this study, human $\delta^{13}C$ values were compared to associated fauna. Bone collagen $\delta^{13}C$ largely reflects protein intake because dietary amino acids are preferentially used for the construction of collagen[51,65]. For individuals from Pastoral Neolithic sites in Kenya, we would therefore expect alignment between the $\delta^{13}C$ of domestic livestock (sheep, goats, and cattle) and humans. Furthermore, we would anticipate that human $\delta^{15}N$ would be indicative of the consumption of terrestrial protein sources due to the dominance of animal products in the diet of pastoralists.

Results

All isotope results are summarised in Supplementary Data 8 and Supplementary Data 9. Identifications of the faunal remains are presented in Supplementary Table 6 and Supplementary Data 7. Isotope samples were subjected to a series of quality controls, these included a C/N ratio of 2.9-3.6, %C of ca.15-48%, and %N of ca. 5-17%[42,66,67]. Thirteen out of fourteen bone samples passed quality checks and were carried forward for analysis.

Kadruka 1

A bulk hair sample from Kadruka 1 Skeleton 68 was sampled for dating at Centre for Isotope Research (CIO) Groningen. Additionally, a subsample was analysed using stable carbon and nitrogen isotopes to explore dietary intake. The results ($\delta^{13}C$ -17.0‰, $\delta^{15}N$ 12.0‰) broadly indicate the consumption of C_3-based dietary sources (animals feeding on C_3 resources or C_3 plants).

Lukenya Hill (GvJm202 and GvJm184)

Two individuals from Lukenya Hill (GvJm202) had BLG derived from Bovinae/*Ovis*. For these individuals it was also possible to analyse tooth enamel (Supplementary Fig.9, Supplementary Data 8). Their $\delta^{13}C$ enamel values of -3.7‰ and -0.3‰ are higher than the $\delta^{13}C$ value of -6.7‰ from the Thomson's gazelle (*Eudorcas thomsonii*), a mixed feeder, and suggest an almost entirely C_4-based diet. Bone collagen from an additional three individuals from the site that did not yield BLG proteins display $\delta^{13}C$ values of -6.6‰ to -5.7‰, again supporting a diet of largely C_4 protein sources (Supplementary Fig.10). These are consistent with bulk collagen results from the same site published in[14]. The human $\delta^{13}C$ values were similar to those obtained from animal remains recovered from the site (-6.5‰ to -5.8‰) which were identified as *Bos* sp. using ZooMS (Zooarchaeology by Mass Spectrometry, see Methods). For humans, the $\delta^{15}N$ for is 12.7‰ compared to a mean $\delta^{15}N$ value of 8.0‰ for the *Bos* specimens from Lukenya Hill. *Bos* bone collagen $\delta^{13}C$ values are consistent with the consumption of C_4 grasses (Supplementary Fig.10). While interpretations are somewhat limited due to the absence of any collagen data from wild fauna from Lukenya Hill, when all human and faunal $\delta^{13}C$ and $\delta^{15}N$ values are considered together they suggest a reliance on grazing animal products rather than cereal crops.

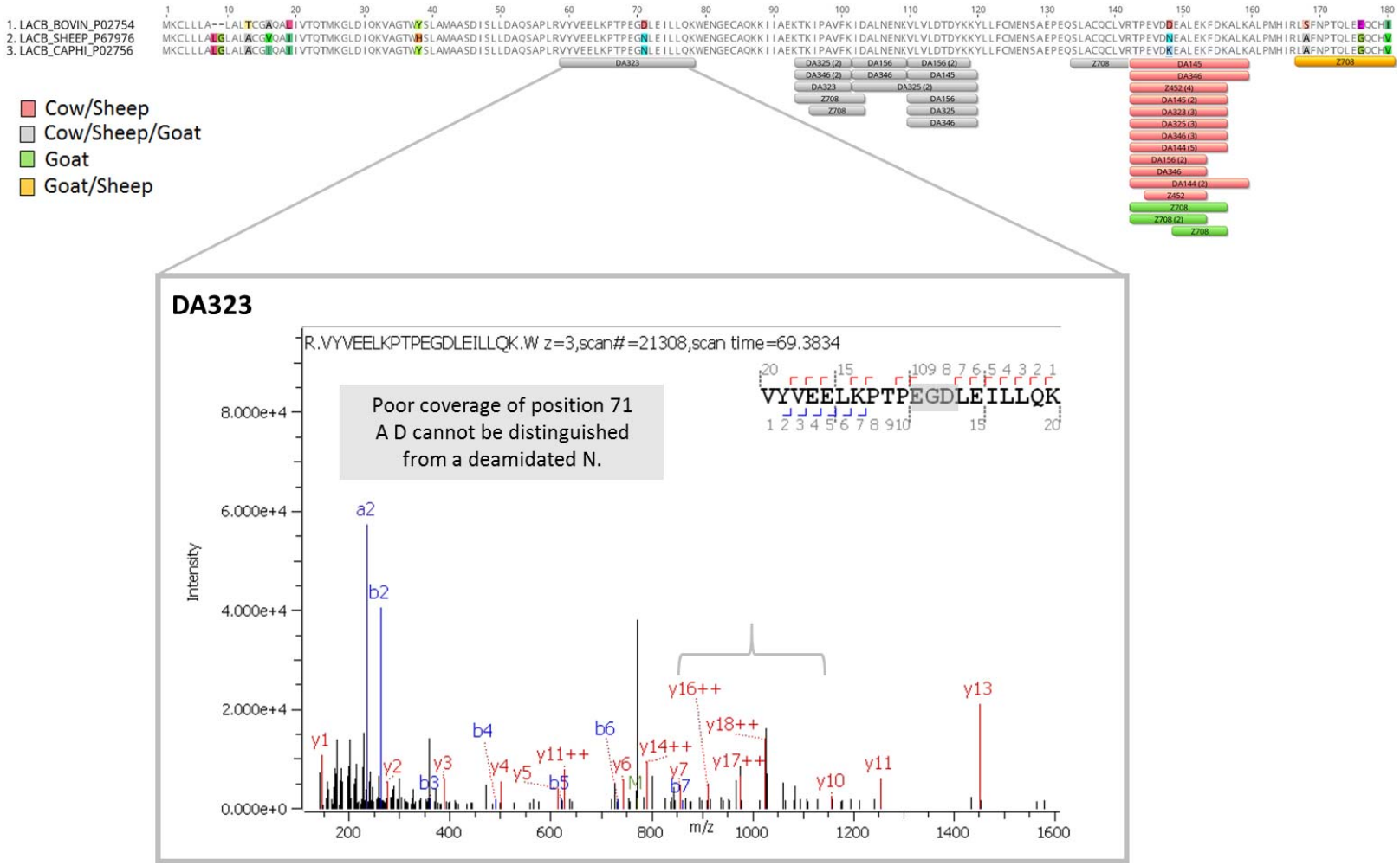
Cole's Burial (GrJj 5a)

Bone collagen from this individual produced a $\delta^{13}C$ value of -4.5‰[16] indicating a diet based on C_4 food protein sources. For this study, the tooth enamel of Individual 1 was analysed

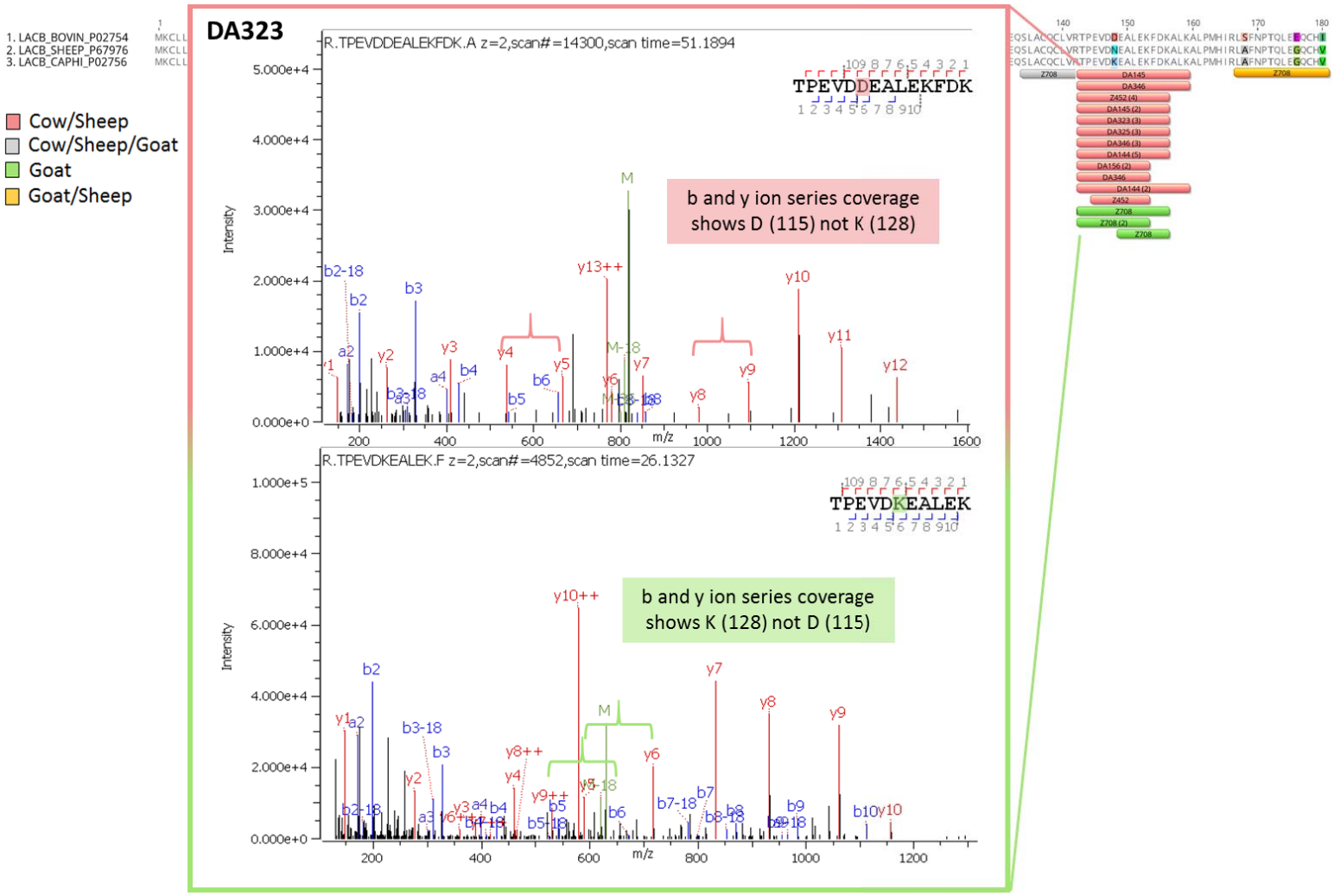
because $\delta^{13}C$ from enamel is more reflective of the whole diet[65]. The individual produced a $\delta^{13}C$ value of -1.2‰, confirming that this individual ate a primarily C_4-based diet. Tooth enamel was also analysed from an isolated tooth and gave a $\delta^{13}C$ -3.4‰ (Supplementary Fig.11) indicative of a diet with a high proportion of C_4 sources. In addition to the humans, tooth enamel was sampled from two rodents (including one mole rat (*Tachyoryctes* sp.)), these were the only fauna available for sampling. The rodents produced $\delta^{13}C$ measurements of -1.2‰ and -4.8‰ that broadly correspond to the values of the humans and, again, suggest the consumption of largely C_4 sources. Bone collagen from two individuals from Cole's Burial was analysed using stable isotopes carbon and nitrogen and reported in[14] and collagen from CB1.01 (in this study Individual 1) produced a $\delta^{13}C$ value of -4.5‰[16]. All three samples produced $\delta^{13}C$ between -4.0‰ and -5.0‰ indicating a high reliance on C_4 sources. Although in this case, distinguishing reliance on animals is challenging given the lack of faunal data.

Molo Cave (GoJi 3)

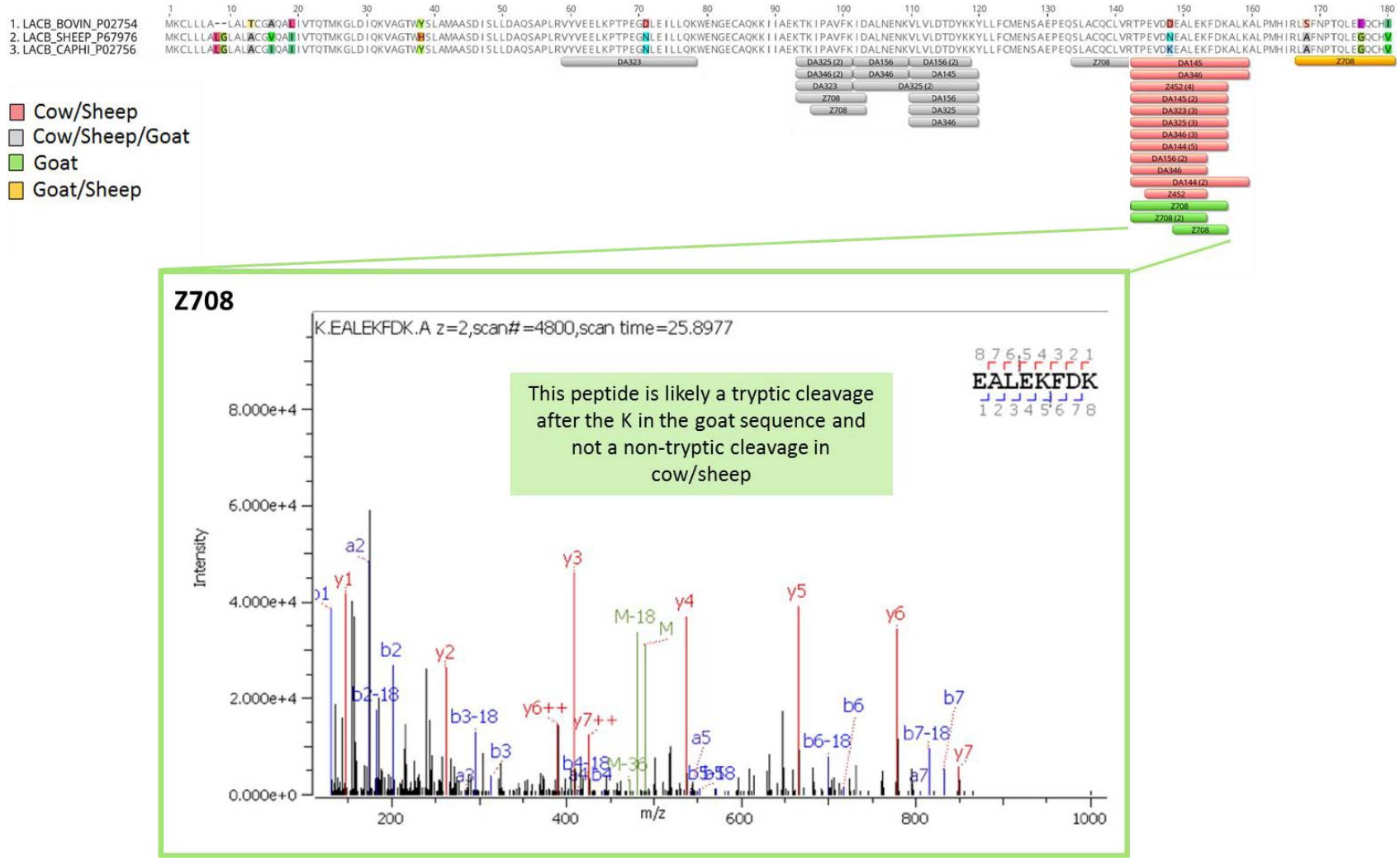
Tooth enamel samples were taken from three human individuals from Molo Cave, including Skeleton 1 that had BLG peptides in their calculus (this study) and is radiocarbon dated to 1415–1320 cal BP[13]. To contextualise the human isotopic results, a range of fauna were sampled from the site representing different feeding niches (arboreal browsers and grazers), which can be separated out according to their $\delta^{13}C$ enamel values. The *Dendrohyrax* and *Heterohyrax*, with $\delta^{13}C$ measurements of -18.3‰ and -14.1‰ respectively, reflect diets comprised largely of C_3 plants. The duiker (*Cephalophus*) $\delta^{13}C$ value (-12.4‰) is consistent with the consumption of largely C_3 sources (Supplementary Fig.12). In contrast, the two *Capra hircus* and one *Bos* specimens ($\delta^{13}C$ ranging from -1.7‰ to 0.5‰) are animals grazing on C_4 grasses. When comparing humans to the fauna, all individuals' (including Skeleton 1) $\delta^{13}C$ values are close to those of the domestic species (*Capra* and *Bos*). To further explore the degree to which individuals buried at Molo Cave relied on animal products (meat and dairy), bone collagen from two individuals was analysed along with associated fauna (Supplementary Fig.13). The humans have similar $\delta^{15}N$ collagen values (11.4‰ and 11.8‰). When considered against the $\delta^{15}N$ of the livestock (*Ovis*: 8.3‰, *Bos* average: 6.6‰), the isotope results suggest that the humans consumed a diet of terrestrial protein. The fact that the humans' isotope values (both $\delta^{15}N$ and $\delta^{13}C$) appear to align with the domestic fauna supports the assertion that these individuals relied on animals or animal products to a large degree.



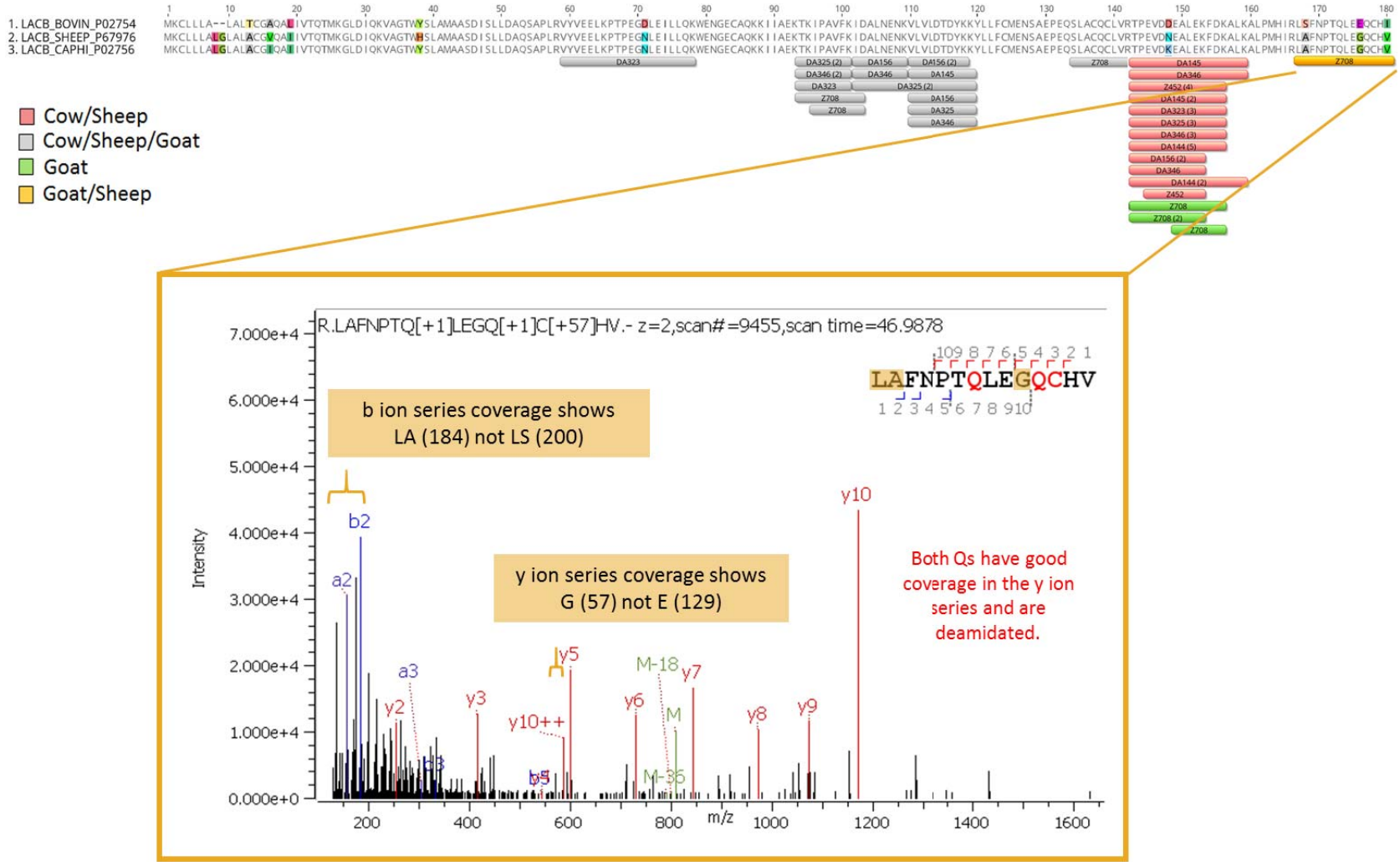
Supplementary Figure 1: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: Annotated spectra for sample DA323. Brackets show B and Y ions difference corresponding to highlighted amino acids.



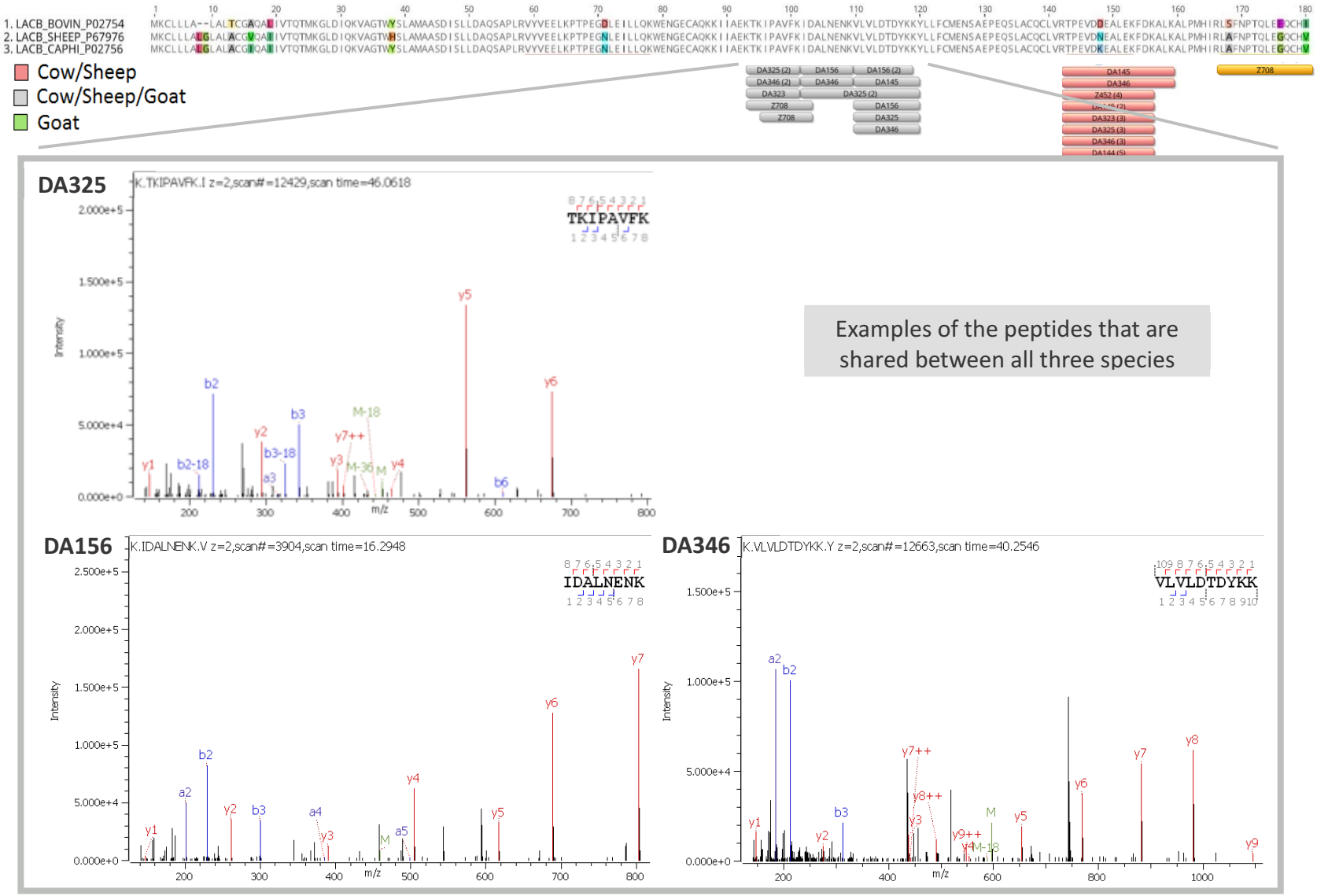
Supplementary Figure 2: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: Annotated spectra for sample DA323. Brackets show B and Y ions difference corresponding to highlighted amino acids.



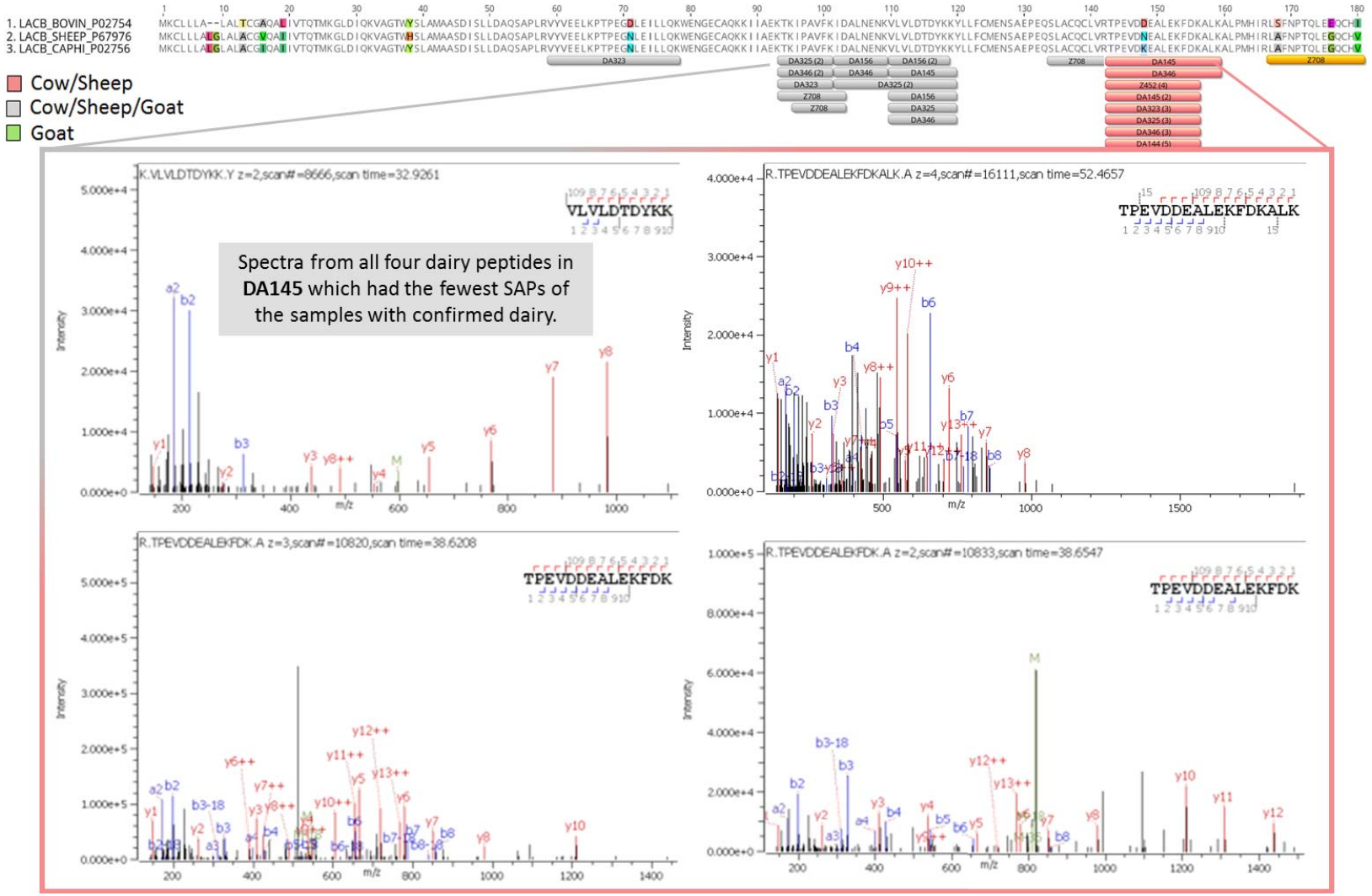
Supplementary Figure 3: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: Annotated spectra for sample Z708.



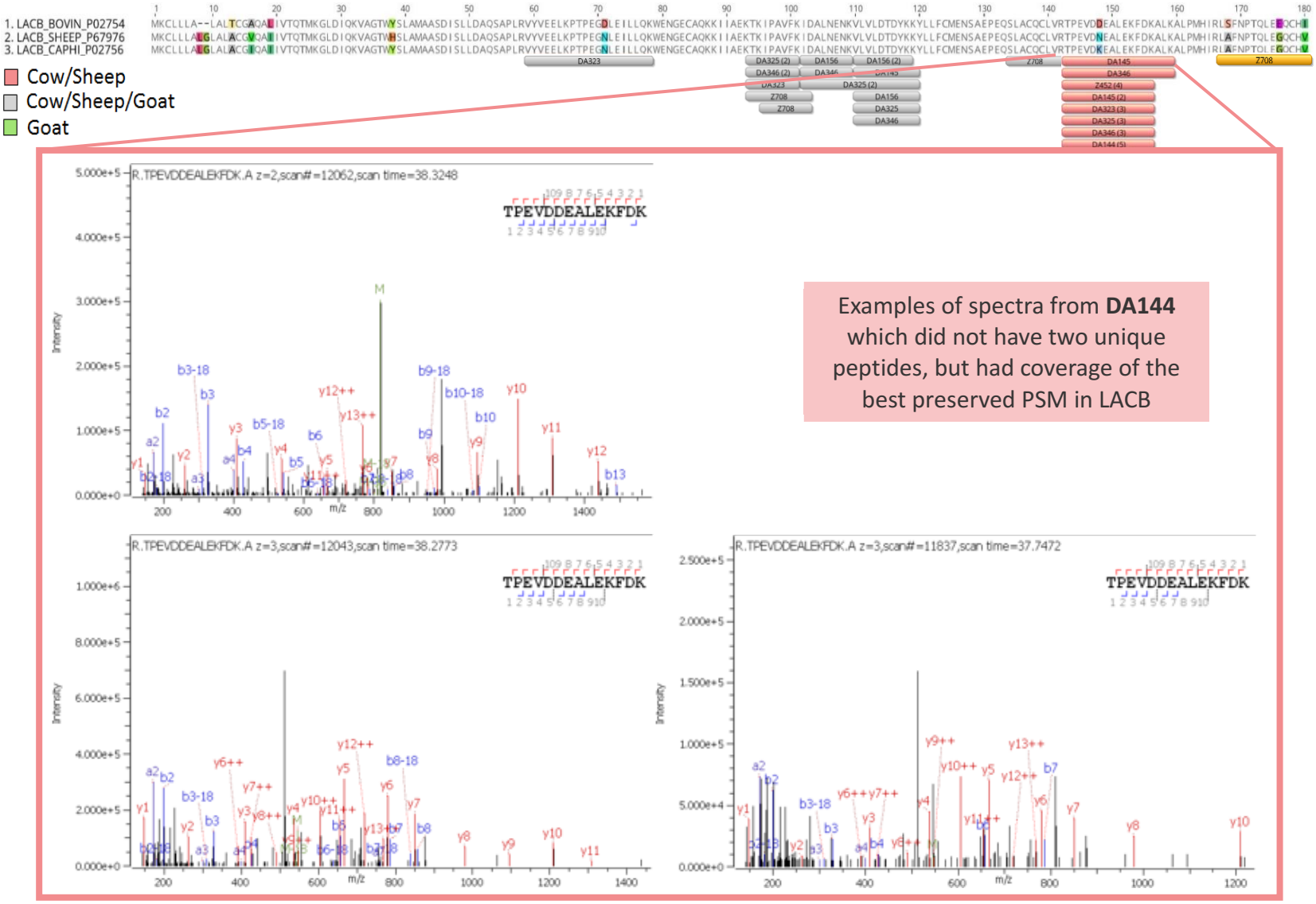
Supplementary Figure 4: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: Annotated spectra for sample Z708. Brackets show B and Y ions difference corresponding to highlighted amino acids.



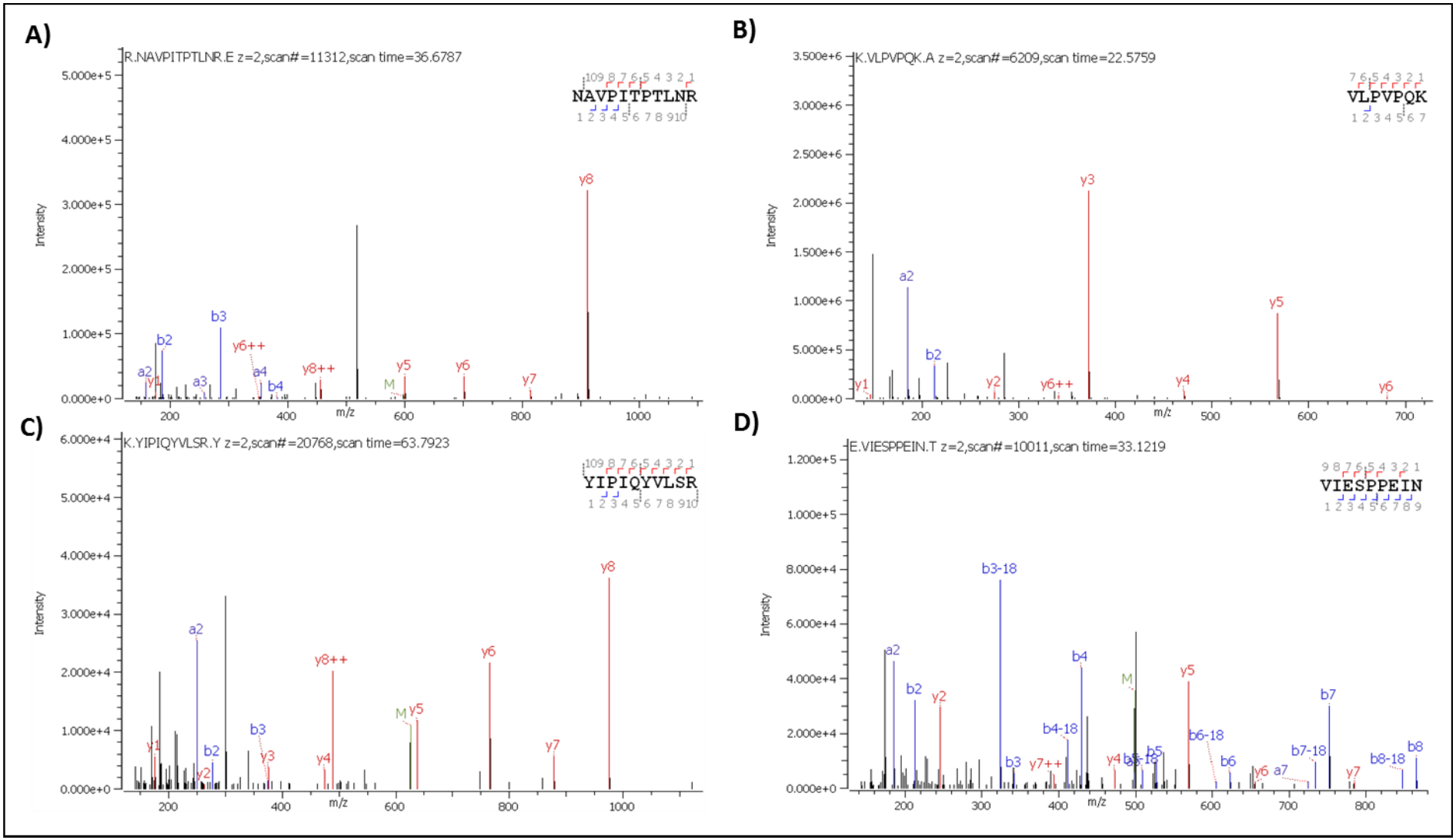
Supplementary Figure 5: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: Annotated spectra for samples DA325, DA156 and DA346.



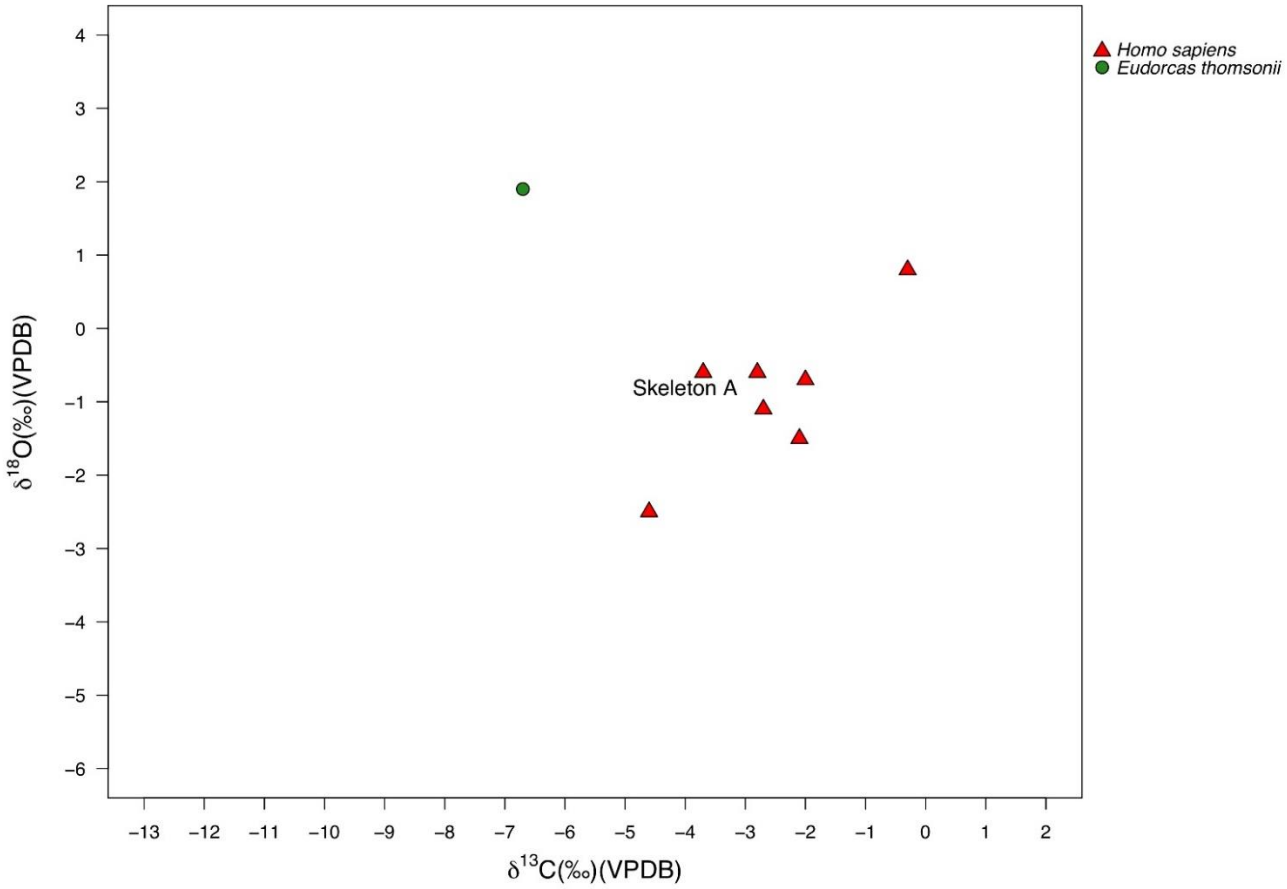
Supplementary Figure 6: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: annotated spectra from all four dairy peptides in sample DA145.



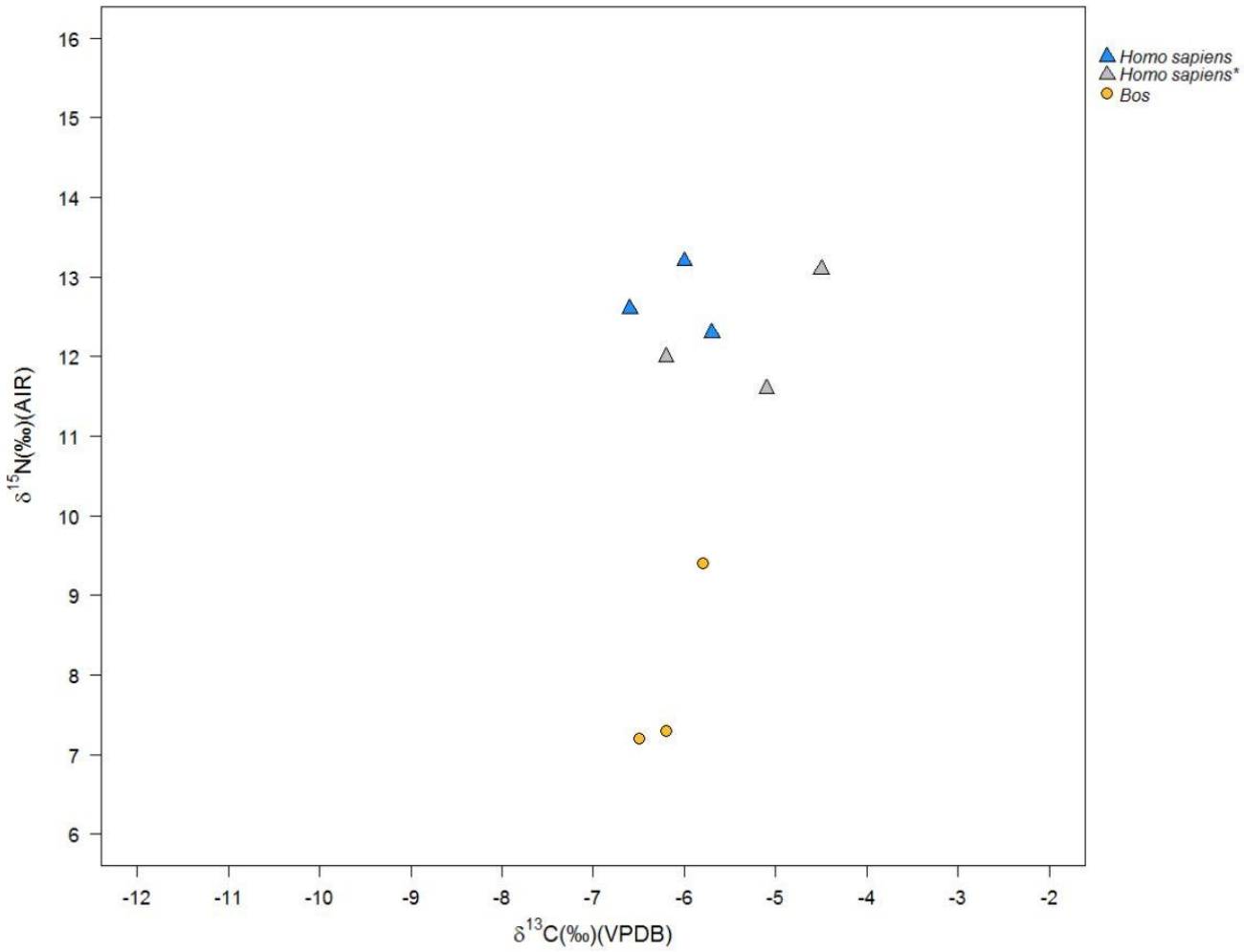
Supplementary Figure 7: Above: Alignment map for all BLG (LACB) peptides by individual. Species-specific information is indicated by different colours. Below: annotated spectra for sample DA144**.**



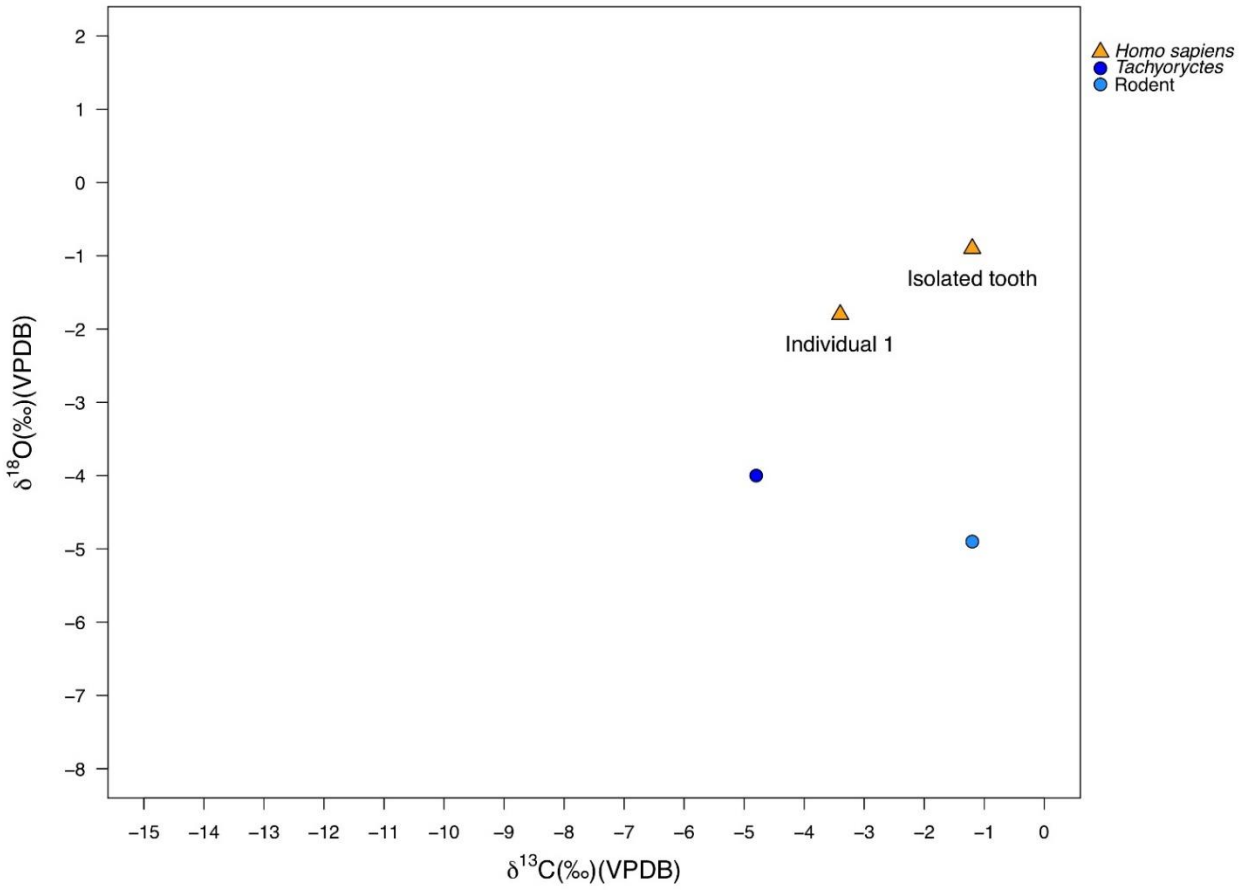
Supplementary Figure 8: Examples of spectra for casein peptides from sample DA156: A) Alpha-S2-casein (CASA2); B) beta-casein (CASB); C) kappa-Casein (CASK); D) kappa-Casein (CASK) identified only with non-tryptic search with Byonic.



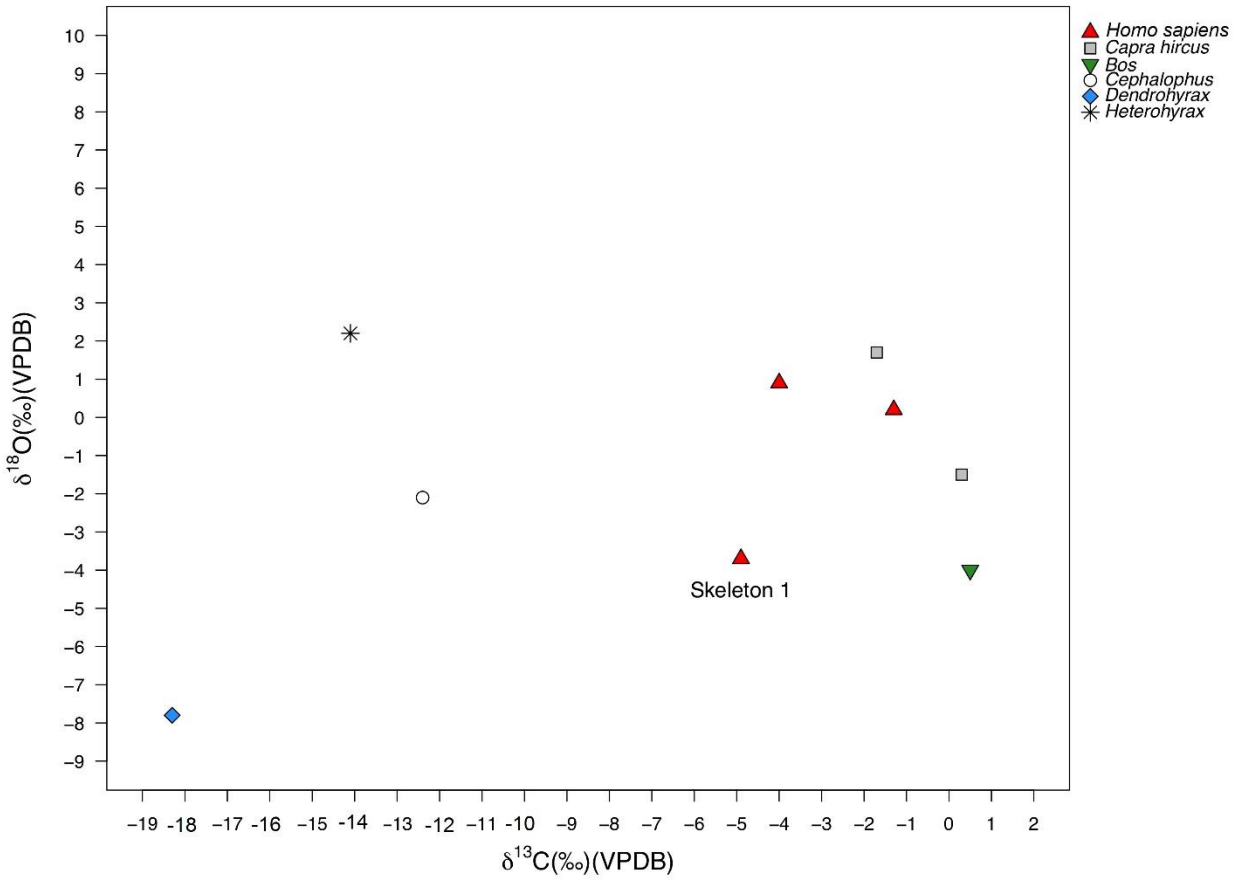
Supplementary Figure 9: $\delta^{13}C$ and $\delta^{18}O$ measurements for tooth enamel samples from humans and a Thomson's gazelle (*Eudorcas thomsonii*) from Lukenya Hill. Skeleton A produced proteomic evidence of milk consumption.



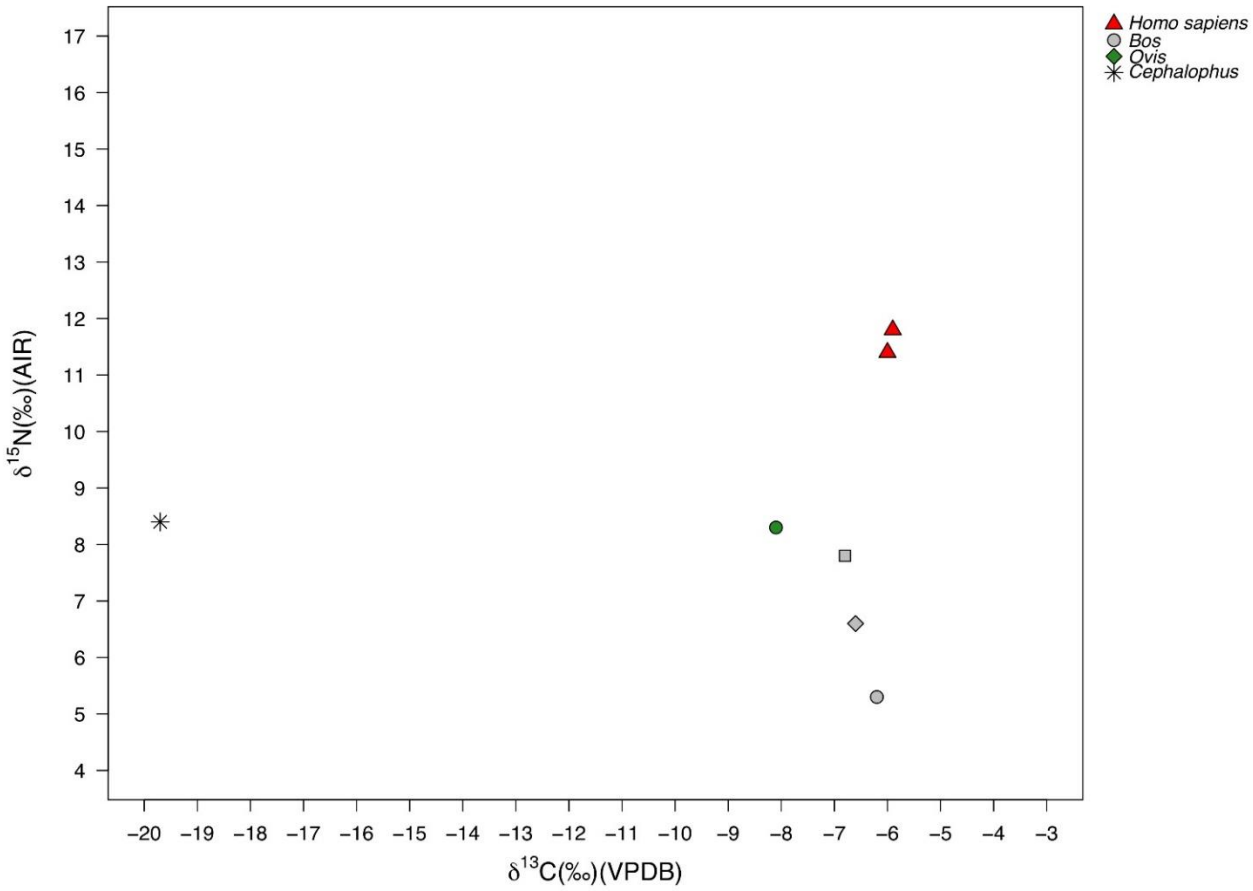
Supplementary Figure 10: $\delta^{15}N$ and $\delta^{13}C$ measurements for human and faunal bone collagen for Lukenya Hill (GvJm202 and GvJm184). *results previously published in[14]



Supplementary Figure 11: $\delta^{13}C$ and $\delta^{18}O$ measurements for tooth enamel samples from humans and rodents from Cole's Burial.

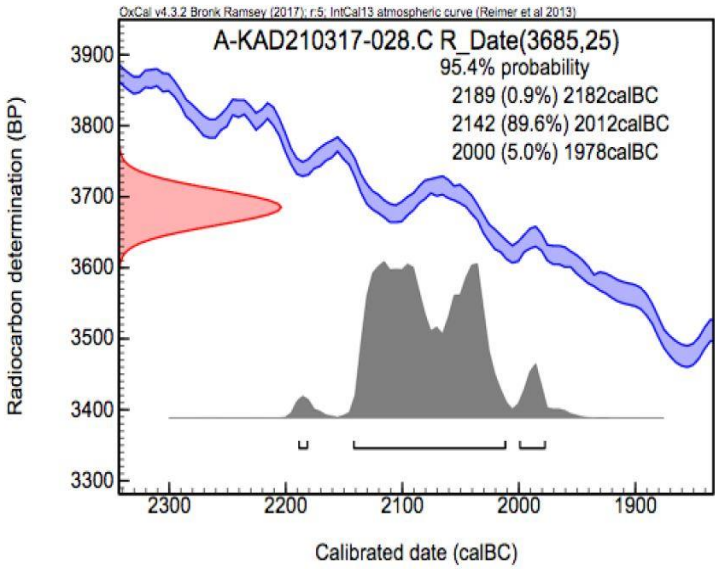


Supplementary Figure 12: $\delta^{13}C$ and $\delta^{18}O$ measurements for tooth enamel samples from humans and fauna from Molo Cave. Skeleton 1 was previously analysed for aDNA (MOL001)[13] and also had milk proteins in their dental calculus.



Supplementary Figure 13: $\delta^{15}N$ and $\delta^{13}C$ measurements for human and faunal bone collagen for Molo Cave.

Sample name	GrM	Calibrated dating result (95.4% probability)
DA-KAD10317-028.C	17738	2189 – 1978 calBC



Supplementary Figure 14: Radiocarbon date for Kadruka 1 SK68. ^{14}C ages were calibrated to calendar years with software program: OxCal, version 4.3[68], using calibration curve: IntCal13[69].

Supplementary Table 1: Summary of all dental calculus samples studied, number that met OSSD criteria. *Radiocarbon dating of these remains was unsuccessful due to insufficient collagen.

Site	Country	Archaeological Period	Individuals/samples analysed	Individuals/samples passed OSSD	Individuals/samples with milk
Kadruka 1	Sudan	Neolithic-Kerma	5/10	3/4	1/1
Kadruka 21	Sudan	Neolithic	5/10	3/4	1/2
Berber Meroitic Cemetery	Sudan	Meroitic	5/5	1/1	1/1
Atbara Setiet West Bank	Sudan	Unknown*	2/2	1/1	0
Roseires East Bank	Sudan	Unknown*	1/1	0	0
Tinga Archaeological Rescue Project	Sudan	Napata -Meroitic	3/3	1/1	0
Kweka cemetery	Sudan	Unknown	2/2	0	0
Kadakol Christian Cemetery	Sudan	Christian Period	1/1	0	0
Lukenya Hill (GvJm202)	Kenya	Pastoral Neolithic	5/5	4/4	2/2
Molo Cave (GoJi3)	Kenya	Pastoral Neolithic	2/2	2/2	1/1
Cole's Burial (GrJj5a)	Kenya	Pastoral Neolithic	3/3	3/3	2/2
Pickford's Site (GvJn14)	Kenya	Pastoral Neolithic	1/1	0	0
Jarigole (GbJ1)	Kenya	Pastoral Neolithic	3/3	0	0
Njoro River Cave (GrJh4)	Kenya	Pastoral Neolithic/Elmenteitan	3/3	1/1	0
Total (individuals/samples)			**41/51**	**19/21**	**8/9**

Supplementary Table 2: Summary of individuals with dairy proteins and radiocarbon dates. Lab codes are only reported for individuals with direct dates as opposed to associated dates from the same site. Dates published in *[13], †[16], ‡Associated date for all burials at GvJm202.

Site	Context	Archaeological Period	Dates cal. BP (Lab code)	Genetic cluster	LP alleles?	Species associated with milk proteins
Kadruka 1	KDK1 SK68	Neolithic-Kerma	4139-3928 (GrM 17738)	Analysis failed	n/a	*Capra*, Caprinae, Bovidae, Pecora
Kadruka 21	KDK21 SK129	Neolithic	Analysis failed	Analysis failed	n/a	Bovinae/*Ovis*, Bovidae
Berber Meroitic Cemetery	BMC 2015 T38 B	Meroitic	n/a	n/a	n/a	Pecora, Bovinae/*Ovis*, Bovinae, Bos, Bovidae
Lukenya Hill (GvJm202)	Skeleton A 533	Pastoral Neolithic	3610–3460*‡	n/a	n/a	Pecora, Bovinae/*Ovis*
Lukenya Hill (GvJm202)	703; West rocky sect; DD 75.5-85.5	Pastoral Neolithic	3610–3460*‡	n/a	n/a	Bovidae, Bovinae/*Ovis*
Molo Cave (GoJi3)	Skeleton 1, 55	Pastoral Neolithic	1415–1320 (OxA-37, 359)*	East Africa Pastoral*	No	Bovinae/*Ovis*
Cole's Burial (GrJj5a)	Individual 1, 107	Pastoral Neolithic	3351-3180 (PSU I8874)†	Pastoral Neolithic**	No	Pecora, Bovidae, Bovinae/*Ovis*
Cole's Burial (GrJj5a)	21, 5a, isolated tooth	Pastoral Neolithic	n/a	n/a	n/a	Pecora, Bovidae, Bovinae/*Ovis*

Supplementary Table 3: Number of possible deamidation sites for milk proteins identified in this study per individual. For two samples (DA356 and DA324) milk proteins were identified but they did not meet the criteria (see methods). These were therefore not reported as evidence of milk consumption in the main text.

Site	Sample	Protein	Total per individual (without species-specific sites)	Deamidated	Site-specific (in all cases these are the deamidated versions)
Kadruka 1	Z708	LACB	3	3	3
Kadruka 21	Z452	LACB	0	0	5
	DA351	CASB	2	0	0
Berber Meroitic Cemetery	DA156	LACB, CASA2, CASB, CASK	17	1	5
Lukenya Hill	DA145	LACB	0	0	3
	DA323	LACB	1	0	4
	DA356	*LACB*	*0*	*0*	*3*
Cole's Burial	DA325	LACB	4	3	3
	DA346	LACB	2	0	5
	DA324	*LACB*	*0*	*0*	*2*
Molo Cave	DA144	LACB	0	0	7

Supplementary Table 4: Summary of species information for peptide sequences. Pass for BLAST is 100% homology and 100% coverage to only the desired protein.

Protein	Start Position	Sequence	BLAST	Geneious
CASA2	130	NAVPITPTLNR	Pass	Bovinae
CASA2	153	TVDMESTEVFTK	Pass	Bovinae
CASA2	48	ENLCSTFCK	Pass	Bovinae
CASB	192	AVPYPQR	Pass	Bovinae
CASB	185	VLPVPQK	Pass	Bovidae
CASB	208	YQEPVLGPVRGPF	Pass	Bovidae
CASK	46	YIPIQYVLSR	Pass	Bovidae
CASK	108	SCQAQPTTMAR	Pass	Bovinae
CASK	90	SPAQILQWQVLSNTVPAK	Pass	*Bos*
CASK	173	VIESPPEIN	Pass	Bovinae
LACB	57	VYVEELKPTPEGDLEILLQK	Pass	Bovidae
LACB	92	TKIPAVFK	Pass	Bovidae
LACB	94	TKIPAVFKIDAL	Pass	Bovidae
LACB	94	TKIPAVFKID	Pass	Bovidae
LACB	96	IPAVFKID	Pass	Pecora
LACB	100	IDALNENK	Pass	Pecora
LACB	100	IDALNENKVLVLDTDYKK	Pass	Pecora
LACB	108	VLVLDTDYKK	Pass	Pecora
LACB	108	VLVLDTDYK	Pass	Pecora
LACB	110	VLVL	Fail	Fail
LACB	134	SLACQCLVR	Pass	Pecora
LACB	141	TPEVDDEALEKFDKALK	Pass	Bovinae or *Ovis*
LACB	141	TPEVDDEALEKFDK	Pass	Bovinae or *Ovis*
LACB	141	TPEVDDEALEK	Pass	Bovinae or *Ovis*
LACB	143	TPEVDKEALEKFDK	Pass	*Capra*
LACB	143	TPEVDKEALEK	Pass	*Capra*
LACB	149	EALEKFDK	Fail	Fail
LACB	167	LAFNPTQLEGQCHV	Pass	Caprinae
LACB	167	LAF	Fail	Fail

Supplementary Table 5: Taxonomic information for species for casein proteins and Beta-lactoglobulin.

Order	Suborder	Infraorder	Family	Subfamily	Genus	Species	CASA2	CASB	CASK	LACB
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Bovinae	*Bos*	*mutus*	x	x	x	x
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Bovinae	*Bos*	*taurus*	x	x	x	x
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Bovinae	*Bos*	*indicus*		x	x	
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Bovinae	*Bubalus*	*bubalis*	x	x	x	x
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Ovis*	*aries*	x	x	x	x
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Ovis*	*orientalis*			x	
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Ovis*	*vignei*			x	
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Capra*	*hircus*	x	x	x	x
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Pantholops*	*hodgsonii*	x			
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Naemorhedus*	*goral*			x	
Cetartiodactyla	Ruminantia	Pecora	Bovidae	Caprinae	*Oreamnos*	*americanus*			x	
Cetartiodactyla	Ruminantia	Pecora	Cervidae		*Rangifer*	*tarandus*				x
Cetartiodactyla	Tylopoda		Camelidae		*Camelus*	*bactrianus*	x	x	x	
Cetartiodactyla		Suina	Suidae		*Sus*	*scrofa*	x	x	x	x
Cetartiodactyla	Odontoceti		Delphinidae		*Tursiops*	*truncatus*		x	x	x
Perissodactyla			Equidae		*Equus*	*asinus*	x	x		x
Perissodactyla			Equidae		*Equus*	*caballus*	x	x	x	x
Primates			Hominidae		*Homo*	*sapiens*		x	x	
Carnvora			Canidae		*Canis*	*lupus*				x
Carnvora			Felidae		*Felis*	*catus*				x

Supplementary Table 6: Identification of faunal remains for isotope analysis using morphometrics and ZooMS.

Type	Site	MPI-SHH Database	Lab ID	Element	Morphological ID	ZooMS ID	Final ID
Bone	Lukenya Hill (GvJm 184)	DA-LUKI0317-002	LUKF001	Humerus CYL	Bovid 3	*Bos*	*Bos*
	Lukenya Hill (GvJm 202)	DA-LUK0317-012	LUKF003	Long bone fr	Mammal ≥3	*Bos*	*Bos*
	Lukenya Hill (GvJm 202)	DA-LUK0317-014	LUKF004	Femur shfr.	Bovid 3	*Bos*	*Bos*
	Molo Cave (GoJi3)	DA-MOL0317-017	MOLF003	Left mandible	Bovid 1 (neonate)	*Bos*/Bison/*Cephalophus*	*Cephalophus* sp.
	Molo Cave (GoJi3)	DA-MOL0317-004.B	MOLF005	R rib angle fr	Bovid 3	*Bos*	*Bos*
	Molo Cave (GoJi3)	DA-MOL0317-007	MOLF008	Non ID fragment	Mammal ≥3	*Bos*	*Bos*
	Molo Cave (GoJi3)	DA-MOL0317-023	MOLF010	Rib proximal fr	Bovid 2	*Ovis*	*Ovis*
	Molo Cave (GoJi3)	DA-MOL0317-008a	MOLF011	Upper limb bone fragment	Mammal ≥3	*Bos*	*Bos*
Tooth	Lukenya Hill (GvJm 202)	DA-LUK0317-013	LUKF002	LUM1	*cf. Eudorcas thomosonii*	n/a	*Eudorcas thomosonii*
	Cole's Burial	DA-COL0317-033	COLF002	RLI1	Rodent indet	n/a	Rodent
	Cole's Burial	DA-COL0317-004	COLF003	LLI1	*Tachyoryctes*	n/a	*Tachyoryctes*
	Molo Cave (GoJi3)	DA-MOL0317-011	MOLF001	LLM3	*Capra hircus*	n/a	*Capra hircus*
	Molo Cave (GoJi3)	DA-MOL0317-009	MOLF002	LUP0	*cf. Bos*	n/a	*Bos*
	Molo Cave (GoJi3)	DA-MOL0317-017	MOLF003	LLdp4	Bovid 1 (neonate)	n/a	*Cephalophus* sp.
	Molo Cave (GoJi3)	DA-MOL0317-004.A	MOLF004	LLM2	*Capra hircus*	n/a	*Capra hircus*
	Molo Cave (GoJi3)	DA-MOL0317-010.A	MOLF006	RUP2	*Dendrohyrax*	n/a	*Dendrohyrax*
	Molo Cave (GoJi3)	DA-MOL0317-022	MOLF007	RLP4	*Heterohyrax*	n/a	*Heterohyrax*

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9. Manuscript C

Isotopic and Microbotanical Insights into Iron Age agricultural reliance in the Central African rainforest

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Isotopic and microbotanical insights into Iron Age agricultural reliance in the Central African rainforest

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The emergence of agriculture in Central Africa has previously been associated with the migration of Bantu-speaking populations during an anthropogenic or climate-driven 'opening' of the rainforest. However, such models are based on assumptions of environmental requirements of key crops (e.g. *Pennisetum glaucum*) and direct insights into human dietary reliance remain absent. Here, we utilise stable isotope analysis ($\delta^{13}C$, $\delta^{15}N$, $\delta^{18}O$) of human and animal remains and charred food remains, as well as plant microparticles from dental calculus, to assess the importance of incoming crops in the Congo Basin. Our data, spanning the early Iron Age to recent history, reveals variation in the adoption of cereals, with a persistent focus on forest and freshwater resources in some areas. These data provide new dietary evidence and document the longevity of mosaic subsistence strategies in the region.

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or the past half a century, if not longer, the processes for the dispersal of Bantu-speaking communities from Western Central Africa have been a major focus of African archaeological, linguistic, and genetic research[1–4]. While there has been an increasing departure from notions of a single sweeping 'Bantu Expansion', the degree to which the movement of people, languages, and the emergence of farming are linked across Africa continues to be forcefully debated[5–7]. Central Africa is at a key location for developing existing models for the spread of farming[8] yet investigations of the emergence of food production, particularly in the rainforest, have been limited[9]. Assumptions that tropical rainforests represent substantial barriers to agriculturalists[10] have been used to rationalise a relatively late arrival of farming in the region, c. 2500 years ago, during a period of climate- or human-induced deforestation[11–13] (Supplementary Note 1). However, unlike other parts of Africa[14–16], there have been few studies directly testing changes in human dietary reliance on agricultural crops, relative to local freshwater, bushmeat, and tropical forest plant resources[17], from the first arrival of domesticates in the region through to the present day.

Linguistic, material culture, and radiocarbon analyses have now shown that human arrival throughout the Congo Basin was a complex and time-transgressive occurrence, potentially with the interaction of different populations occurring[10,18–25]. Furthermore, ideas relating to the inability of farming populations to occupy the tropical rainforests of Central Africa have come under renewed scrutiny[26]. Experimental research has demonstrated that pearl millet (*Pennisetum glaucum*) can be grown in forested portions of the Inner Congo Basin[27]. This suggests that discoveries of pearl millet (c. 2330–330 BP) at Iron Age sites across Central Africa, regions presently covered in tropical rainforest[18,28], need not represent a time of mass 'rainforest crisis'[29,30]. Not only that, but Iron Age expansions into the various tributaries of the Congo River continued well after the supposed peak in rainforest decline 2500 years ago, suggesting more complex, ongoing processes of agricultural adaptation, and settlement. Together, these developments make it essential to build more integrated, multidisciplinary, and context-specific insights into changes in diet and land use in different parts of Central Africa through time, as different agricultural populations negotiated their tropical surroundings.

Here, we present new, direct dietary information from Iron Age sites in the Democratic Republic of Congo (DRC) using the stable carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), and oxygen ($\delta^{18}O$) isotope analysis of human and animal remains. Isotopic results were obtained for human burials from the sites of Imbonga (IMB; $n = 1$), Longa (LON; $n = 1$), Bolondo (BLD; $n = 18$), and Matangai Turu Northwest (MTNW; $n = 1$). In addition, bone collagen ($n = 10$) and enamel ($n = 6$) were analysed for a range of fauna from BLD to create an isotopic baseline. The sites studied represent different geographic and temporal contexts (Fig. 1). IMB is the type site for the earliest pottery tradition of the central equatorial rainforest and the individual analysed, indirectly dated to ~2050 BP (Supplementary Note 2), would have been a member of a group representing already established agriculture in the region after its initial settlement by sedentary immigrant populations a few centuries earlier. In contrast, individuals from LON and BLD represent subsistence practices during the Late Iron Age, when populations were spreading further across the Congo Basin. Finally, isotopic results from the individual from MTNW, previously identified as a likely hunter-gatherer[31], offers new evidence about the intricacies of subsistence, cultural, and genetic identities further to the eastern edge of the Basin (Supplementary Note 2). Collectively, the samples analysed span the period following the first arrival of food producers in this region (~2050 BP) through to relatively recent occupation (~130 BP; Supplementary Note 2, Supplementary Tables 1 and 2, and Supplementary Figs. 1, 3–7).

$\delta^{13}C$ analysis of human tissues has long been demonstrated to provide insights into reliance on plants with different photosynthetic pathways (namely C_4 versus C_3) and their animal consumers (Supplementary Note 3)[32–34]. Significantly, in Central Africa, wild, as well as potentially domesticated (e.g., yams), forest plants are C_3, while incoming cereal crops (e.g., pearl millet, sorghum, and, for later periods, maize) are C_4. $\delta^{13}C$ measurements of wild plants and animals from the rainforests of the DRC show that these forests are largely composed of C_3 vegetation[35]. Moreover, they show a recognisable 'canopy effect' on this C_3 vegetation that results in lower $\delta^{13}C$ among plants, and their animal consumers, living under dense canopies compared to those living in more open areas[35], something that has been well-documented in many other tropical regions[34,36].

The sites of IMB, LON, and BLD are located on tributaries of the Congo River in the western DRC (Fig. 1 and Supplementary Note 2) an area presently covered in dense C_3-dominated evergreen and semi-deciduous forest[37]. Stable carbon measurements of faunal tooth enamel from BLD, which largely reflect the proportions of C_3/C_4 plants consumed, reflect local palaeoecology, as well as providing baseline values for human diet. The final site, MTNW, is situated in the closed-canopy forest of the Ituri Region of the Northeast Congo Basin with palaeoenviromental proxies, suggesting a predominance of tropical forest tree taxa during the time of occupation[38,39].

$\delta^{15}N$ analysis provides insights into the positions of humans within their trophic web and their potential consumption of aquatic resources[40,41], while $\delta^{18}O$ measurements reflect water sources and environments[42,43] (see "Methods" section or Supplementary Note 3 for full details). To the best of our knowledge, this is the first time that multi-tissue stable isotope analysis of prehistoric humans and animals has been applied in the Congo Basin, in order to provide long-term insights into changing human reliance on different resources. We also present results of $\delta^{13}C$ and $\delta^{15}N$ analysis of charred lumps interpreted as food residues from BLD, as well as microparticle analysis of dental calculus recovered from MTNW, to obtain more detailed insights into prepared and consumed foods, respectively.

Results

Faunal and human bone collagen. Bone collagen results were assessed using established indicators of preservation, including a C/N ratio between 2.9–3.6, %C of ca.15–48%, and %N of ca. 5–17% (ref. [44–47]). Two results generated for human burials from BLD were excluded from final analysis as they produced a %N < 5 and a %C < 15 % (Supplementary Table 3).

The fauna from BLD fall broadly into four groups: wild browsers (antelope and duiker, $n = 2$), domesticated browsers (goats, $n = 2$), mammalian carnivores ($n = 2$), and aquatic species (fish and crocodile, $n = 4$). $\delta^{13}C$ values from BLD mammals ($n = 6$) are consistent with a largely C_3-based diet with measurements ranging from −23.7 to −18.2‰, although the goats, the dog, and the fox-sized carnivore could potentially have some C_4 component to the diet (Fig. 2 and Supplementary Table 3). The common duiker (*Sylvicapra grimmia*, BLD 83/2-8) and small antelope (BLD 83/2-6 + 7) have $\delta^{15}N$ values (5.9 and 7.3‰, respectively) consistent with herbivorous diets. In contrast, the fox-sized carnivore (13.7‰) and dog (12.3‰) display higher $\delta^{15}N$ that is consistent with consumption of animal protein. Three of the aquatic species sampled had higher $\delta^{15}N$ values than the catfish ($\delta^{15}N$ 9.4‰), the crocodiles gave $\delta^{15}N$ of 11.1 and 11.4‰, and the bichir (*Polypterus* sp.), a fish known to consume other fish and small vertebrates[48], produced a $\delta^{15}N$ measurement of 12.7‰.

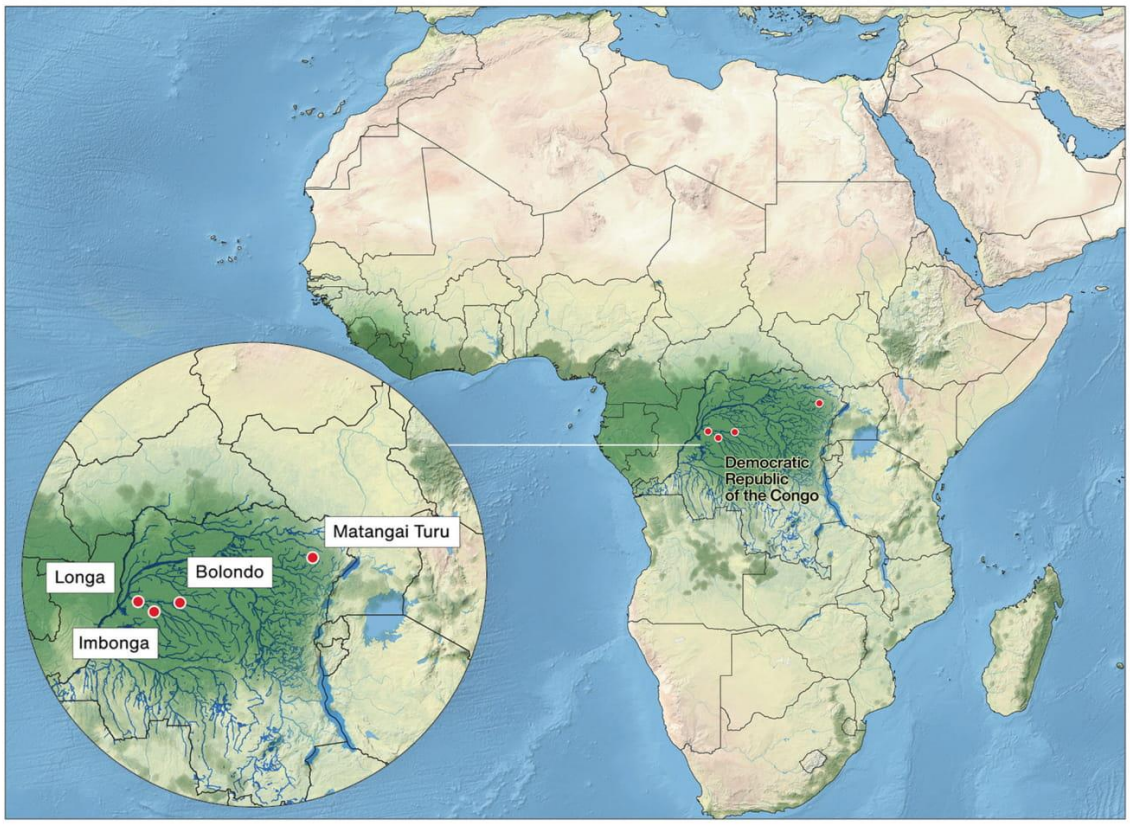

Fig. 1 Map showing the location of the archaeological sites of study in the Democratic Republic of the Congo. Imbonga (IMB), Longa (LON), Bolondo (BLD), and Matangai Turu Northwest (MTNW). Tropical rainforest is shown in dark green. The map was created for this study by Hans Sell (Graphic Designer for the Max Planck Institute for the Science of Human History, Jena, Germany) using QGIS 3.12 https://qgis.org/en/site/ and the Natural Earth Database from https://www.naturalearthdta.com/downloads/. To increase accuracy, river locations are based on OpenStreetMap data provided by GEOFABRIK http://download.geofabrik.de/africa/congo-democratic-republic-latest-free.shp.zip. Final adjustments to colour saturation and site labels were made using Adobe Illustrator and Photoshop.

The accompanying $\delta^{13}C$ value of −24.7‰ for the bichir somewhat overlaps with that expected for C_3 plants and animals, demonstrating the need to examine both $\delta^{15}N$ and $\delta^{13}C$, in order to separate freshwater fish and forest resources.

The $\delta^{13}C$ values for the bone collagen of humans from BLD ($n = 11$) dating to between 1426 and 1942 years cal. AD (Supplementary Figs. 3–7) are quite variable, ranging from −21.0 to −16.3‰. Alongside the individual from LON (directly dated to 1642—after 1938 cal. AD), these values are generally consistent with reliance on C_3 plants, C_3 plant-consuming wild and domestic animals, and freshwater resources. The average $\delta^{15}N$ value of 14.5‰ for the human individuals from BLD in comparison to the average for the goats (9.9‰) is within the range of values reported for diet–collagen spacing, potentially indicating reliance on these domesticates[41,49]. However, $\delta^{15}N$ values ranging from 13.4 to 16.9‰, and three individuals with $\delta^{15}N$ values higher than 15‰, as well as the riverine setting and modern and historical evidence that the site was a fishing camp (Supplementary Note 2), indicate that freshwater fish was also a major part of human diets at BLD. Nevertheless, it is still evident that all humans and domestic animals, as well as the fox-sized carnivore, have higher $\delta^{13}C$ values than the available wild C_3 or freshwater fauna, indicating the consumption of an additional resource enriched in $\delta^{13}C$.

A visible negative correlation between human $\delta^{15}N$ and $\delta^{13}C$ suggests that this was, in fact, a plant food (Fig. 2), although a Pearson's rank test (correlation coefficient = −0.600, d.f. = 9, $p = 0.05$) produced results at the limit of statistical significance. This is likely a product of the small sample sizes available. The metabolic routing bias of bone collagen $\delta^{13}C$ towards protein components of the diet (at the expense of carbohydrate and fat inputs), and importance of high protein freshwater fish in diets of the measured individuals, means that we can expect that consumption of this low-protein plant resource was actually greater than it appears in the bone collagen values of Fig. 2. Thus, although somewhat underrepresented in the present bone collagen isotope results, there is a signal indicative of some C_4 plant component, which must have contributed to the diet of Late Iron Age humans and domestic animals in the Inner Congo Basin from at least the 15th century cal. AD onwards.

Faunal and human tooth enamel. Tooth enamel is widely regarded as the archaeological material of choice in the tropics. Stable carbon and oxygen isotopes of tooth enamel have been shown to robustly preserve ecological variation, even in tropical regions, from the Miocene to the Late Pleistocene[34,50,51]. Tooth enamel was sampled from human second and third molars

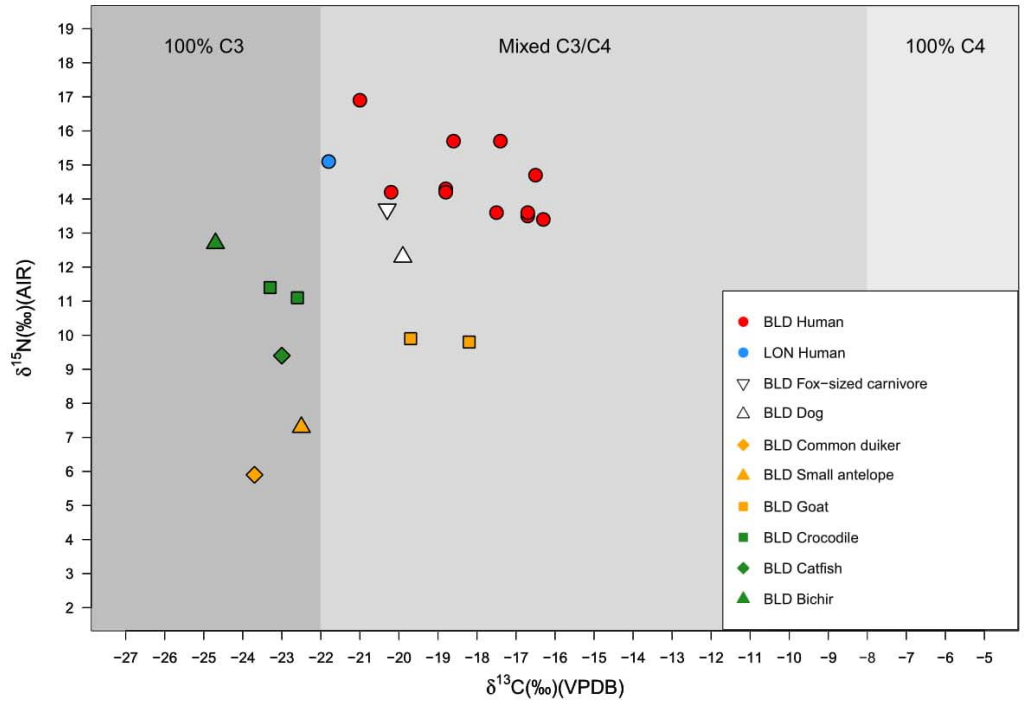

Fig. 2 Human and faunal bulk bone collagen $\delta^{13}C$ and $\delta^{15}N$ results for BLD and LON. Shading indicates estimated bone collagen $\delta^{13}C$ for individuals consuming 100% C_3, mixed C_3/C_4, and 100% C_4 sources[33].

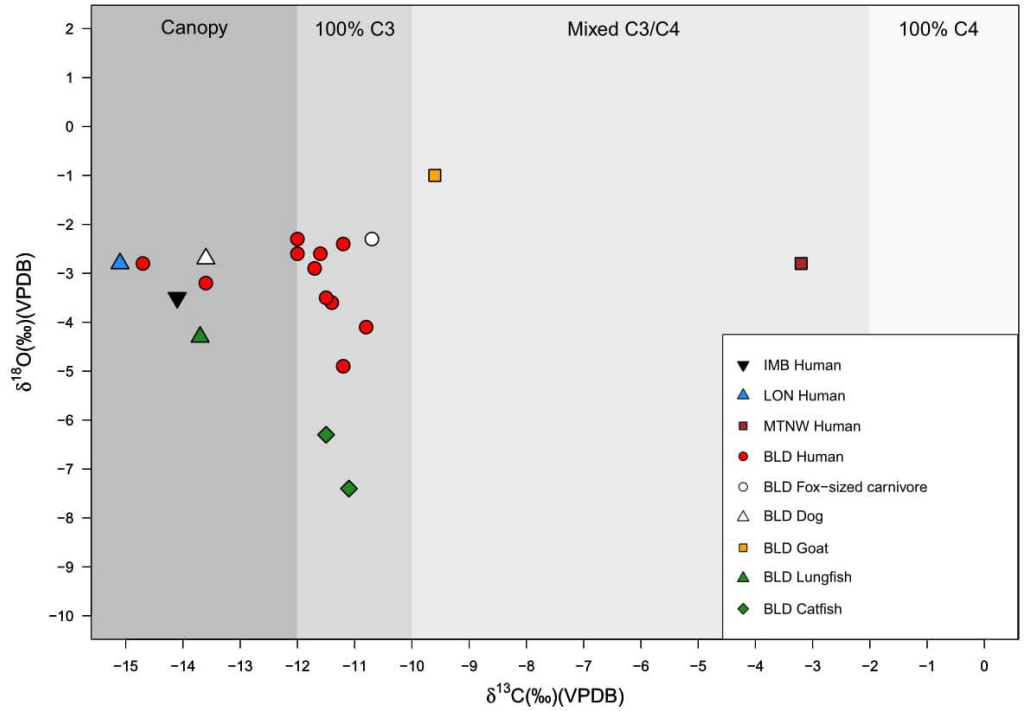

Fig. 3 Human and faunal bulk tooth enamel $\delta^{13}C$ and $\delta^{18}O$ for BLD, IMB, LON, and MTNW. Shading indicates estimated tooth enamel $\delta^{13}C$ for individuals living under dense canopy and consuming 100% C_3, mixed C_3/C_4, and 100% C_4 sources based on literature[34,51].

enabling the investigation of diet during late childhood–early adolescence. Furthermore, given that tooth enamel $\delta^{13}C$ reflects the isotopic composition of the whole diet (including carbohydrates, fats, and proteins), as opposed to the dominance of the protein signal in bone collagen, tooth enamel $\delta^{13}C$ enables additional dietary resolution (see Supplementary Note 3).

Faunal and human $\delta^{13}C$ enamel results from IMB, LON, BLD, and MTNW (Fig. 3 and Supplementary Table 4) reveal diverse subsistence strategies that can be divided into three broad groups: wild forest resources, forest and freshwater resources, and more open environments, including C_4 food sources. The individual from the earliest site mentioned here, IMB, has a $\delta^{13}C$ value of −14.1‰ indicating reliance on a mixture of tropical rainforest and freshwater resources, while the individual from LON shows clear evidence for reliance on tropical rainforest resources with a $\delta^{13}C$ value of −15.1‰. Two individuals from BLD gave $\delta^{13}C$ values of −14.7 and −13.6‰ also suggesting a heavy reliance on tropical or freshwater resources. The $\delta^{13}C$ values of remaining individuals from BLD (−12.0 to −10.8‰) are indicative of a dominance of C_3 food sources, possibly yams, plantain, or oil palm grown in slightly more open conditions[52,53], or perhaps a mixed diet of closed canopy and freshwater resources. There is no clear evidence for dietary reliance on C_4 plants in any of the human tooth enamel samples analysed from IMB, LON, or BLD.

As enamel carbonate is reflective of all food sources it would not be affected by a high degree of fish consumption, making this lack of evidence for C_4 consumption somewhat surprising given the collagen data. For four individuals from BLD, it was possible to analyse both bone collagen and tooth enamel to investigate tissue-specific or age-related dietary differences. One individual (BLD 83/3 individual 2) demonstrates a predominant reliance on C_3 or freshwater food sources from mid-late childhood through to adulthood giving a $\delta^{13}C_{enamel}$ of −11.7 and $\delta^{13}C_{coll}$ value of −21.0‰. Interestingly, however, BLD 83/1 individual 1 $\delta^{13}C_{enamel}$ value (−13.6‰) is consistent with a reliance on tropical rainforest and freshwater resources, but the accompanying $\delta^{13}C_{coll}$ value (−17.4‰) reflects a C_3/C_4-based protein diet. A similar shift is also seen when comparing enamel and collagen results for BLD 83/8 ($\delta^{13}C_{enamel}$ −11.4‰, $\delta^{13}C_{coll}$ −20.2‰) and BLD 83/10 ($\delta^{13}C_{enamel}$ −10.8‰, $\delta^{13}C_{coll}$ −16.7‰). These distinctions could be a product of either (i) the fact that low-protein rainforest plant resources are more visible in the tooth enamel, while higher-protein animals eating C_4 plants (e.g., goats) or higher $\delta^{13}C$ freshwater resources, with higher $\delta^{13}C$ values are more visible in collagen or (ii) greater consumption of C_4-based resources in adulthood compared to childhood.

In sharp contrast to the western DRC samples, the M3 from the individual from MTNW has a $\delta^{13}C$ enamel value of −3.2‰ that clearly indicates that C_4 resources made the dominant contribution to the diet of this individual. Unfortunately, it was not possible to analyse bone collagen from this individual, but multiple teeth (M1–M3) were analysed to investigate diet throughout childhood. The results (Supplementary Fig. 8) demonstrate that this individual relied upon C_4 food sources throughout childhood and as a juvenile.

Microbotanical remains dental calculus. Dental calculus was analysed from three mandibular molars (M1, M2, and M3) from the MTNW individual and a total of 38 starch granules and 9 phytoliths were retrieved (Supplementary Table 5). Microcharcoal is very common (Fig. 4a, aq–as). The calcium phosphate matrix was decontaminated prior to decalcification, as per a published protocol[54] in which calculus is immersed in sodium hydroxide of 2% w/v solution for 24 h. As expected for ancient starch granules, the discovered calculus starch displays signs of damage to their semicrystalline matrix, having partially or totally lost their native birefringence. Other signs of diagenesis include fissuring, pitting, granulation, and implosion of the hilum. The taxonomic identification of starch granules depends on whether they represent unique morphometric identifiers that can be compared to published reference collections. In this respect, the mixture of polygonal, orbicular, and quadratic granules found derive from a grass seed ($n = 26$), but cannot specifically be assigned to pearl millet (*Pennisetum glaucum*), wild finger millet, (*Eleusine africana*), or domestic finger millet (*Eleusine coracana*), as they all have in common compound granules and/or markedly polygonal shapes with mean metrics <10 µm (Supplementary Fig. 9a–c).

In contrast, starch granules from *Sorghum bicolor* are polymorphic with roughly polygonal, orbicular, and quadratic shapes, a centric hilum often creased or slit (Supplementary Fig. 9d–h), and can be uniquely identified by prismatic–polygonal shapes with mean maximum length 18–30 µm (ref. [55]). Another common starch granule type identified is parabolic and/or oblong elongate ($n = 11$; Fig. 4). In our reference collection, the best possible match for this cohort is in the Dioscoreaceae, which in sub-Saharan Africa produces the highest number of unique identifiers in granule morphometrics and overwhelmingly associates with wild yams[55].The remaining type (ovate, $n = 1$; Fig. 4) is also tentatively associated with an underground storage organ, and similar granules occur in the Asphodelaceae family[55]. With regards to phytoliths, all phytoliths ($n = 9$) come from one tooth (M1) and they are characterised as medium to large, brown globular bodies, with tuberculate to echinate projections. These large, brown phytoliths with variably tuberculated to echinated spines are referenced in the nutshell of *Elaeis guineensis* (Supplementary Fig. 9i–n), whose charred remains were also discovered at the site.

Charred food fragments from Bolondo. The charred food fragments recovered from contexts at BLD ($n = 8$) fall into three groups based on their $\delta^{13}C$ values: those with $\delta^{13}C$ values < −27‰; a single food fragment with an intermediate $\delta^{13}C$ value of −24‰; and two food fragments with $\delta^{13}C$ values around −9‰ (Supplementary Table 6). $\delta^{13}C$ values ranging from < −27 to −24‰ are likely indicative of food fragments consisting of C_3 or aquatic resources, while $\delta^{13}C$ values of −9‰ indicate that the primary content was likely C_4 resources. The charred food fragments from BLD contained between 0.6 and 2.5% N, which means that for all samples apart from BE06, the N_2 peak was too small for reliable determination of their $\delta^{15}N$ values. BE06, a sample with a high $\delta^{13}C$ value (−9.3‰), has a relatively high $\delta^{15}N$ value (7.8‰) compared to those of herbivores from the site, perhaps indicating that C_4 plant resources like pearl millet and sorghum consumed by humans were growing in different soil conditions compared to the plants eaten by wild and domestic herbivores.

Discussion

The discovery of pearl millet *(Pennisetum glaucum)* in Iron Age pits in Southern Cameroon[18] was one of the most significant findings of the past two decades in Central African archaeology. The discovery sparked debate over the environmental context for early agriculture[26,56], contributing to an increasingly complex narrative for the settlement of the Central Africa rainforest[10,21,57,58]. Yet direct assessments about the degree to which these early farming communities, particularly in the DRC, relied on C_4 resources are limited[17]. Our data enable direct exploration of the adoption of agriculture at different points during the Iron Age in the DRC and highlight substantial regional

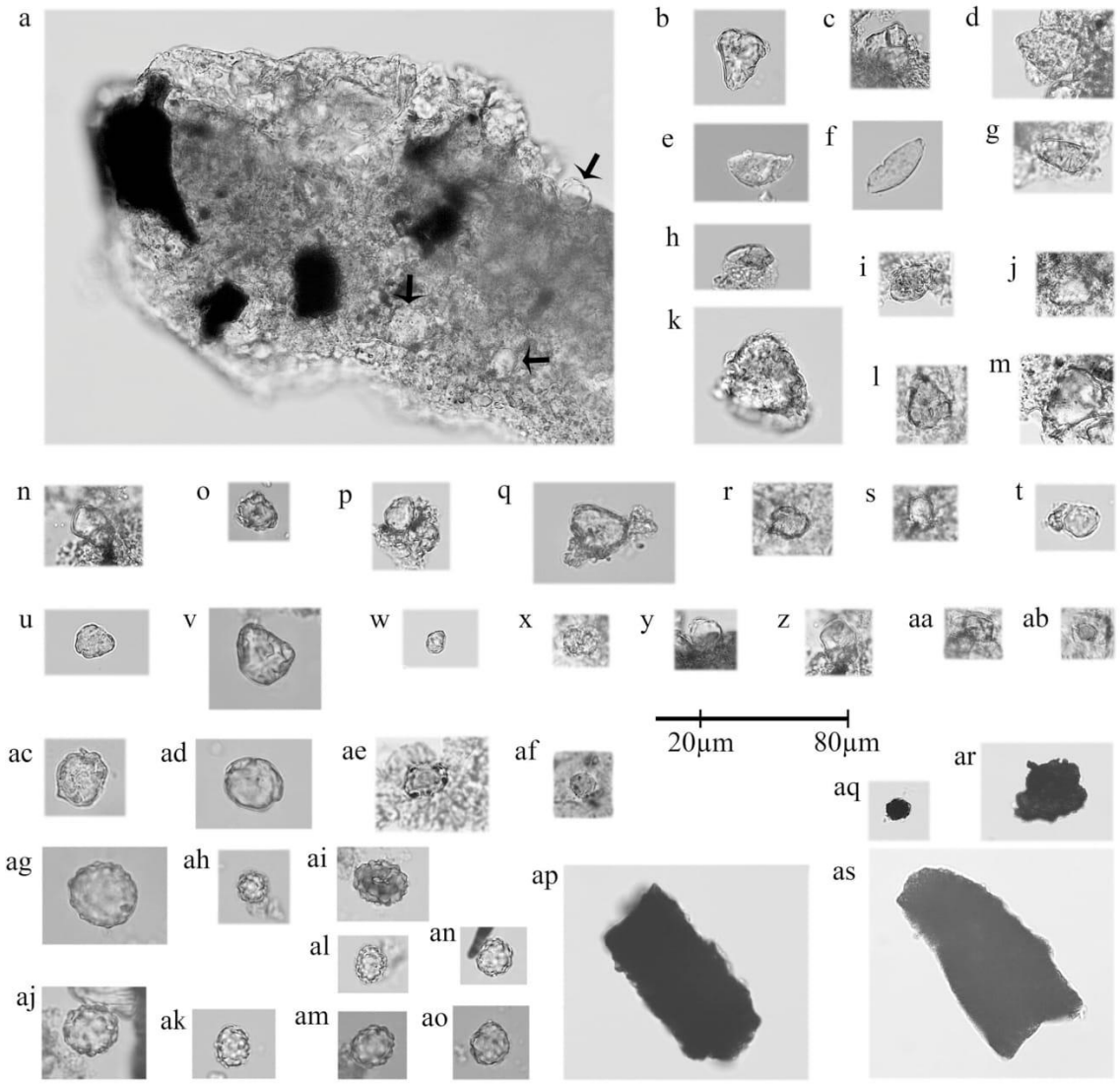

Fig. 4 Selected microbotanical materials extracted from the dental calculus of MTNW. a Starch and microcharcoal entrapped within calcified matrix, M2. Starch granules: **b**–**d** parabolic, M2; **e** oblong elongate, M2; **f** oblong elongate, M3; **g**, **h** parabolic, M2; **i** ovate, M2; **j**–**m** parabolic, M2; **n** quadratic, M2; **o** polygonal, M2; **p** orbicular, M3; **q** polygonal, M3; **r** orbicular, M2; **s** quadratic, M2; **t** polygonal, M2; **u** orbicular, M2; **v** polygonal, M1; **w** orbicular, M3; **x** orbicular, M2; **y** orbicular, M2; **z** polygonal, M2; **aa** quadratic, M2; **ab** orbicular, M2; **ac** polygonal, M2; **ad** orbicular, M1; **ae** orbicular, M2; **af** orbicular, M2; phytoliths: ag–ao globular tuberculate to echinate, M1; microcharcoal: **ap**–**as** M2.

variability, particularly with regards to uptake of C_4 crops into the diet. The IMB individual, indirectly dated to ~2050 BP (Supplementary Note 2), provides a snapshot of potentially early agricultural groups entering the region. While this period is commonly associated with the arrival of cereal cultivation, this individual shows a heavy reliance on C_3 closed rainforest or freshwater resources rather than C_4 crops. Moving to the Late Iron Age and ongoing 'Bantu expansion' into a number of tributaries of the Congo River, individuals from LON and BLD show no clear evidence for a dietary reliance on C_4 plants in tooth enamel samples. Collagen values, however, do suggest some degree of C_4 consumption in addition to a core reliance on C_3 closed rainforest and more open C_3 resources, perhaps including plantain, oil palm and yams or manioc, and freshwater resources. This interpretation is supported by zooarchaeological research at BLD that highlights a dominance of fish, as well as the apparent importance of riverine locations for these settlements.

These findings are particularly interesting given that there is clear archaeobotanical evidence, at BLD in particular, for the presence of pearl millet. $\delta^{13}C$ data from two charred food fragments from flotation samples from BLD, as well as identified charred pearl millet grains, definitively show that millet was processed in this western portion of the DRC during the Iron Age. However, it did not apparently dominate the diets of buried individuals at the same site, though its exact importance in adulthood is somewhat masked by protein representation of bone collagen. This potentially implies that millet was used in a different context in the Iron Age of this region and, instead of being a staple, was possibly used in feasting, brewing, or prestige contexts[59]. Such an interpretation could be supported by the fact that

multi-tissue $\delta^{13}C$ analysis of humans at BLD indicates increased C_4 contributions in adult life relative to childhood or juvenile diets, though this remains tentative at present. Regardless, results encourage a shift away from broad linguistic and genetic models for the 'Bantu expansion' when studying agricultural adaptations in Central Africa, and necessitates further direct, context-specific multidisciplinary analyses in order to understand the adoption and incorporation of C_4 cereal crops in Iron Age subsistence in the western DRC. An experimental study has demonstrated that it is possible to grow pearl millet within the Inner Congo Basin today[27]. While pollen evidence suggests the existence of a 'rainforest crisis' c. 2500 years ago[60,61], this study and our data demonstrate that this was not necessary for the initial, or indeed subsequent, cultivation of millet in the region.

The importance of undertaking a multidisciplinary, contextual approach to the emergence of food production in Central Africa is further indicated by the data from the northeastern DRC site of MTNW. In contrast to the western DRC sites, the stable isotopic data from this individual highlight a clear overall dietary reliance on C_4 food sources throughout childhood and into teenage years. The location of Matangai Turu in proximity to migrating farming populations in eastern Africa[62], observed genetic affinity of the sampled individual to these groups, as well as hunter-gatherer populations[63], and the identification of Poaceae starch (likely sorghum) in the dental calculus of this individual indicate that this C_4 signal represents the use of sorghum possibly acquired through interactions with agricultural groups in eastern Africa. Nevertheless, the human remains date to 813 ± 35 BP, a time when the region is believed to have been covered by lowland Guineo-Congolain rainforest[31,35,38,64]. Moreover, the presence of starches from Dioscoreaceae and Asphodelaceae in the dental calculus, as well as the association with wild rainforest fauna[54,64] indicate ongoing contributions of forest resources to the lifeways of this Late Iron Age population. Evidently, the adoption of cereal crops into subsistence economies across tropical Africa was not uniform, displaying regional variation and incorporation into existing, dynamic lifeways.

The results of this study reveal a diversity of subsistence practices spanning the Central African Iron Age that involved the incorporation of incoming C_4 crops to varying degrees. For the Matangai Turu individual this likely reflects complex forager–farming interactions and exchanges during this time, which have been increasingly recognised in genetic studies[65,66]. In contrast, there is presently no evidence for pre-Iron Age indigenous settlement in the Inner Congo Basin, though the local agency of the Late Iron Age populations adapting to the western portion of the region should not be underestimated. Tropical forests in the DRC, as elsewhere[67], were home to diverse groups who developed a range of strategies to procure and produce food. We can only begin to look at these dynamic, contextually dependent strategies if we move away from sweeping narratives based on genetic or linguistic data to focus on direct evidence from archaeology, archaeobotany, archaeozoology, and biomolecular methodologies relating to the actual significance of different resources to diets across time and space.

In this way, we can also begin to properly understand the wider significance of ongoing agricultural adaptations in Central Africa. The Congolese portions of the Central African rainforest are considered some of the most vulnerable to climate change[68–70] and are increasingly appreciated as essential, but now threatened, carbon 'sinks' for the continental and global carbon cycle[37,71]. Consequently, there has been substantial discussion about the relative sustainability and antiquity of intensive agricultural land use[72,73] versus mixed agricultural, agroforestry, and hunting strategies in the region[74,75]. Despite frequent NGO or government calls to focus on productive cereal monoculture to meet growing populations in West and Central Africa[76], it is clear that mixed use of C_4 plants, wild resources, rainforest economic plants, and freshwater resources have characterised subsistence practices in the western DRC for over 2000 years. Indeed, the continuation of heterogeneous food production strategies could prove crucial in ensuring long-term food security in tropical Central Africa, as well as the survival of Congolese environments crucial to the global carbon cycle[77] pan-African precipitation[78], and global biodiversity[79].

Methods

Four Iron Age sites were selected for study from across the DRC (Fig. 1 and Supplementary Note 2): IMB, LON, BLD, and MTNW. Bulk bone collagen $\delta^{13}C$ and $\delta^{15}N$ measurements were obtained for human burials from LON ($n = 1$), BLD ($n = 11$), and MTNW ($n = 1$). Totals reflect results taken forward for interpretation. For BLD, two results were excluded from final analysis due to poor preservation (for full details see: "Results" section and Supplementary Table 3). Bone collagen was also analysed from a range of faunal remains from BLD ($n = 10$) to establish a dietary baseline, including domestic browsers (goats), wild browsers (duiker), and fish. To further explore dietary intake, human tooth enamel was sampled from IMB ($n = 1$), LON ($n = 1$), BLD ($n = 11$), and MTNW ($n = 1$). Due to differential preservation, it was only possible to generate both a bulk collagen and tooth enamel results for four human burials from BLD, and the single individual from LON. In addition, animal tooth enamel from BLD ($n = 6$) was analysed for $\delta^{13}C$ and $\delta^{18}O$ to aid the interpretation of human values.

BLD is a c. 660 BP to present site located in the western Interior Congo Basin on the floodplain of the Tshuapa River. The site was first excavated in 1983 with the most recent field season taking place in 2016, with financial support of the Deutsche Forschungsgemeinschaft[80]. Owing to partly waterlogged conditions, the level of organic preservation at BLD is far higher than at sites located above the floodplain, and a number of human burials have been excavated[80] (Supplementary Fig. 2). In addition, tooth enamel samples were analysed from one human individual from each of the sites of IMB and LON. Both sites were excavated by Manfred Eggert in the 1980s. IMB is the type site for the oldest pottery of the equatorial forest and is located on the Momboyo River, and LON is located on the Ruki River[20,81].

Finally, a single individual dating to 813 ± 35 BP was analysed from MTNW, a Later Stone Age rockshelter located in the Ituri rainforest of the Eastern Congo Basin[31].This individual was previously identified as a likely hunter-gatherer based on morphological evidence, associated lithics, presence of wild fauna, and absence of domesticated plants[31]. However, the presence of a large assemblage of ceramics, iron slag, and iron objects means it is impossible to say definitively whether the individual was from a primarily foraging group or associated with Bantu-speaking groups[31]. Unfortunately, it was not possible to sample any associated fauna from MTNW for this study, but a range of forest taxa were present at the site, including porcupines, antelopes, primates, small bovids, and snails[64]. While there are clear geographical and ecological differences between MTNW and BLD the fauna at both sites are indicative of a closed forest environment. For the individual from Matangai Turu, it was possible to sample all three permanent molars to track dietary consumption throughout childhood. The tooth enamel $\delta^{13}C$ of human molars is influenced by dietary intake during the time of tooth formation with the first molar forming between 2 months prior to birth to 4 years after birth, the second molar between 4 and 7 years, and third molar between 9 and 16 years (Supplementary Note 3).

Stable isotope analysis of bone collagen. Human and faunal bone collagen was extracted using a modified Longin[82] method. Bone samples (~1 g) were broken into small pieces and adhering soil was removed by abrasion using a sandblaster. Samples were demineralised by immersion in 0.5 M HCl for 1–7 days. Once demineralisation was complete, samples were rinsed three times with ultra-pure H_2O. The residue was gelatinised in pH 3 HCl at 70 °C for 48 h, and the soluble collagen solution Ezee-filtered to remove insoluble residues[83]. Samples were lyophilised in a freeze dryer for 48 h. Where sufficient material was available, ~1.0 mg of the resulting purified collagen was weighed, in duplicate, into tin capsules for analysis.

The $\delta^{13}C$ and $\delta^{15}N$ ratios of the bone collagen were determined using a Thermo Scientific Flash 2000 Elemental Analyser coupled to a Thermo Delta V Advantage mass spectrometer at the Isotope Laboratory, MPI-SHH, Jena. Isotopic values are reported as the ratio of the heavier isotope to the lighter isotope ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) as δ values in parts per mill (‰) relative to international standards, VPDB for $\delta^{13}C$ and atmospheric N2 (AIR) for $\delta^{15}N$. Results were calibrated against international standards of (IAEA-CH-6: $\delta^{13}C = -10.80 \pm 0.47$‰, IAEA-N-2: $\delta^{15}N = 20.3 \pm 0.2$‰, and USGS40: $\delta^{13}C = -26.38 \pm 0.042$‰, $\delta^{15}N = 4.5 \pm 0.1$‰) and a laboratory standard (fish gelatin: $\delta^{13}C = \sim -15.1$‰, $\delta^{15}N = \sim 14.3$‰). Based on replicate analyses long-term machine error over a year is ±0.2‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{15}N$. Overall measurement precision was studied through the measurement of repeats of fish gelatin ($n = 80$, ±0.2‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{15}N$). The faunal ($n = 8$) and human ($n = 12$) bone collagen results from BLD and

LON are presented in Supplementary Table 3. Samples with a C/N ratio between 2.9–3.6, %C of ca.15–48, and %N of ca.5–17% were carried forward for interpretation[44–46].

Stable isotope analysis of tooth enamel. Approximately 10 mg of tooth enamel powder was obtained from sampled teeth using gentle abrasion with a diamond-tipped drill along the full length of the buccal surface and transferred to a microcentrifuge tube. Teeth were sampled from both humans and fauna (where available) across the four sites. Second and third molars were preferentially sampled from humans providing a long-term insight into juvenile diet, and avoiding the weaning effect potentially visible in first molars. Tooth enamel samples were pretreated with 1 mL 1% NaClO for ~60 min. The samples were rinsed three times with ultra-pure H_2O and centrifuged before 1 mL 0.1 M acetic acid was added for 10 min. After this, samples were rinsed with ultra-pure H_2O, for a total of three washes[84,85]. After the final rinse, each tube was placed in a freeze drier for 4 h. In addition, an in-house standard of equid tooth enamel was processed alongside the samples of this study. Approximately 2 mg of the pretreated sample was weighed out into 12 mL borosilicate glass vials for analysis.

Following reaction with 100% phosphoric acid at 70 °C, sample CO_2 evolved and was analysed for stable carbon ($^{13}C/^{12}C$) and oxygen isotopic ratio ($^{18}O/^{16}O$) composition using a Thermo Gas Bench 2 connected to a Thermo Delta V Advantage Mass Spectrometer. Carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) stable isotope values were calibrated against international standards IAEA NBS 18 ($\delta^{13}C$ −5.014 ± 0.032‰, $\delta^{18}O$ −23.2 ± 0.1‰), IAEA 603 ($\delta^{13}C$ +2.46 ± 0.01‰, $\delta^{18}O$ −2.37 ± 0.04‰), IAEA CO8 ($\delta^{13}C$ −5.764 ± 0.032‰, $\delta^{18}O$ −22.7 ± 0.2‰), and USGS44 ($\delta^{13}C = \sim -42.1$‰) registered by the International Atomic Energy Agency. Machine error based on the analyses of standards is ±0.1‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{18}O$. Overall measurement precision was assessed through repeat measurements of MERCK $CaCO_3$ ($n = 20$, ±0.2‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{18}O$, $\delta^{13}C = \sim -40.6$‰, $\delta^{18}O = \sim -13.3$‰) and an in-house equid tooth standard ($n = 10$, ±0.3‰ for $\delta^{13}C$ and ±0.2‰ for $\delta^{18}O$).

Microparticle analysis of dental calculus from MTNW. Dental calculus was processed from three mandibular molars (M1, M2, and M3) from the MTNW individual. Images of the mineralised plaque prior to removal from the teeth, as well as those from contaminant starch granules and phytoliths are published elsewhere (see ref. [54]; Fig. 2). The elemental breakdown includes carbon, oxygen, calcium, and phosphorus, with small quantities of aluminium, silicon, nitrogen, sodium, and chlorine[54], and the Ca:P ratio was 2:1–1:7 indicating hydroxyapatite. We present microbotanical materials released from the calcified matrix after thorough decontamination protocols and decalcification in a cleanroom laboratory[54], as well as microbotanicals still trapped in the calculus matrix, but visible enough to have their two dimensional morphology identified. Identifications were made according to published morphometric classification criteria for the identification of ancient starch from sub-Saharan plants[55].

Stable isotope analysis of charred food fragments. Charred fragments classified as prepared food remains during archaeobotanical analysis at BLD were retrieved from flotation samples after sorting under a binocular microscope. A total of 2–3 mg of each sample was weighed into tin capsules for stable carbon and nitrogen isotope analysis. The $\delta^{13}C$ and $\delta^{15}N$ ratios of the charred food fragments were determined, using a Thermo MAT 253 continuous flow isotope ratio mass spectrometer coupled to a Thermo Flash 1112 Series elemental analyser in the Institut für Geowissenschaften, Goethe-Universität, Frankfurt am Main, Germany. Isotopic data are provided in Supplementary Table 6.

The carbon contents of the samples were calculated based on the area under the CO_2 peak relative to the weight of the sample, calibrated using IAEA-CH-7. Stable carbon isotope values were calibrated to the VPDB scale using IAEA-C-7 ($\delta^{13}C$ −32.15 ± 0.05‰) and IAEA-USGS24 ($\delta^{13}C$ −16.05 ± 0.04‰). Measurement uncertainty in $\delta^{13}C$ values was monitored using three in-house standards: LEU (DL-leucine, $\delta^{13}C$ −28.3 ± 0.1‰), GLU (DL-glutamic acid monohydrate, $\delta^{13}C$ −10.4 ± 0.1‰), and MIL (millet flour from a single panicle from a plot in Senegal, $\delta^{13}C$ −10.2 ± 0.1‰; Supplementary Data 1). Precision ($u(R_w)$) was determined to be ±0.06‰, accuracy or systematic error ($u(bias)$) was ±0.11‰, and the total analytical uncertainty in $\delta^{13}C$ values was estimated to be ±0.13‰, using the equation presented in Supplementary material (Supplementary Data 1).

The nitrogen contents of the samples were calculated based on the area under the N_2 peak relative to the weight of the sample, calibrated using IAEA-N2. Stable nitrogen isotope values were calibrated to the AIR scale using IAEA-N-1 ($\delta^{15}N$ 0.4 ± 0.2‰) and IAEA-N-2 ($\delta^{15}N$ 20.3 ± 0.2‰). Measurement uncertainty in $\delta^{15}N$ values was monitored using three in-house standards: LEU (DL-leucine, $\delta^{15}N$ 6.5 ± 0.4‰), GLU (DL-glutamic acid monohydrate, $\delta^{15}N$ −1.9 ± 0.1‰), and MIL (millet flour from a single panicle from a plot in Senegal, $\delta^{15}N$ 3.1 ± 0.6‰). $u(R_w)$ was determined to be ±0.18‰, $u(bias)$ was ±0.59‰, and the total analytical uncertainty in $\delta^{15}N$ values was estimated to be ±0.61‰.

AMS dating. Bone samples from five individuals from BLD, and the single individual from LON were sent for radiocarbon dating at the Scottish Universities Environmental Research Centre AMS Laboratory, Glasgow (SUERC, Lab ID: GU), in order to improve understanding of their chronology. Samples were pretreated using previously published methods[86]. Radiocarbon ages were calibrated to calendar timescale using OxCal 4 (ref. [87]) and IntCal13 atmospheric calibration curve[88] (Supplementary Note 2 and Supplementary Figs. 1, 3–7).

Statistics and reproducibility. Sample size was determined by archaeological sample preservation and availability. Data for human bone collagen were analysed using Pearson's r R (version 3.5.3).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All of the data reported in the paper are presented in the main text or in the Supplementary Information, Tables, and Figures. Human and faunal skeletal samples have the following site-specific prefixes: 'IMB' for Imbonga, 'BLD' for Bolondo, 'LON' for Longa, and 'MTNW' for Matangai Turu Northwest. All human and faunal sample IDs are provided in Supplementary Tables 3 and 4. Charred food remains from BLD have sample codes starting 'BE' and full IDs are provided in Supplementary Table 6. The majority of individuals analysed in this study are from BLD and were excavated in 1983 by Manfred Eggert (Supplementary Note 2, and Supplementary Tables 3 and 4). Individuals from LON and IMB were also excavated by Manfred Eggert in the 1980s (Supplementary Note 2). The individual from MTNW was excavated by Julio Mercader and colleagues in the late 1990s (Supplementary Note 2). Additional faunal remains from 2016 excavations at BLD were selected for study based on species identifications (Supplementary Tables 3 and 4) and degree of preservation. Human and faunal skeletal material remaining from the stable isotope analyses, from individuals excavated at BLD, LON, and IMB, are currently housed at the Stable Isotope laboratory, Department of Archaeology, Max Planck Institute for the Science of Human History, Jena, Germany. Charred food remains from BLD are currently stored at the Institute for Archaeological Sciences, Goethe University, Frankfurt am Main, Germany. The skeletal remains of the individual from MTNW are housed at the Department of Biological Sciences, Complutense University of Madrid, Madrid, Spain. Dental calculus from the same individual is stored at the Department of Anthropology and Archaeology, University of Calgary, Canada. All data supporting the findings of this study are available in existing publications or upon request from the corresponding authors.

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Author contributions

M.B. and P.R. designed the study. H.-P.W., B.E., J.M., and V.L. provided materials and provenancing information. M.B., J.Z., S.M., B.F., and P.R. performed isotope analysis of human and faunal remains. A.S. performed isotopic analysis of charred food remains. J.M., M.S., J.I., and S.C. facilitated and conducted dental calculus analysis. M.B., P.R., and H.-P.W. wrote the manuscript with input from all authors.

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Competing interests

The authors declare no competing interests.

Additional information

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Supplementary Information for:

Isotopic and Microbotanical Insights into Iron Age agricultural reliance in the Central African rainforest

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Supplementary Note 1: Food Production during the Central African Iron Age

For decades, the palaeoenvironmental context for the spread of farming and Bantu-speaking groups across sub-Saharan Africa has been an important topic of debate [1–4]. The spread of agriculture across Africa has been dominated by theories surrounding the "Bantu Expansion" and the palaeoenviromental conditions that coincided, and possibly facilitated, the migrations of these Bantu-speaking populations. Two significant palaeoenvironmental events have been argued to have fundamentally altered the vegetation and local ecosystems of the Central African rainforest during the Holocene. The first, around 4,000 cal. BP saw the contraction of rainforest along its northern and southern periphery, including the coastal regions of southern Cameroon and Gabon [5,6]. The second phase, at *c.* 2,500 cal. BP, is characterised by the fragmentation and deforestation of the core evergreen rainforest zones (the Late Holocene rainforest crisis or LHRC), resulting in the rapid expansion of pioneer vegetation and creation of more open environments [7]. Although these changes have been long discussed, they remain relatively poorly characterised and understood. Indeed, more recent palaeoenvironmental studies of lake basin records and marine cores have argued that, as this latter deforestation event was contemporaneous with the arrival of Bantu speaking groups, it could represent significant human alteration of the landscape, rather than natural forest retreat [8–10], with major implications for how we view the impact of early farmers in Central Africa. There have been, however, significant counter-arguments against a strong human impact on the forests during this phase, in particular, a discrepancy between timing of clear settlements and large-scale vegetation changes [11,12].

It has been proposed that the opening up of a 'Savannah Corridor' *c.* 2,500 cal. BP facilitated the movement of Bantu speaking farmers, from their hypothesised homeland at the present-day Nigeria-Cameroon border area, eastwards and southwards across the rest of the African continent [3,10,13]. Since the opening up of the landscape has commonly been considered to have supported the introduction and cultivation of new crops, the equatorial forest of Central Africa has been viewed as a barrier for agricultural expansion. While agricultural practices became increasingly diverse and intensified in western Africa during the Iron Age, in the core forested regions of Central Africa small-scale food production, hunting, gathering, and fishing have been argued to have persisted [14,15]. Linguistic models suggest the migration of Bantu speaking groups into the equatorial rainforest was delayed by as much as 300 years in comparison to rapid dispersals into savannah-type environments [3]. Therefore the discovery of domesticated pearl

millet (*Pennisetum glaucum),* a crop of Sahelian origin [16,17], at Early Iron Age sites in Cameroon [18] stimulated notions that its presence was indicative of more open environments and seasonal rainfall [1,2]. There have, however, since been calls to re-examine the hypothesised environmental context for the early cultivation of pearl millet at sites in Central Africa [20].

Modern experiments have shown it can be successfully grown in the Inner Congo basin even during periods of high annual rainfall [20], challenging previously proposed environmental constraints for millet cultivation that form the basis of many broad models of farming expansion into Central Africa. The presence of pearl millet at Early Iron Age sites in Central Africa not only raises questions about the nature of the environmental setting in which it was cultivated but also about how communities were utilising this new crop. The paucity of domestic C_4 cereal finds at African rainforest sites makes it challenging to explore this archaeologically however, and the importance of forest C_3 crops, including yams and fruit trees such as *Canarium*, and of oil palm (*Elaeis guineeensis*) exploitation, should not be overlooked [1,21–23]. Charred endocarps of *Canarium schweinfurthii* recovered from rockshelters in the Ituri rainforest attest to consumption of wild fruits throughout the Holocene [24]. Oil palm endocarp remains in particular, are abundant at rainforest sites [25], and likely provided a major source of fat. Tubers such as wild and domesticated yams are still difficult to trace in archaeobotanical records though ethnographic evidence shows the importance and efficiency of wild yams exploitation for human nutrition in the Central African rainforest (e.g. [26,27]), including species which also occur in the Inner Congo Basin. The potential role of domesticated yams during the Iron Age so far remains unresolved. Furthermore, archaeobotanical remains only show detailed snapshots of specific plants, in contrast, the isotope analysis of human and faunal tissue can provide an assessment of overall dietary reliance.

Supplementary Note 2: Sites of study, chronology and new AMS dates

All human bone samples dated in this study were sent to the Scottish Universities Environmental Research Centre AMS Laboratory, Glasgow (SUERC, Lab ID: GU). Radiocarbon ages were calibrated to calendar timescale using OxCal 4 [28] and IntCal13 atmospheric calibration curve [29].

Imbonga

Imbonga (IMB) is a waterside village located on the Momboyo River. It is the type-site for the earliest pottery tradition of the central equatorial rainforest. Several excavations were carried out here in the 1980s by Manfred Eggert [30–32], and a number of radiocarbon dates were published (Supplementary Table 1). For the present study, enamel was sampled from a human second molar discovered in sediment contained in a ceramic vessel at IMB 81/11 (Supplementary Table 1). It is a second molar of an individual between 9–12 years of age. No other skeletal remains were found in this context. The vessel, a richly decorated flat-based bowl attributable to the Early Iron Age Inganda style, had been found isolated but in spatial proximity to a number of Early Iron Age pottery deposits. Although possibly not *in situ* at discovery, the vessel was found upside down, i.e. in a position typical of Early Iron Age ritual pottery deposits known from the Inner Congo Basin. Two holes intentionally knocked into opposite sides of the vessel wall likewise suggest ceremonial burial. IMB 81/11 has not been directly dated. However, Inganda pottery is known to have been produced in the 2nd and 1st centuries cal. BC.

Assuming contextual integrity of the find, the tooth sample may therefore be regarded as by far the oldest evidence included in this study representing one of the earliest periods of the regional Early Iron Age, 200 years more recent, at the most, than regional Iron Age beginnings associated with Imbonga ceramics. Imbonga pottery has been found at some 60 sites across the western parts of the Inner Congo Basin. It is not associated with any stone artefacts [32] but with a sedentary way of life, an advanced iron metallurgy, and a food production system. Reconstructing the subsistence practices of these early pottery-making communities has been extremely challenging due to poor conditions of preservation and limited datasets. Charred pearl millet remains have been found in Imbonga period and slightly more recent Early Iron Age contexts at the sites of Iyonda on the Congo River and Boso-Njafo on the Lulonga River, dating between *c*. 2330–1960 BP [33]. Other archaeobotanically documented contemporaneous food

plants include cowpea and, possibly, tubers. It has also been hypothesised that yams and plantain were likely important staples during this time. However, the respective contributions of these plants to Early Iron Age human diets remain largely unknown.

Longa

Longa (LON) is situated on the Ruki River. Archaeological survey and excavations were carried out by Manfred Eggert in the 1970s and 1980s [30,34], and three radiocarbon dates were published (Supplementary Table 1). In the context of the present study, a bone fragment from one human individual (LON 81/2) was directly dated to between 1642 and >1938 cal AD (95% probability) (Supplementary Figure 2; Supplementary Table 2).

Bolondo

Bolondo (BLD) is situated on the floodplain of the Tshuapa River, within the zone of seasonal inundation. Today, it is a small, year-round fishing camp inhabited by a few families who live in lightweight houses erected on artificial clay and refuse mounds. According to ethnography (e.g. [35]) such camps (locally called *nganda*) used to be seasonally inhabited special-function sites, each associated with a particular nearby dryland village where only a tiny fraction of the total population would be present at the pertaining *nganda* during the low-water fishing season. However, due to recent overpopulation and food shortage at urban centres, that has created new demands and sales chances for smoked fish, a great many *nganda* have shifted from seasonal subsistence fishing to perennial inhabitation and increasingly market-oriented fishing. In any case, freshwater fish has remained a conspicuously dominant foodstuff for *nganda* residents to the present day.

Excavations at the site first took place in 1983 as part of Manfred Eggert's former River Reconnaissance Project, with further excavations, pollen coring, and new radiocarbon dating in 2016 financed by the *Deutsche Forschungsgemeinschaft* [32]. Excavations have revealed a neatly stratified sequence from *c*. cal. years AD 1330 to today (Supplementary Table 1) featuring house remains, stake holes, midden deposits, and a number of human inhumation burials as well as botanical and faunal remains. Animal bones include mainly freshwater fish [36] but also crocodile, antelope, domestic dog and goat [37]. Most significant among the archaeobotanical finds are charred remains of Pearl millet (*Pennisetum glaucum*) and banana phytoliths.

The human and faunal remains in this study came from excavations in 1983 (BLD 83/*) and 2016 (BLD 16/*). Due to the waterlogged conditions, there was good overall organic

preservation (Supplementary Figure 2). For stable isotope analysis, bones and tooth enamel were sampled from 11 human individuals. While it was impossible to obtain both a bone and enamel sample from every individual there is direct crossover between the two sets of tissues for five individuals.

For five individuals buried at Bolondo it was possible to obtain new direct AMS dates from bone collagen (Supplementary Table 2). While calibration plateaus impede precise calendar dating in all instances but one (SUERC-89290, BLD 83/5), it appears this individual in addition to 83/1 individual 2 and BLD 83/4 are from an earlier period and burials 83/1 and 83/8 are from a later period (Supplementary Figures 3-7). Considered together, the entire suite of dates may be regarded as representing a time span between the second half of the 15th and the early 19th century cal AD. Therefore, representing pre-colonial funerary evidence well before imperial regulations widely inhibited human interment at fishing camps.

None of the Bolondo burials were associated with ceramic finds interpretable as grave goods. However, all of them were found in stratigraphic contexts representing intermediate and recent layers of the site stratigraphy. These are characterised by settlement refuse including large quantities of pottery fragments belonging to the following style groups of the regional Later Iron Age Tshuapa Tradition (in stratigraphic sequence from earliest to most recent): Bolondo, Bokone, Bolombi, and Ilemba-Bokonda [32].

Matangai Turu Northwest

Matangai Turu Northwest (MTNW) is a granite rock shelter with a floor area of 47 m^2 located in the Ituri region, under a mixed, lowland rainforest. Mercader and colleagues [38] retrieved a human skeleton from level five, in a sandy clay loam with slightly acidic pH, 5% water content, and directly dating to 813 ± 35 ^{14}C BP (1218-1277 AD, 1 sigma: UtC-5074). This same level yielded charcoal fragments and endocarps from Guineo-Congolian trees such as *Canarium schweinfurthii* (Burseraceae) and the African oil palm (*Elaeis guineensis*, Arecaceae) [39]. From a faunal perspective, all taxa contemporaneous with the burial are forest taxa ranging from snails (Achatina, Limicolaria) to bovids, primates, rodents, and carnivores. Unfortunately, none of this fauna could be located, and thus sampled, for this study. No domesticated fauna was found, and the overall palaeoenvironment at the time of occupation, as established by phytolith analysis, is a closed-canopy forest [40].

Dental pathologies include dental calculus and enamel hypoplasias but no caries. Previously published results report intentional incisor mutilation [38], a practice known among local agriculturalists. The stature of the buried individual averaged 156-159 cm not allowing us to conclude if this person was Mbuti-related. Contextually, the human remains associate with LSA lithics [41], Late Iron Age ceramics stylistically connected to those from the Western branch of the East African Rift System [42], and one isolated iron bar, without evidence of smelting or forging [43].

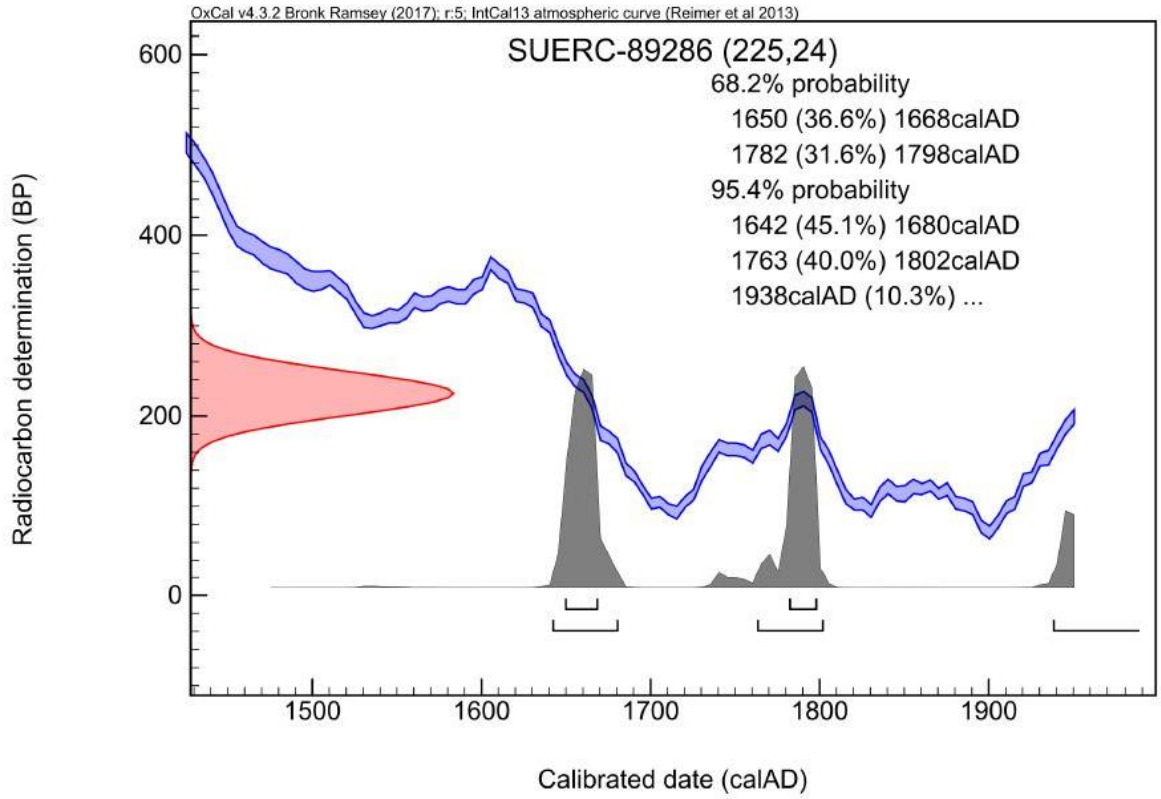
Supplementary Note 3: Reconstructing subsistence practices in tropical environments using stable isotopes

The stable light isotope analysis of human and animal tissues and food residues are widely applied methodologies for palaeodietary reconstruction [44–46]. $\delta^{13}C$ and $\delta^{15}N$ analysis of bulk bone collagen is one of the most commonly applied approaches, and enables exploration of the degree to which humans were reliant on plants following the two main photosynthetic pathways (C_3 and C_4), and their animal consumers, as well as their position within the foodchain. The archaeological sites of this study currently represent tropical forest environments largely dominated by C_3 forest species, as well as oil palm (*Elaeis guineensis)*. Phytolith evidence for MTNW has confirmed a similar environmental context (closed canopy forest) was present at the time of occupation [40]. These C_3 plants that dominate tropical forest environments, as well as domesticated oil palm and yams, have a $\delta^{13}C$ between -35‰ and -19‰ making them distinguishable from C_4 plants, which include wild grasses, millets and sorghum, which have a $\delta^{13}C$ between -13‰ to -8‰ [47–50]. C_3 plants growing under a closed canopy, as well as their consumers, have even lower $\delta^{13}C$ due to low light and recycled the CO_2 the well-documented so-called 'canopy effect' [51,52]. The distinct $\delta^{13}C$ of these groups of plants is passed into the tissues of their consumers with a known fractionation effect [54]. $\delta^{15}N$ of bone collagen provides information relating to the trophic level of consumers, being elevated *c.* 3-5‰ with each step in a foodchain [54]. Longer food chain lengths in aquatic systems mean that aquatic resources tend to also have higher $\delta^{15}N$ values, enabling their distinction from terrestrial resources – something that can be difficult on the basis of $\delta^{13}C$ alone [55,56].

The $\delta^{13}C$ of bone collagen largely reflects protein intake averaged over a number of years and consequently plant consumption can be under-represented [53,57,58]. In contrast, $\delta^{13}C$ of the hydroxyapatite of tooth enamel is more reflective of the whole diet of an individual (proteins, fats, and carbohydrates) and can provide dietary information at the time of tooth development that will vary depending on the tooth sampled [59]. For example, while femur bone collagen $\delta^{13}C$ represents approximately the last 10 years of protein intake in the diet [58], the tooth enamel $\delta^{13}C$ of human 3rd molars is influenced by the whole diet intake between 9-16 years of age [60]. Tooth enamel also offers the opportunity to measure $\delta^{18}O$ that reflects water and food sources, and can provide insight into the broader environment experienced by an individual including evaporative potential and, indirectly, insights into canopy density [61,62]. The primary influence on tooth enamel $\delta^{18}O$ will depend on whether the animal studied is an obligate drinker (gets the

majority of its water from open water sources) or a non-obligate drinker (gets a major portion of water from consumed foods) [63]. Tooth enamel has been the material of choice for palaeodietary studies in the tropics due to its high levels of preservation, and the fact that dietary contributions of low protein plant resources are more visible [64–66].

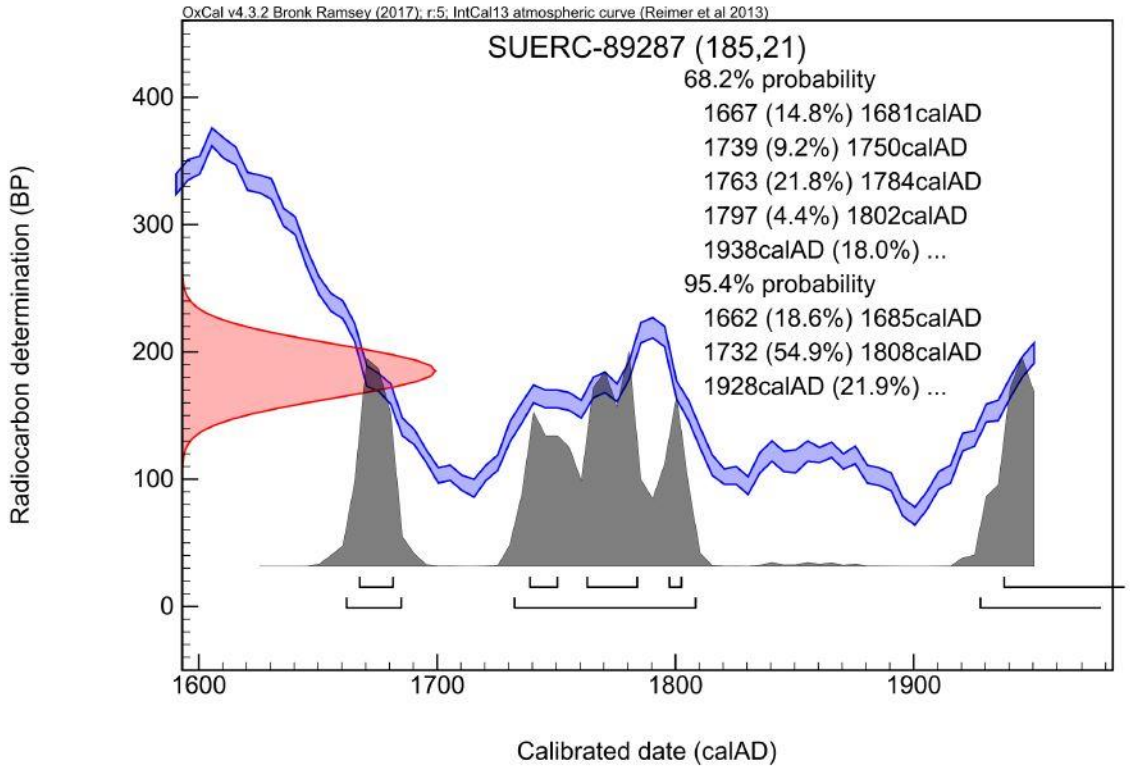
The stable isotopic analysis of human tissues can provide insights into the overall reliance of an individual on different food groups, while the analysis of food or charred foodstuffs is more likely to provide information about subsistence practices at a community or site-level. Integrated isotopic approaches using compound-specific carbon isotopes and bulk stable isotopes have detected C_4 crops, such as millet and maize, in archaeological ceramics [67–69]. However, the samples in this study were isolated finds of charred food fragments recovered through flotation and have no direct association with ceramics. In recent years, there has been growing recognition that such charred foodstuffs have great potential for providing insights into diet, food processing and cooking, with foodstuffs such as bread, doughs or porridge being identified in the archaeological record [70,71]. The δ^{13} results in this study suggest that two of the food fragments with visible millet remains indeed originate from pearl millet pointing to cereal-based food, whereas the others with a smoother vesicular porridge-type texture yielded C_3 signals pointing to non-cereal food.



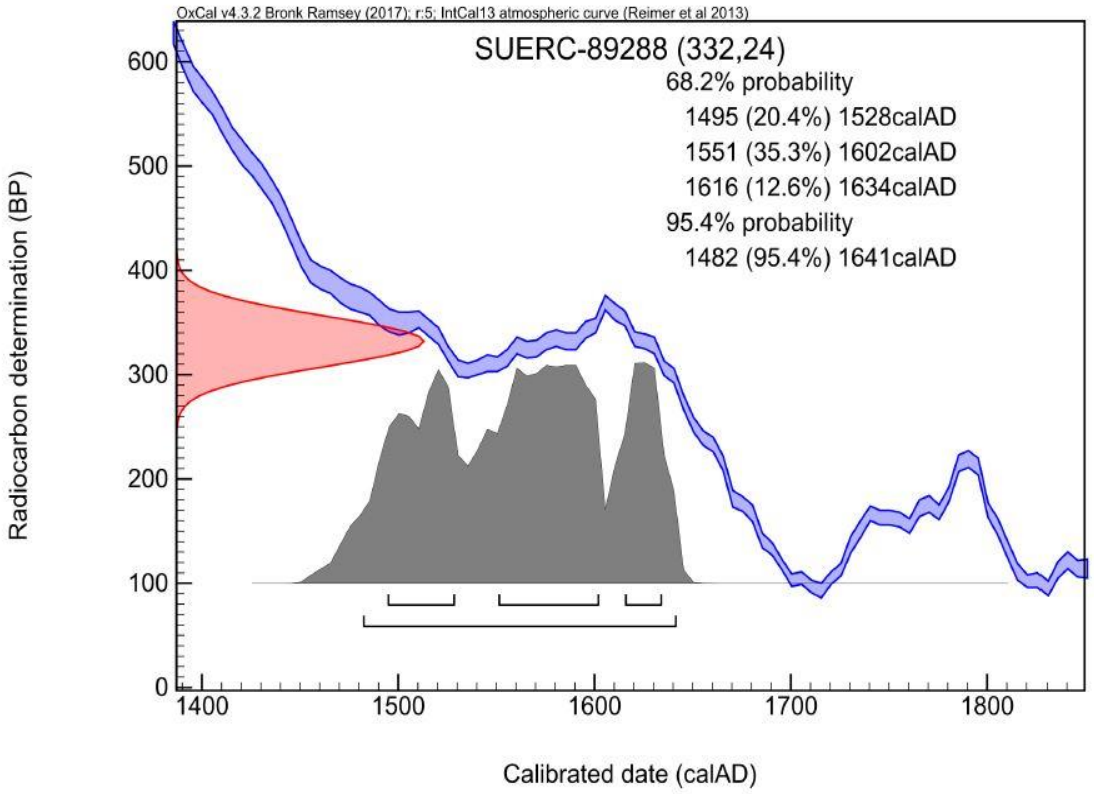
Supplementary Figure 1: Calibration of radiocarbon measurement for sample LON81/2 (SUERC-89286/GU53256)

Supplementary Figure 2: Human bone samples from Longa (LON) and Bolondo (BLD) showing the degree of organic preservation

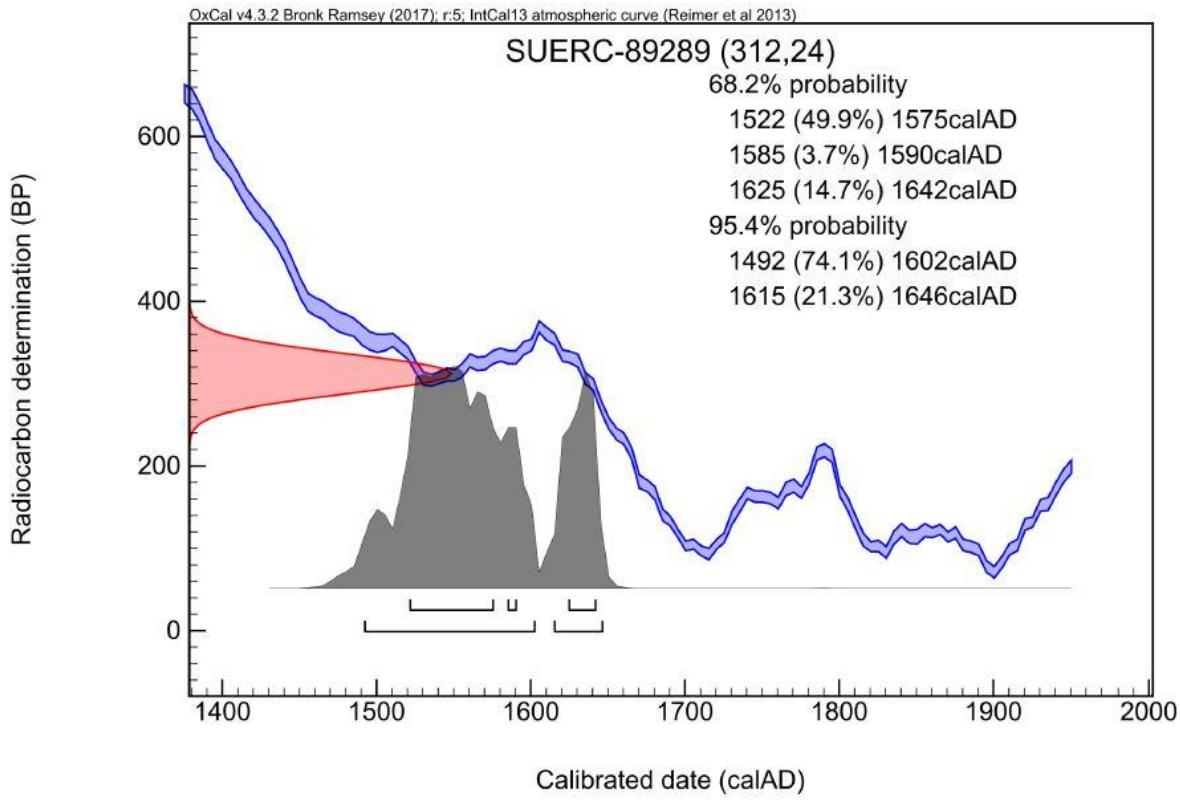
Left: Part of the postcranial skeletal remains from LON 81/2. Right: The bulk of the human bones from BLD 83/4.



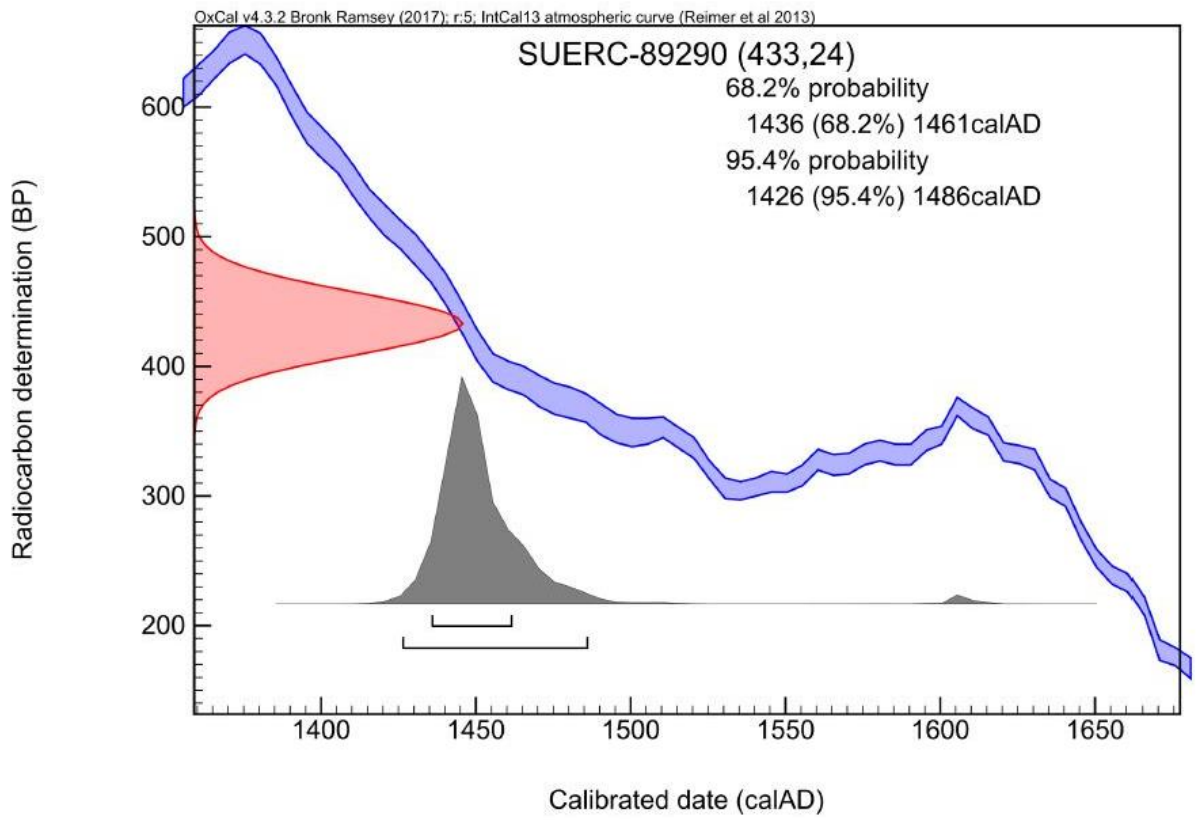
Supplementary Figure 3: Calibration curve for sample BLD 83/1 Individual 1 (SUERC-89287/GU53257)



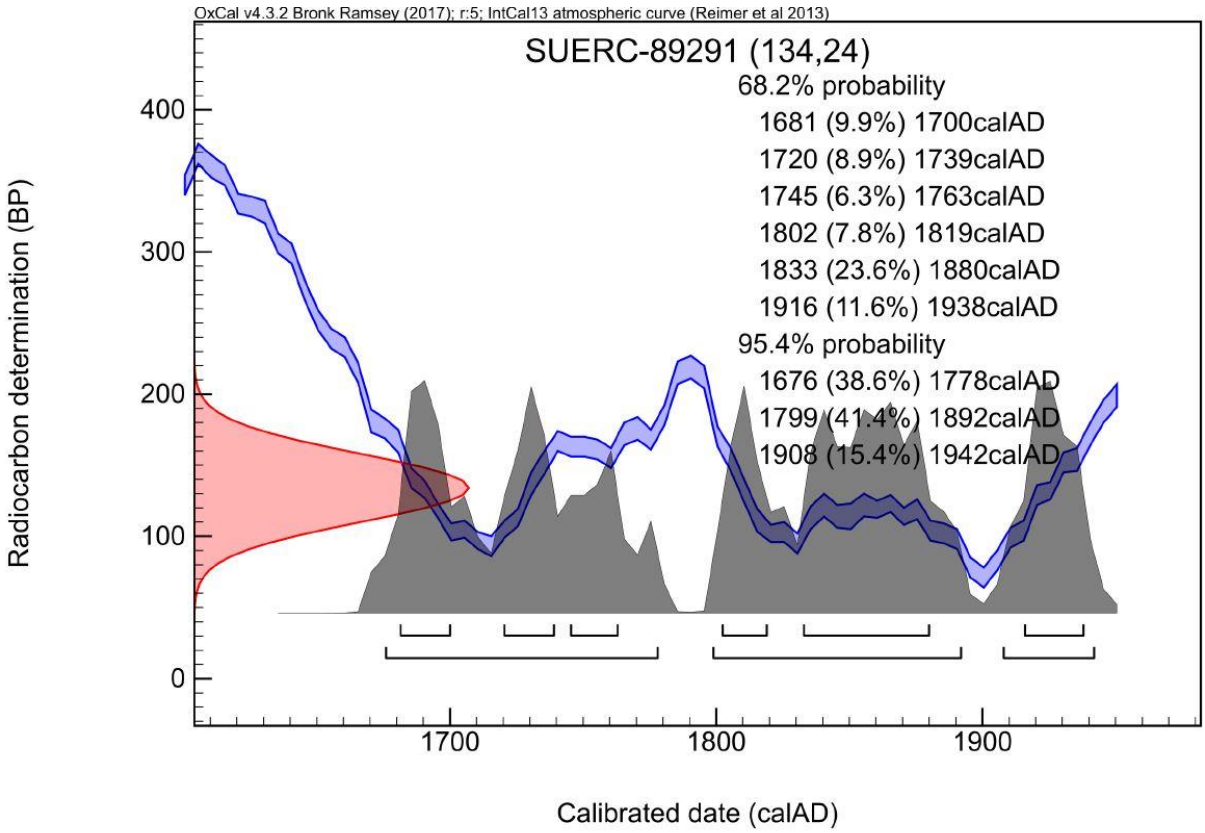
Supplementary Figure 4: Calibration curve for sample BLD 83/1 Individual 2 (SUERC-89288/GU53258)



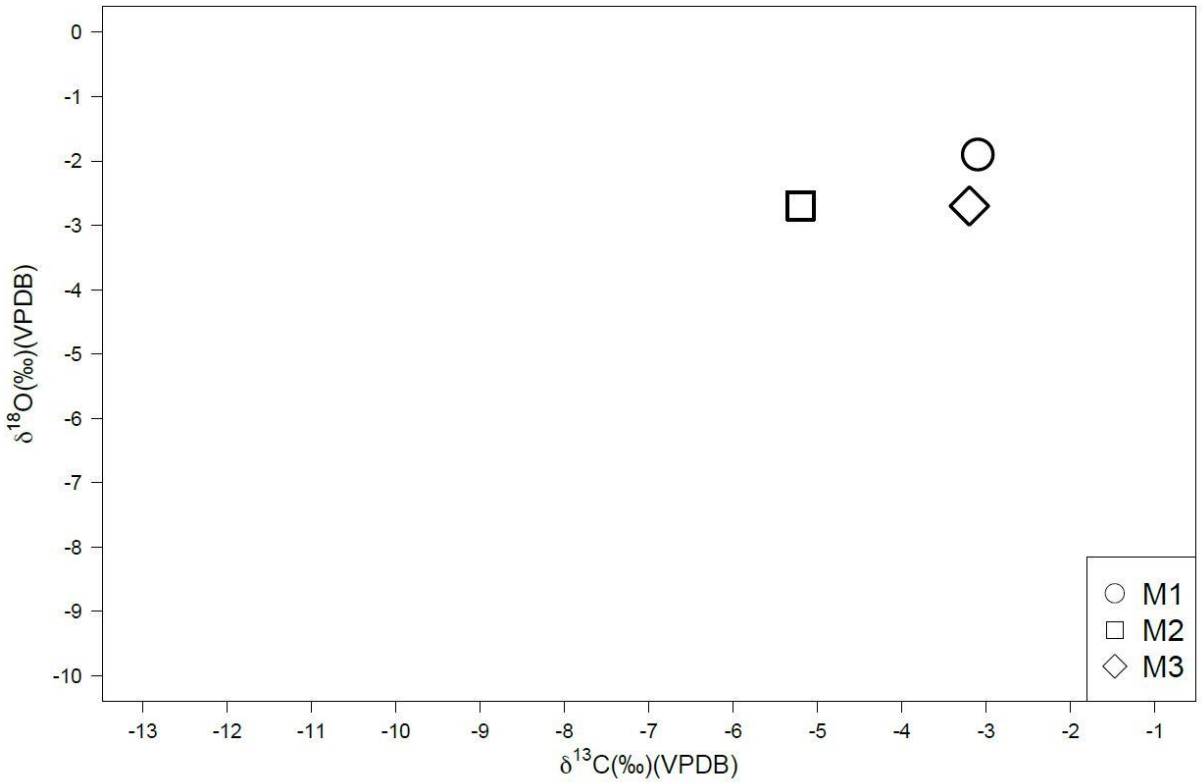
Supplementary Figure 5: Calibration curve for sample BLD 83/4 (SUERC-89289/GU53259)



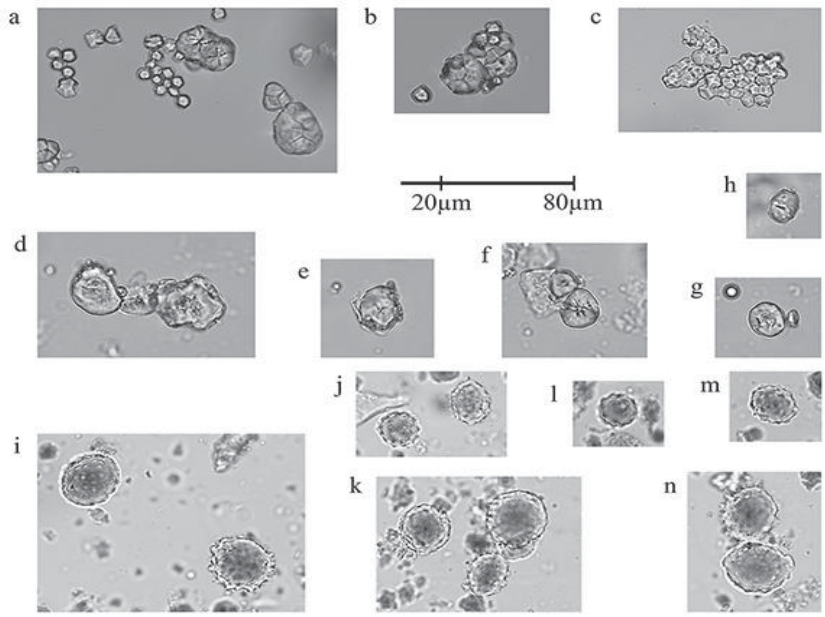
Supplementary Figure 6: Calibration curve for sample BLD 83/5 (SUERC-89290/GU53260)



Supplementary Figure 7: Calibration curve for sample BLD 83/8 (SUERC-89291/GU53261)



Supplementary Figure 8: Tooth enamel results (M1-M3) for individual from MTNW



Supplementary Figure 9: Reference collection

Cereal starch: a) *Eleusine coracana*, b) *Eleusine africana*, c) *Pennisetum glaucum*, d-h) *Sorghum bicolor.* Phytoliths*:* i-n) *Elaeis guineensis* (nutshell).

Supplementary Table 1: Summary of previously published dates from IMB, LON and BLD.

(GrN: Groningen, Laboratory for General Physics. Hv: Hannover, Lower Saxony State Office for Soil Research. KI: Kiel, C14-Laboratory of the Institute for Pure and Applied Nuclear Physics. KN: Universität Köln, Labor für C14-Datierung, Institut für Ur- und Frühgeschichte. – Note that Hannover (Hv) results are considered unreliable [32,72].

Site	Context	Material	Radiocarbon date	Calibrated date (95%)	Lab no.
Imbonga	IMB 81/3	Charcoal	2900 ± 285 BP	1450–800 cal BC	Hv-11576
	IMB 83/1	Charcoal	2665 ± 110 BP	920–790 cal BC	Hv-12614
	IMB 81/9/1	Charcoal	2160 ± 90 BP	375–100 cal BC	KI-2428
	IMB 81/9/1	Charcoal	3775 ± 105 BP	2453–2039 cal BC	Hv-11574
	IMB 81/1	Charcoal	2860 ± 280 BP	1420–800 cal BC	Hv-12207
	IMB 81/1	Charcoal	2130 ± 125 BP	380–10 cal BC	Hv-11575
Longa	LON 81/1	Charcoal	730 ± 75 BP	1236–1289 cal AD	Hv-11571
	LON 81/1	Charcoal	500 ± 90 BP	1322–1454 cal AD	GrN-13586
	LON 81/1	Charcoal	260 ± 120 BP	1470–1955 cal AD	KN-4205
Bolondo	BLD 83/1	Charcoal	1725 ± 95 BP	213–416 cal AD	Hv-12624
	BLD 83/2	Charcoal	1195 ± 70 BP	720–896 cal AD	Hv-12619
	BLD 83/2	Charcoal	1175 ± 210 BP	650–1030 cal AD	Hv-12618
	BLD 83/1	Charcoal	915 ± 105 BP	1010–1230 cal AD	Hv-12625
	BLD 83/1	Charcoal	660 ± 80 BP	1270–1395 cal AD	GrN-13078
	BLD 83/2	Charcoal	230 ± 110 BP	1514–1955 cal AD	KN-4203

Supplementary Table 2: New radiocarbon dates for human burials from Longa (LON) and Bolondo (BLD)

Dates provided by the Scottish Universities Environmental Research Centre (SUERC), University of Glasgow. Calibrated date ranges were obtained by means of the Oxford Radiocarbon Accelerator Unit calibration program OxCal v4.3.2 [28] using the IntCal13 atmospheric calibration curve [29]

Context	Material	Radiocarbon date	Calibrated date (95%)	Lab no.
LON 81/2	Bone	225 ± 24 BP	1642–after 1938 cal AD	SUERC-89286 (GU53256)
BLD 83/1, Indv. 1	Bone	185 ± 21 BP	1662–after 1928 cal AD	SUERC-89287 (GU53257)
BLD 83/1, Indv. 2	Bone	332 ± 24 BP	1482–1641 cal AD	SUERC-89288 (GU53258)
BLD 83/4	Bone	312 ± 24 BP	1492–1646 cal AD	SUERC-89289 (GU53259)
BLD 83/5	Bone	433 ± 24 BP	1426–1486 cal AD	SUERC-89290 (GU53260)
BLD 83/8	Bone	134 ± 24 BP	1676–1942 cal AD	SUERC-89291 (GU53261)

Supplementary Table 3: $\delta^{13}C$ and $\delta^{15}N$ values for human and faunal bone collagen from BLD and LON.

*indicates samples that were not carried forward for interpretation as %N was <5% and %C was <15% indicating poor sample quality.

Sample ID	Site	Calibrated Dates (95% confidence)	Species	Element	$\delta^{13}C$‰ VPDB	%C	$\delta^{15}N$‰ AIR	%N	C/ N
BLD 16/5-46 HV	BLD		Bichir (*Polypterus* sp.)	Vertebra	-24.7	41.5	12.7	13.4	3.6
BLD 16/5-53	BLD		*Crocodylidae*	Tibia	-22.6	38.5	11.1	13.8	3.3
BLD 16/5-55	BLD		*Crocodylidae*	Femur	-23.3	36.6	11.4	13.0	3.3
BLD 16/5-71	BLD		Catfish (*Clarias* sp.)	Pectoral spine	-23.0	39.8	9.4	13.5	3.5
BLD 16/5-53	BLD		*Canis familiaris*	Mandible	-19.9	41.5	12.3	14.9	3.3
BLD 16/5-13	BLD		*Capra hircus*	Scapula	-19.7	38.8	9.9	13.9	3.3
BLD 83/2-8	BLD		*Sylvicapra grimmia*	Metacarpus	-23.7	36.5	5.9	12.4	3.4
BLD 83/2-6+7	BLD		Small antelope	Tibia	-22.5	28.9	7.3	9.8	3.4
BLD 83/2-10	BLD		Fox-sized carnivore	Humerus	-20.3	24.3	13.7	8.0	3.5
BLD 83/2-10	BLD		*Capra hircus*	Mandible	-18.2	43.6	9.8	15.8	3.2
LON 81/2	LON	1642–1938 cal AD	*Homo sapiens*	Rib	-21.8	24.2	15.1	8.7	3.2
BLD 16/5-35	BLD		*Homo sapiens*	Rib	-16.7	33.7	13.6	12.0	3.3
BLD 16/5-31	BLD		*Homo sapiens*	Metatarsal	-16.3	39.6	13.4	14.2	3.2
BLD 83/4	BLD	1492–1646 cal AD	*Homo sapiens*	Scapula	-17.5	28.5	13.6	10.4	3.2
BLD 83/3: Individual 2	BLD		*Homo sapiens*	Bone fragment	-21.0	42.4	16.9	15.6	3.2
BLD 83/6: Individual 2	BLD		*Homo sapiens*	Metacarpal	-18.8	29.5	14.3	10.5	3.2
BLD 83/1: Individual 2	BLD	1482–1641 cal AD	*Homo sapiens*	Rib	-16.5	35.7	14.7	13.4	3.1
BLD 83/1: Individual 1	BLD	1662–1928 cal AD	*Homo sapiens*	Rib	-17.4	40.5	15.7	15.1	3.1
BLD 83/5	BLD	1426–1486 cal AD	*Homo sapiens*	Femur	-18.8	25.0	14.2	9.2	3.2
BLD 83/8	BLD	1676–1942 cal AD	*Homo sapiens*	Long bone	-20.2	26.5	14.2	9.7	3.2
BLD 83/10	BLD		*Homo sapiens*	Rib	-16.7	29.0	13.5	10.4	3.2
BLD 83/6/I	BLD		*Homo sapiens*	Bone fragment	-18.6	32.2	15.7	11.5	3.3
*BLD 83/6 Individual 1	BLD		*Homo sapiens*	Rib	-19.6	10.4	12.9	3.5	3.5
*BLD 83/7	BLD		*Homo sapiens*	Rib	-17.1	10.2	14.0	3.4	3.3

Supplementary Table 4: $\delta^{13}C$ and $\delta^{18}O$ values for human and faunal tooth enamel from BLD, IMB, LON and MTNW

Sample ID	Site	Species	Tooth	$\delta^{13}C$‰ VPDB	$\delta^{18}O$‰ VPDB
IMB 81/11	Imbonga	*Homo sapiens*	M2	-14.1	-3.5
LON 81/2	Longa	*Homo sapiens*	M3	-15.1	-2.8
MTNW	Matangai Turu Northwest	*Homo sapiens*	M3	-3.2	-2.8
BLD 83/7, Individual 2	Bolondo	*Homo sapiens*	M3	-12.0	-2.6
BLD 83/7, Individual 1	Bolondo	*Homo sapiens*	M3	-11.2	-2.4
BLD 83/4-2	Bolondo	*Homo sapiens*	M2	-14.7	-2.8
BLD 83/3, Individual 2	Bolondo	*Homo sapiens*	M3	-11.7	-2.9
BLD 83/3, Individual 1	Bolondo	*Homo sapiens*	M3	-11.6	-2.6
BLD 83/6, Individual 1	Bolondo	*Homo sapiens*	M2	-12.0	-2.3
BLD 83/6, Individual 6	Bolondo	*Homo sapiens*	M2	-11.2	-4.9
BLD 83/1, Individual 1	Bolondo	*Homo sapiens*	M3	-13.6	-3.2
BLD 83/8	Bolondo	*Homo sapiens*	M2	-11.4	-3.6
BLD 83/10	Bolondo	*Homo sapiens*	M3	-10.8	-4.1
BLD 16/1-13	Bolondo	*Homo sapiens*	M3	-11.5	-3.5
BLD 16/5-53, Bot ID 394	Bolondo	*Canis familiaris*	Molar	-13.6	-2.7
BLD 83/2-10	Bolondo	Fox-sized carnivore	Molar	-10.7	-2.3
BLD 83/2-10, No. 7	Bolondo	*Capra hircus*	Molar	-9.6	-1.0
BLD 16/5-71, Bot ID 412	Bolondo	Lungfish *(Protopterus* sp.*)*	Upper jaw	-13.7	-4.3
BLD 16/5-46, HV Bot ID 383	Bolondo	Catfish *(Clarias* sp.*)*	Upper jaw	-11.1	-7.4
BLD 16/5-46, HV Bot ID 383	Bolondo	Catfish *(Clarias* sp.*)*	Upper jaw	-11.5	-6.3

Supplementary Table 5: Summary table of number of starch granules and phytoliths observed from dental calculus samples from M1-M3 of the individual from MTNW.

		M1	M2	M3	Total
Starch	Parabolic	0	8	1	9
	Ovate	0	1	0	1
	Oblong elongate	1	0	1	2
	Polygonal	3	4	1	8
	Orbicular	6	7	2	15
	Quadratic	0	3	0	3
Phytolith	Globular tuberculate/echinate	9	0	0	9

Supplementary Table 6: $\delta^{13}C$ and $\delta^{15}N$ values of charred food remains from BLD

*N_2 peak was too small for reliable determination of $\delta^{15}N$ values

Sample ID	Site	Archaeological ID	Botanical ID	$\delta^{13}C$‰ VPDB	%C	$\delta^{15}N$‰ AIR	%N	C/N
BE01	Bolondo	BLD 16/1-41	377	-27.9	51.2	*4.7**	0.9	69.9
BE02	Bolondo	BLD 16/1-38	371	-27.7	68.4	*2.6**	0.6	138.6
BE03	Bolondo	BLD 16/1-39	373	-28.7	51.6	*6.3**	0.6	108.1
BE04	Bolondo	BLD 16/1-36	367	-28.7	51.6	*4.2**	0.6	94.2
BE05	Bolondo	BLD 16/1-37	369	-28.4	38.4	*3.9**	0.6	75.4
BE06	Bolondo	BLD 16/5-61	402	-9.3	42.0	7.8	2.5	19.7
BE07	Bolondo	BLD 16/5-59	400	-9.3	42.0	*7.1**	2.5	20.0
BE08	Bolondo	BLD 16/5-59	400 (2)	-24.1	31.2	*8.4**	1.5	24.8

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10. Discussion

10.1 Pastoralism and dairying in eastern Africa

In Manuscript A, we critically examined the existing evidence for human use of animal milk and milk products in ancient Africa. We identified some key challenges in exploring foodways during the Holocene including unequal archaeological investigation across the continent and insufficient engagement between different disciplines. Despite these challenges, we were able to find some common themes. Firstly, dairying emerged at different rates across the African continent reflecting flexible pastoral lifeways which incorporated sheep/goat and cattle herding to varying degrees. Multidisciplinary methods, including proteomics and stable isotopes analysis, can help capture this diversity which was crucial for many communities to survive. We emphasize that environmental conditions constrained and supported the development of specialized dairy-centred pastoralism, for example the bimodal rainfall pattern that emerged in Kenya supported two birthing seasons a year leading to increased milk availability [7,54]. While efforts have been made to compare pastoral lifeways and paleoenvironmental records [130,131] we believe generating new, dated evidence for milk consumption is an important first step for testing theories of climatic and zoonotic stressors and developing regional chronologies for dairying in eastern Africa.

To further explore our hypothesis that milk was a critical food source in arid environments we analysed dental calculus from pastoral communities in Sudan and Kenya (Manuscript B). Through the identification of milk proteins in calculus we demonstrate that communities were consuming milk 6,000 years ago. We also identify the first direct evidence of goat's milk consumption from the Kerma Period confirming that small-stock, as well as cattle, were important dairy animals. Goats are less efficient milk producers than cattle, but are better suited to arid environments and remain key dairy animals in Sudan today. In 2018, Sudan was the second largest producer of goat's milk in the world, producing around 1.2 million tons [132]. We also show that herders were consuming milk as they first moved into southern Kenya supporting our hypothesis that milk was an important food source and may have acted as a buffer against environmental stressors. Additionally, we show that one individual from Kenya dating to c. 1350 cal. BP was consuming milk despite not having any known lactase persistence alleles. This supports global trends, based on

ancient genetic and archaeological studies, that milk was being consumed before the emergence of lactase persistence.

10.1.1 Methodological potentials and challenges

10.1.1.1 Identifying milk consumption using palaeoproteomic methods

Prior to the application of proteomic methods to ancient dental calculus from Africa (Manuscript B) evidence for milk consumption has primarily derived from dairy lipids recovered from ceramics. While residue analysis provides insights into ancient cuisine and food processing and lipids are often well preserved even under challenging conditions [48,50,133,134], there are some important limitations to consider. Firstly, the presence of dairy fats on ceramics cannot necessarily be used to directly assess the contribution of milk to human diets. Although an increase in the number of vessels used for storing or processing milk through time at a particular site could support the intensification of dairying, the evidence itself is derived from material culture and therefore is only a proxy for consumption. Secondly, the storage of milk in organic containers which is common in the ethnographic literature [37,135] means that evidence from ceramics may under-represent dietary reliance on milk [136]. Furthermore, the mixing of dairy with plant lipids could result in signals consistent with ruminant fats [98] and so local faunal and human isotopic baselines are crucial for forming accurate interpretations. The results of this thesis show how proteomics can provide direct evidence of milk consumption at an individual-level and, in some cases, species-specific evidence about the animals used for dairying.

As with lipid residue analyses, studies of dairying using ancient dental calculus also have inherent limitations in regards to establishing dietary reliance on dairy products and these must be taken into account when formulating hypotheses and research frameworks. The absence of milk proteins does not necessarily mean dairying was not an important component of an individual's diet. Furthermore, the incorporation of food-derived proteins into calculus is not uniform, varying within one deposit as well as between those from different teeth of the same individual. It is therefore difficult to establish true dairy-based dietary reliance using proteomics alone. As demonstrated by Manuscript B, it is only through the combination with other methods such as isotopic analysis of human tissues or lipid residues, that milk consumption and dairying can be examined at different scales to more fully assess overall community reliance on dairy products.

While our proteomic data provides direct evidence for milk consumption and in some cases species-specific information, there is an emerging picture that there are biases in the recovery of specific milk proteins [85]. Despite the increasing number of proteomic studies of ancient dental calculus we are only just beginning to understand how dental calculus differs from modern plaque [137], how proteins are incorporated into calculus, and why specific proteins and peptides are preserved. β-lactoglobublin (BLG), the most commonly recovered milk protein in archaeological calculus, is resistant to heat denaturation and enzymatic digestion [138–140] but the mechanisms behind its preservation over thousands of years are yet to be fully established.

The extraction method employed may also introduce bias. There are currently three main methods for protein extraction from ancient dental calculus (GASP, FASP and SP3, see section 4.1) and while there have been studies comparing these methods for modern cellular proteins [141] and modern and fossilised bone [142,143], tests for archaeological calculus are limited [85]. In Manuscript B, we applied two extraction methods to a subset of 10 calculus samples from a cemetery complex in Sudan. Differences were observed in the total number of proteins identified as well as which specific milk peptides were recovered. For example, one individual from Kadruka had beta-caseins (CASB) identified from the sample pretreated using SP3 method but not in the associated sample prepared with FASP. Due to the small sample size we are unable to make any meaningful conclusions at this stage but further testing could identify potential biases relating to laboratory methods.

10.1.1.2 Data analysis and establishing authenticity for low-abundance samples

In conducting the first proteomic study of prehistoric dental calculus from Africa we were able to identify several key methodological challenges. A primary concern was the authenticity of the results as samples were largely derived from extensively handled museum collections. One method of assessing whether proteins are truly “ancient” is estimating the deamidation rates of glutamate and asparagine as a proxy for overall protein degradation [144,145]. However, this method has limited applications to the samples presented in this study. The total number of endogenous proteins is very low meaning that, collectively, there are an insufficient number of deamidation sites for the types of statistical modelling that has been successfully applied to archaeological bone and well-

preserved calculus from British and Mongolian archaeological contexts (see [47,108,145]). While deamidation studies hold great potential these methods cannot be applied universally until further tests are carried out on a range of archaeological substrates (calculus, bone, residues) originating from different temporal and geographical contexts.

To further explore the proteomic profile of ancient calculus samples we created a custom-made Oral Signature Screening Database (Manuscript B). The database consists of core oral bacteria and human immune response proteins based on published results of ancient dental calculus and unpublished datasets generated by the Proteomics Laboratory at the Max Planck Institute for the Science of Human History in Jena. While this tool can never authenticate whether proteins are endogenous or ancient, our aim was to provide an additional screening method that could be used to quickly identify possibly problematic samples. As an open-access, publicly available database we hope it can be continually tested and updated as new calculus datasets become available.

The archaeological human and animal remains studied in this thesis derived from both recent excavations and collections curated for many years in museums. While less common today, historically, animal-derived glues and consolidants have been applied to archaeological human remains in order to protect and stabilize the bones. These glues include shellac secreted by female lac insects (*kerria lacca*) as well as bovine glue [146]. Efforts were made to avoid sampling visibly treated bones and teeth but in some cases there were no strict records for how remains were curated. Although not part of this thesis we conducted an additional study [147] whereby a range of glues used in archaeological conservation were applied to modern sheep bone and proteomic extractions performed. The results generated were used to create an additional glue/consolidant database against which archaeological samples could be run. We hope this will complement the existing cRAP (common Repository of Adventitious Proteins; The Global Proteome Machine) which lists common laboratory-related proteins, proteins added through handling and proteins commonly used in mass spectrometry.

10.1.1.3 Identifying milk consumption using bulk collagen isotope analysis

Stable carbon and nitrogen isotope analyses of archaeological human and animal remains have provided important insights into herd management strategies, mobility and pastoralist

diets across Africa [131,148–150]. However, nitrogen stable isotope analysis of bone collagen can only provide limited information about human reliance on dairy products since $\delta^{15}N$ values cannot distinguish between different animal products, such as meat or milk, derived from the same animal. Since fermented dairy products were likely important to early pastoral communities investigations into potential isotopic differences between milk and dairy products have also been undertaken [151], however they show no significant differences in $\delta^{15}N$ meaning that elevated $\delta^{15}N$ human values cannot be attributed to consuming large amounts of dairy products. Nevertheless, in specific contexts, isotopes can still aid explorations of the emergence of specialized forms of pastoralism in certain regions. For example, differences in human stable carbon isotopic measurements were used to investigate the transition to cattle-based pastoralism in southernmost Africa [152]. After 1000 AD, humans appear to be consuming greater quantities of C_4-based foods, likely reflecting the increased consumption of products from C_4-feeding cattle [152]. By the 16th-century cow's milk was a well-established dietary staple of the Khoekhoe people in this region [153] it is likely the shift in stable isotope values represents the start of the transition to cattle-based economies. Furthermore, differences in the $\delta^{13}C$ and $\delta^{15}N$ of human bone collagen from sites across Africa have been used to distinguish between pastoral and farming populations [148]. Herders exhibit higher $\delta^{13}C$ and $\delta^{15}N$ values reflecting a greater reliance on grazing animals consuming C_4 grasses [27,148,149,154].

In Manuscript B, bulk bone collagen analysis was used to examine human dietary intake. When possible, wild and domestic animals were also analysed from each site to create a dietary baseline. By comparing $\delta^{13}C$ and $\delta^{15}N$ measurements for humans and associated fauna it was possible to assess the consumption of different C_3/C_4 plant sources, or animals feeding of these different plants, as well as protein intake (terrestrial vs aquatic). Human stable isotopic values fell within the range of domestic animals suggesting humans were relying on animal protein sources. Viewed in isolation the human isotope results cannot be attributed to milk consumption but when considered against the proteomic evidence they suggest that dairying was a component of pastoral subsistence economies at the sites of study.

10.1.2 Implications for the spread of dairying and pastoralism

Large-scale climate change has long-been recognised as playing a transformative role in the physical and social landscape of Holocene Africa. However, there is a growing recognition of the importance of smaller scale, regional environmental risks, particularly in East Africa [130,131,155]. Our results provide a snapshot of pastoral lifeways during two key periods. Firstly, we identified milk consumption at sites in Sudan towards the end of the African Humid phase (c. 6000 years ago) a time at which increased aridity has been argued to have caused pastoralists to move to well-watered regions of the Nile Valley and eastern Sahel [8]. Secondly, we provide evidence of milk consumption from two sites in southern Kenya (c. 3500-3200 cal. BP) which coincide with the emergence of more favourable environmental conditions in East Africa and an increase in pastoral sites [7,27,130]. The identification of milk consumption at this time tentatively suggests dairying may have been a component of pastoral diet as communities first began to settle in the area. As discussed, determining true reliance is challenging but proteomic, isotopic datasets in addition to the presence of livestock remains confirm as a reliance on animal protein and/or secondary products. As successfully demonstrated by studies combining isotopic and palaeoecological data for pastoral settlements around Lake Victoria and Lake Turkana in Kenya [131], our new proteomic data could be combined with environmental data to further explore the relationship between herding and inland local ecologies.

We hypothesize that dairying in Africa reflects a global pattern of milk consumption occurring before the widespread genetic adaptation to digest lactose. Due to variable preservation, only one individual in Manuscript B had proteomic evidence of milk consumption and full genome sequencing showing the absence of any known LP alleles [45]. Although it should be noted that other individuals without proteomic evidence for milk consumption come from sites which have other individuals without known LP variants [44,45]. The expansion and integration of proteomic evidence and ancient DNA data would provide further clarification as to whether early pastoral communities in Africa had the genetic adaptation to digest milk or not. In addition, the analysis of individuals from more recent periods in Africa might help refine estimations about when LP first emerges. In the absence of LP, it is possible that ancient communities, like modern communities in Africa, were processing raw milk into fermented products with a lower lactose content [156,157]. It has also been hypothesised that bacteria both in naturally produced dairy products and

the human gut, such as *Lactobacillus* and *Bifidobacterium*, may have supported the digestion of milk products without discomfort [158–160].

Finally, the proteomic data generated in this study for eastern Africa may contribute more broadly to proteomics method development. As the first published data for ancient calculus samples from Africa it provides insights into overall preservation and protein recovery for dental calculus from new contexts. Through the Oral Signature Screening Database and subsequent study on conservation glues and consolidants, we hope to complement the growing literature on protein extraction methods [103,143], peptide identification and analysis [161,162], and authentication of ancient proteins [145,163].

10.2 The development of agriculture in Central Africa

In Manuscript C we investigated dietary reliance in the Congo Basin during the Iron Age. Our data demonstrate that pearl millet (*Pennisetum glaucum*) played a relatively limited role in the western portions of the Congo Basin, with populations relying on available forest and freshwater resources. By contrast, an individual from the northeast of the Democratic Republic of the Congo, with genetic affiliations with eastern African pastoralist groups and local hunter-gatherer populations, demonstrates significant dietary reliance on C_4 crops (either sorghum or millet). We argue that early Iron Age populations in Central Africa adapted to their forest environments, making use of diverse freshwater and plant resources, with millet having a more limited dietary role. This raises the possibility that these early Iron Age populations are not solely the product of external population movements, but are a combination of incoming crops and technologies and local adaptive knowledge and preferences.

10.2.1 Methodological potentials and challenges

10.2.1.1 Stable isotope approaches to agricultural reliance in Central Africa

For the first time, we conducted a multi-tissue isotope study of human and faunal remains to directly examine the degree to which early Iron Age inhabitants of the Congo Basin relied upon incoming Sahelian crops, such as pearl millet, relative to local wild resources. In the context of tropical Central Africa, the application of stable isotope analyses is

particularly powerful due to the non-overlapping carbon isotopic signatures between local wild forest plant sources (C_3) and domesticated cereals such as pearl millet (C_4). Consequently, isotope analysis can provide new insights into dietary reliance in rainforest contexts which have historically been seen as barriers to the spread of agriculture and millet cultivation in Africa [9]. However, proposed environmental constraints for millet cultivation have been questioned [77] with modern experiments showing millet can be grown today in the Inner Congo basin under humid conditions and high annual rainfall [76]**.** Archaeobotanical studies also show the presence of pearl millet in Central Africa from around 2,300 years ago [74,164] yet information about how communities may have used millet in the Inner Congo Basin is limited. Our results demonstrate that C_4 foods were integrated into existing, diverse subsistence systems throughout the Central African Iron Age.

Another strength of isotopic dietary reconstructions is the possibility of looking at diet at different points in an individual's lifetime. Due to differences in the time taken for various tissues to form and remodelling rates it is possible to examine diet in childhood and adulthood by analysing tooth enamel and bone collagen respectively. In the case of Manuscript C, we used these differences to demonstrate differences between the diet of individuals during childhood and adulthood. The collagen results showed an increase in the consumption of C_4 sources in later life. This was intriguing given the metabolic routing bias of bone collagen $\delta^{13}C$ towards protein components of the diet (as opposed to plant sources). Yet, there remains a signal indicative of some C_4 plant component. Our findings may tentatively support the hypothesis that millet had a more restricted, perhaps ceremonial use such as beer brewing or feasting [165].

10.2.1.2 The importance of local baselines

Establishing faunal baselines are essential for accurately reconstructing human dietary intake. As animals have unique feeding regimes it is important to compare the isotopic measurements of humans, who could be feeding on these animals, to local fauna [166]. For sites in tropical environments this can be particularly difficult as organic preservation levels may be low meaning it may not be possible to get a representative sample of animals or extract enough high-quality collagen for analysis. Fortunately, for the study presented in this thesis, waterlogged conditions and the sheltered conditions of a rockshelter resulted in

the survival of organic remains. The faunal baseline in Manuscript C enabled the identification of different feeding regimes (wild browser, domesticated browser, mammalian carnivores and aquatic species) enabling comparisons of human diets against both wild and domestic animals. Nevertheless, we were only able to construct a faunal baseline for one specific site which limited the exploration of human reliance on specific animals across space and time.

As illustrated by Manuscript C, it can be challenging to interpret human isotopic values when freshwater fish were also likely a major contributor to human diets. Due to enrichment of ^{15}N between trophic levels it is easy to distinguish aquatic species from terrestrial animals with the former generally having higher $\delta^{15}N$ values. The differences between marine and freshwater fish is far smaller but marine fish generally exhibit both higher $\delta^{15}N$ and $\delta^{13}C$ when compared to freshwater fish [167]. As demonstrated by modern ecological studies many factors can alter the nitrogen and carbon isotopic compositions of freshwater food webs, including variations in the sources and cycling of carbon and nitrogen, the availability of oxygen and productivity of primary producers (for full review see [168]). Accounting for such complexity in ancient freshwater systems remains extremely difficult. However for riverine archaeological sites, where inhabitants are likely to have consumed appreciable amounts of fish, sampling multiple aquatic species is an important first step for making dietary interpretations.

10.2.2 Implications for the expansion of agriculture into the Congo Basin

Our results contribute to a growing body of literature which demonstrates that throughout human history communities were able to employ a range of strategies to successfully procure and produce food in tropical environments [61]. We illustrate that traditional “Bantu Expansion” models require critical reassessment when applied to investigating agriculture in Central Africa. The presence of botanical remains of pearl millet and charred food fragments at Bolondo demonstrate that communities were growing and processing crops as ongoing ‘Bantu’ expansions continued into a number of tributaries of the Congo River. However, communities were not reliant on these incoming crops and instead employed dynamic subsistence systems as seen elsewhere in the DRC [169].

The study also highlights the strength of a multidisciplinary context-specific approach to exploring agricultural systems in the Congo Basin. For one individual, isotopic measurements of enamel, as well as starch retrieved from dental calculus, shows that they were consuming C_4 foods throughout childhood and adolescence. However, starches from forest resources were also discovered in their calculus and wild faunal species were present at the site [170,171]. Collectively, this data shows consumption of both wild and domestic plant sources and could tentatively allude to forager-farmer interactions on the fringes of the Congo Basin. Furthermore the differences observed between the enamel and collagen results of individuals from Bolondo shows how analysing multiple tissues can reveal more nuanced narratives for how humans were using different foodstuffs.

10.3 Future directions

To continue the development of in-depth regional models for the expansion of pastoralism and dairying in Africa will require larger proteomic datasets. For the Sahara, lipid residues have already provided important insights into dairying practices [48,50] but analysing ceramics using proteomics could provide more detailed, species-specific evidence [98]. Extending the sites studied southwards from Kenya to Tanzania and Zambia could also aid investigations of the timing of dairy expansion across the African continent more widely, as well as potential environmental and zoonotic constraints facing pastoralists as this subsistence strategy moved into southern Africa [56,57]. To provide further support the hypothesis that milk consumption occurred before the emergence of lactase persistence in Africa would require the integration of dietary and archaeogenetic studies of ancient african populations so that LP status and diet could be directly compared to evidence for dairy consumption across space and time. Increased ancient population genetic studies for Africa could also help investigate whether such strategies were a product of large-scale migration or adoption [43–45].

The data presented in Manuscript C has showcased the potential of isotope analyses to expand our understanding of the adoption of agriculture in Central Africa. While organic preservation may be a limiting factor for sites in modern, and past rainforest settings, the analysis of tooth enamel in particular, which is less susceptible to diagentic change and more likely to preserve, could help build up a more detailed picture of how different communities were using and incorporating incoming C_4 crops. Additionally, the

combination of isotopic results with microparticle and proteomic studies of dental calculus could provide further insights into the utilization of different plants by communities in the tropics [172]. As proteomics develops, perhaps to include the identification of specific plants [85], this may also prove a useful tool in the investigation of diets in tropical settings as well.

Stable isotope methodologies have been employed within archaeology for decades with paleodietary studies growing rapidly in recent years [80]. As isotope approaches are applied to different geographic and temporal contexts, the generation of faunal and plant baselines is fundamental for establishing local signals and interpreting past human diets [173]. Increasing the number of fully reported, published datasets, through open-access online repositories such as IsoArcH and IsoMemo, will also enable the integration of isotope data with archaeological, chronological and palaeoenvironmental records [174,175]. The palaeoproteomic analysis of dental calculus is also becoming more common [46,47,100,106–108] and has offered new insights into dietary variation. However, further explorations of the specific conditions that influence protein preservation [87] are needed to understand biases in protein recovery [85]. In addition, the advancement of extraction methods as well as increasing the number of published proteomes for different animals and plants could improve the total number of proteins extracted and identified[79]. Finally, the continued development of methods assessing whether proteins recovered from archaeological materials are endogenous [145] will help address concerns about authentication. Both palaeoproteomics and isotopic studies have, and will, continue to transform our understanding of the human past. While such approaches are increasingly being applied to new materials and contexts, prioritising method development, establishing local baselines and generating reference data is essential for these disciplines to grow and explore new research questions.

10.4 Conclusion

The papers presented in this thesis demonstrate how a multidisciplinary approach, including proteomics and stable isotope analysis, can refine regional narratives for food production across Africa. We confirm that pastoral communities were consuming milk 6,000 years ago in northeastern Africa and suggest dairying may have been an important buffer against environmental risks. Milk production is an effective way of converting natural forage into a nutritious food source for human consumption [176]. Additionally, unlike meat,

milk is a renewable resource sustaining populations without requiring the slaughtering of valuable animals. For modern pastoral communities in Africa, milk is a dietary staple which accounts for a significant proportion of daily energy intake [177,178] as well as an important source of income [179], in some of the most marginal and rapidly changing environmental settings on the continent.

Pastoralism is an effective risk-spreading strategy to drought due to the ability to move animals to new pasture and water sources and, through the maintenance of high numbers of females, retain milk supply even after significant losses [54]. Both in the past and present, livestock have provided food security in the more arid regions of Africa. Today however, prolonged drought, conflict, loss of rangelands and large-scale agricultural investment is undermining the stability and resilience afforded by pastoral lifeways [180]. Our research confirms the longevity of pastoral economies, while recognizing such systems have, and will, continue to evolve [181] we provide a historical perspective for examinations of the economic, social and cultural implications of present pastoral lifeways in East Africa [179,182,183].

Through a combination of methods, our work illuminates the complex, intricate relationships between human subsistence practices and regional, local environments. Moving to tropical Central Africa, our results break away from simplistic, traditional models for the passive adoption of new C_4 crops revealing dynamic systems in the Inner Congo Basin and tentatively suggest millet may have had a specialised, potentially symbolic use. Today, forest foods and bushmeat are essential sources of nutrients for millions of people living in the tropical forests of Central Africa [184,185]. Balancing the needs of the growing population in rural areas, the demand for timber while also protecting local forest food sources poses a significant challenge [186,187]. We urge the prioritization of further direct assessments of human subsistence practices in order to better understand how domesticates became adapted to these tropical environments and what legacy this has for 21st century food security in the region.

This thesis offers important insights into how ancient communities in eastern and Central Africa, two regions where future food security is becoming increasingly under threat in the face of human-induced climate change and landscape modification or deforestation, successfully adapted to the changing landscape. Appreciating the finely tuned, yet

dynamic, nature of ancient human adaptations to these arid and tropical regions, respectively, offers important perspectives for developing and maintaining sustainable food systems of the future. In particular, it shows that western, capitalist focus on large herds or monoculture to increase wealth are often incompatible with changing extreme environments, and communities have always maintained a diverse approach to subsistence in different parts of the continent. Archaeological science, alongside archaeology and ethnography, promises to ensure that these traditional avenues to food security are brought to bear on modern policy and are not lost forever.

11. References

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12. Summary

This dissertation investigates dietary variation and change in Africa during the Holocene using protein and stable isotope analyses. By focusing on East and Central Africa it provides new insights into pastoral and agricultural adaptations in contrasting spatial, temporal, and environmental contexts. Today, animal milk is a vital food source for millions of people across Africa. However, establishing when communities were first consuming milk and how environmental conditions may have influenced the emergence of specialised pastoralism remains challenging. Meanwhile, in Central Africa, discussions of past diets have focused on the mechanisms for the adoption and spread of a particular crop, pearl millet, often associated with the Bantu expansion and supposed to have restricted environmental tolerances. This dissertation provides new proteomic and isotopic insights into diet in order to address these questions and advance our knowledge of pastoralism, dairying and agriculture in ancient Africa.

Manuscript A gives a comprehensive overview of archaeological and biomolecular evidence for dairying across the entire African continent. It reviews existing zooarchaeological data for the emergence of domestic fauna in the archaeological record as well as mortality profiles indicative of raising animals for dairying. In addition, it considers the merits and limitations of other lines of evidence such as rock art images of milking scenes and the identification of dairy fats on ceramics through lipid residue analysis. The evidence is reviewed with special focus on broad climatic changes during the Holocene as well as regional environmental risks. This study provides an important methodological and theoretical foundation for a growing body of research in regions and archaeological contexts that have been critically under-studied.

Manuscript B presents new proteomic evidence for milk consumption in Sudan and Kenya. By providing direct evidence for milk consumption and species-specific information about animals raised for dairying it overcomes some of the shortcomings of existing datasets for dairying in Africa. The identification of milk proteins in dental calculus confirms individuals were consuming milk in Sudan from 6,000 years ago and in southern Kenya 4,000 years ago. This evidence shows that communities were drinking milk as they were first moving and settling in southern Kenya which could indicate that dairying was an important buffer against environmental stressors. In addition, based on current archaeogenetic data for

Africa, it appears communities were consuming milk despite being lactose intolerant. This adds to the emerging global pattern that dairying occurred *before* the genetic ability to fully digest milk.

Manuscript C presents the results of an isotope study that reconstructs the diets of communities living in the Congo Basin during the Iron Age (~2,000 years ago to 130 years ago). Stable isotope analyses were used to assess dietary reliance on wild or tropical (C_3) food sources opposed to C_4 domestic cereals such as pearl millet. For three sites, stable carbon isotope values demonstrate communities were reliant on tropical wild sources despite new, incoming cereal crops. At one site botanical remains of millet were recovered but it appears to have limited contribution to human diets, perhaps indicating that millet had a more symbolic significance. In contrast, a hunter-gatherer individual from Matangai Turu, with genetic affiliations for eastern African pastoralist groups, shows a heavy reliance on C_4 sources. This project illustrates how communities employed diverse, flexible subsistence practices across the Congo Basin and highlights the strength of using isotope analyses to assess dietary reliance.

This dissertation provides important new evidence about diet in two contrasting contexts in ancient Africa. Overall, these studies illustrate the strength of combining proteomic and isotopic methodologies in order to provide a more nuanced, complementary understanding of pastoral and agricultural lifeways in ancient Africa.

13. Zusammenfassung

In dieser Dissertation werden Variation und Veränderung der Ernährungsweisen in Afrika während des Holozäns mit Hilfe von Protein- und stabilen Isotopenanalysen untersucht. Der räumliche Fokus der Dissertation liegt auf Ost- und Zentralafrika und liefert neue Erkenntnisse über pastorale und landwirtschaftliche Anpassungen in verschiedenen räumlichen, zeitlichen und ökologischen Kontexten. Heute ist tierische Milch eine lebenswichtige Nahrungsquelle für Millionen von Menschen in ganz Afrika. Eine zentrale Forschungsfrage ist immer noch, wann Gemeinschaften anfingen Milch zu konsumieren und wie Umweltbedingungen die Entstehung eines spezialisierten Pastoralismus beeinflusst haben. In Zentralafrika konzentrieren sich die Diskussionen über die Ernährung auf die Mechanismen der Einführung und Verbreitung der Kulturpflanze Perlhirse, welche oft mit der Bantu-Ausdehnung in Verbindung gebracht wird und scheinbar die Toleranz gegenüber der Umwelt eingeschränkte. Die vorliegende Dissertation liefert neue Erkenntnisse, um die Fragen des Wann? und Wo? der Ernährung zu klären und unser Wissen über Pastoralismus, Milchwirtschaft und Landwirtschaft im früheren Afrika zu verbessern.

In Manuskript A wird ein umfassender Überblick über archäologische und biomolekulare Nachweise für die Milchwirtschaft auf dem gesamten afrikanischen Kontinent gegeben. Es werden vorhandene zooarchäologischen Studien zur Entstehung von Haustieren in archäologischen Forschungsarbeiten sowie Untersuchungen von vorhandenen Sterblichkeitsprofilen, die für die Aufzucht von Milchvieh sprechen, präsentiert. Darüber hinaus werden die Vorteile und Limitationen anderer Belege, wie beispielsweise Felsmalereien in denen Melkszenen dargestellt sind, und die Identifizierung von Milchfetten in Keramik durch die Analyse von Lipidrückständen, untersucht. Die Forschungsergebnisse werden mit besonderem Augenmerk auf klimatische Veränderungen während des Holozäns sowie auf regionale Umweltrisiken geprüft. Diese Studie ist eine wichtige methodische und theoretische Grundlage für eine wachsende Zahl von Forschungen in Regionen und archäologischen Kontexten, die kritisch unterschätzt wurden.

Manuskript B präsentiert neue Proteomiks Resultate zum Milchkonsum im Sudan und in Kenia. Der direkte Nachweis des Milchkonsums und artspezifische Informationen über

Milchvieh, ergänzen bestehende Datensätze der Milchviehhaltung in Afrika. Die Identifizierung von Milchproteinen im Zahnstein weist daraufhin, dass Individuen im Sudan seit 6.000 Jahren und im Süden Kenias seit 4.000 Jahren Milch konsumierten. Die neuen Forschungsergebnisse zeigen, dass Bevölkerungsgruppen bereits Milch tranken, als sie zum ersten Mal nach Südkenia migrierten und sich dort niederließen. Dies könnte darauf hindeuten, dass Milchwirtschaft ein wichtiger Puffer gegen Umweltstressoren gewesen ist. Darüber hinaus scheint es, dass Menschen Milch konsumierten, obwohl sie Laktose intolerant waren, wie neueste archäogenetische Daten für Afrika zeigen. Die Resultate unterstützen vorhandene Studien, welche belegen, dass Milchproduktion bereits vor der genetischen Fähigkeit, Milch vollständig zu verdauen, stattfand.

In Manuskript C werden stabil Isotopenergebnisse zu den Ernährungsgewohnheiten von Bevölkerungsgruppen, welche im Kongobecken während der Eisenzeit (vor ca. 2000 bis 130 Jahren) lebten, präsentiert. Die Studie hat zum Ziel, die Abhängigkeit der Ernährung von wilden oder tropischen (C3) Nahrungsquellen im Gegensatz zu C4-Heimgetreide wie Perlhirse zu untersuchen. Für drei Standorte zeigen die ^{13}C Resultate, dass die Gemeinschaften trotz neuer Getreidequellen auf tropische Wildquellen angewiesen waren. An einem Standort wurden botanische Überreste von Hirse gefunden. Allerdings, scheint die Hirse nur einen geringen Beitrag zur menschlichen Ernährung geleistet zu haben, was vermuten lässt, dass die Hirse nur symbolische Bedeutung hatte. Im Gegensatz dazu zeigt die Isotopenanalyse eines Jägers und Sammlers aus Matangai Turu, der genetische Verbindungen zu ostafrikanischen Hirtengruppen hat, eine starke Abhängigkeit von C4-Quellen. Das Forschungsprojekt veranschaulicht, wie Bevölkerungsgemeinschaften im gesamten Kongobecken unterschiedliche, flexible Subsistenzpraktiken praktizierten, ebenso wie das Potential der stabil Isotopenanalysen zur Beurteilung der Abhängigkeit von Nahrungsquellen.

Die vorliegende Dissertation präsentiert bedeutende neue Erkenntnisse über die Ernährung in zwei gegensätzlichen Kontexten im alten Afrika. Die Studie veranschaulicht das Potential der Kombination von Proteomiks- und Isotopenmethoden, um ein detailreiches, komplementäres Verständnis der pastoralen und landwirtschaftlichen Lebensweise im alten Afrika zu erhalten.

14. Declaration of Honour (Eigenständigkeitserklaerung)

In accordance with the doctoral degree regulations of the Faculty of Biology and Pharmacy at the Friedrich Schiller University Jena, I hereby declare:

(a) that I am aware of the applicable doctoral regulations,

(b) that I have written the doctoral thesis myself and that I have not taken any text sections from another author or from my own examination papers without indicating them and that I have indicated all tools and sources used by myself in this work,

(c) that I have mentioned all persons who have supported me in the selection and evaluation of the material as well as in the production of the manuscript,

(d) that I have not used the assistance of a commercial doctoral advisor and that third parties have neither directly nor indirectly received monetary benefits from me for work that is related to the content of the submitted doctoral thesis,

(e) that I have not yet submitted this doctoral thesis as an examination paper for an academic examination,

(f) that I have submitted neither the same thesis nor an essentially similar thesis, nor a different thesis as a doctoral thesis at another university.

Jena, 16.03.2020

Madeleine Bleasdale

15. Curriculum Vitae

Madeleine Bleasdale

Max Planck Institute for the Science of Human History,
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EDUCATION

2016 – 2020	**Max Planck Institute for the Science of Human History, Jena, Germany** PhD to be awarded by Friedrich-Schiller-Universität Jena
2015 – 2016	**University of York, United Kingdom** MSc. Bioarchaeology (Distinction) *The Queen's Chapel of the Savoy: A dietary reconstruction of a British post-medieval population using stable isotope analysis*
2011-2014	**University College London, United Kingdom** BSc. Archaeology (First class) *TB or not TB? An investigation into the representation of Tuberculosis in Medieval skeletal collections and the challenges of an osteological approach to the disease*

PUBLICATIONS

2018	**Bleasdale, M.,** & Alexander, M. M., Preliminary isotopic analysis of bone collagen. In: Sibun, L, Ponce, P. In Life and Death: Archaeological excavations at the Queen's Chapel Savoy, London. Spoilheap Publications, Monograph 17.
2019	**Bleasdale, M.,** Ponce, P., Radini, A., Wilson, A. S., Doherty, S., Daley, P., Brown, C., Spindler, L., Sibun, L., Speller, C., & Alexander, M. M. Multidisciplinary investigations of the diets of two post-medieval populations from London using stable isotopes and microdebris analysis. *Archaeological and Anthropological Sciences,* 11(11), 6161–6181
2020	Wilkin, S., Miller, A. V., Taylor, W. T. T., Miller, B. K., Hagan, R. W., **Bleasdale, M.,** Scott, A., Gankhuyg, S., Ramsøe, A., Uliziibayar, S., Trachsel, C., Nanni, P., Grossmann, J., Orlando, L., Horton, M.,

Stockhammer, P. W., Myagmar, E., Boivin, N., Warinner, C., & Hendy, J. (2020). Dairy pastoralism sustained eastern Eurasian steppe populations for 5,000 years. *Nature Ecology & Evolution,* 4(3), 346–355.

2020 Wilkin, S., Ventresca Miller, A., Miller, B. K., Spengler, R. N., Taylor, W. T. T., Fernandes, R., Hagan, R. W., **Bleasdale, M.,** Zech, J., Ulziibayar, S., Myagmar, E., Boivin, N., & Roberts, P. Economic Diversification Supported the Growth of Mongolia's Nomadic Empires. *Scientific Reports*, 10 (1), 3916

Accepted **Bleasdale, M.**, Goldstein, S., Hendy, J., & Boivin, N. The Archaeology of Dairying in Holocene Africa. Target Journal: *Journal of World Prehistory*. Accepted: 11/2019

Accepted Wang, K., Goldstein, ST., **Bleasdale, M.,** Bostoen, K., Clist, B., Buck ,LT., Deme, A., McIntosh, R.J., Mercader, J., Ogola, C., Petraglia, M., Power, RC., Sawchuk, E., Willmsen, EN., Crowther, A., Ndiema, E., Roberts, P., Krause, J., Boivin, N., Schiffels, S. Ancient genomes from eastern and southern Africa reveal patterns of coexistence and interaction. *Science Advances.* Accepted: 03/2020.

In Review **Bleasdale M.**, Wotzka, HP., Eichorn, B., Mercader, J., Styring ,A., Zech, J., Soto, M., Inwood J., Clarke, S., Marzo, S., Fiedler, B., Boivin, N., Roberts, P. Isotopic and Microbotanical Insights into Iron Age agricultural reliance in the Central African rainforest. *Communications Biology*. Submitted: 02/2020.

In Review **Bleasdale, M.,** Richter, KK., Janzen, A., Brown, S., Scott, A., Zech, J., Wilkin, S., Wang, K., Schiffels, S., Desideri, J., Besse, M., Reinold, J., Saad, M., Babiker, H., Power RC., Ndiema, E., Ogala, C., Manthi, FK., Zahir, M., Petraglia, M., Trachsel, C., Nanni, P., Grossmann, J., Hendy, J., Crowther, A., Roberts, P., Goldstein ST., Boivin, N. Ancient proteins provide direct evidence of dairy consumption in eastern Africa. *Nat. Comms.* Submitted: 03/2020.

CONFERENCE PRESENTATIONS

2019 Scientific Advisory Board, Jena, "*Using proteomics to track the spread of dairying in Africa"*

2019 UKAS19, Manchester, *"The proteomic analysis of ancient dental calculus from eastern Africa: Current applications, pitfalls and future prospects"*

2018 ISBA8, Jena, *"Proteomic evidence of milk consumption in Neolithic Sudan."*

2018 Ancient Proteins@20, Copenhagen, *"Protoemic evidence of dairying in Neolithic Sudan"*

2018 SAfA 24th Biannual Meeting, Toronto, *"Dairying in Mid-Holocene Africa: insights from the proteomic analysis of dental calculus"*

FIELDWORK AND TRAINING

2018 Transcriptomics and proteomics training course, Functional Genomics Centre, Zurich

2017 Sampling project leader, Tayma, Saudi Arabia

2017 Sampling project leader, Qurayyah, Saudi Arabia

2017 Sampling assistant, University of Edinburgh, United Kingdom

2017 Sampling project leader, University of Geneva, Switzerland

2017 Sampling assistant, National Museums of Kenya, Kenya

2016 Sampling assistant, National University of Mongolia, Mongolia

2013 Excavator, Sima de las Palomas, Spain

2013 Excavator, Poulton Medieval Cemetery, United Kingdom

2012 Excavator, Tel Bet Yerah, Israel

Jena, 16.03.2020

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