Chapter 6
The Potential of Dental Calculus as a Novel Source of Biological Isotopic Data

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Abstract Stable isotope analysis has become an essential tool in investigations of ancient migration and paleodietary reconstruction. Because the biogeochemistry of bone collagen and apatite is well known, current methods rely almost exclusively on analyses of bones and teeth; however, dental calculus represents a potentially additional biological source of isotopic data from ancient skeletons. Dental calculus is a mineralized bacterial biofilm that forms on the surfaces of teeth. Sampling dental calculus does not damage the dentition and thus can be used in cases where it is not possible to perform destructive analyses of conventional mineralized tissues. Like bone and dentine, dental calculus contains both inorganic and organic components, allowing measurement of C, N, O, H, and Sr isotopes. Additionally, dental calculus forms as serial, non-remodeling laminar accretions on the tooth surface, opening up the possibility of analyzing discrete time points during the lifetime of an individual. However, as a microbial biofilm and not a human tissue, the biochemistry of dental calculus is complex, containing multiple calcium phosphate mineral phases, organic

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and inorganic food remains, hundreds of human and bacterial proteins, and diverse biomolecules from thousands of endogenous bacterial taxa. Isotopic investigation of dental calculus is still in its infancy, and many questions remain regarding its formation and processes of diagenesis. This chapter (1) reviews the unique advantages presented by dental calculus as a novel source of biological isotopic data, (2) critically evaluates published isotopic studies of dental calculus, and (3) explores the current challenges of dental calculus stable isotope analysis through a case study of an Ancient Puebloan Basketmaker II population from the American Southwest.

Keywords Dental calculus · Dietary reconstruction · Carbon stable isotopes · Nitrogen stable isotopes · Oral microbiome

6.1   Introduction

Dental calculus is a long-term reservoir of dietary information in the archaeological record. Microscopic investigation of plant microremains (e.g., phytoliths and starch grains) preserved within dental calculus is now a well-established and powerful approach for reconstructing the use and consumption of plants by ancient peoples (Power et al., 2014, 2015a). This direct approach has broadened our understanding of the plant consumption and subsistence ecology of archaic hominins (Henry et al., 2011, 2012; Power et al., 2015b; Salazar-Garcia et al., 2013), as well as more recent human populations in geographic locations as varied as Oceania (Dudgeon & Tromp, 2012), the Caribbean (Mickleburgh & Pagan-Jimenez, 2012), Africa (Leonard et al., 2015) and Europe (Warinner et al., 2014a).

In parallel to this approach, the recent discovery that ancient biomolecules, such as DNA, proteins, and alkaloids, also preserve within dental calculus has provided valuable and novel information on the diet, health, and behavior of past populations. Genetic investigations of medieval German dental calculus have detected trace amounts of preserved dietary DNA from both plant and animal food resources (Warinner et al., 2014a), and recent proteomic investigations of prehistoric European and northern Southwest Asian human dental calculus have identified beta-lactoglobulin, a diagnostic and species-specific milk protein that attests to the expansion of cattle, sheep, and goat dairying economies since the Bronze Age (Warinner et al., 2014b). Moreover, both genetic and proteomic approaches allow reconstruction of the native bacterial communities of the oral cavity, the oral microbiome, which provides additional useful information for understanding the health status of ancient populations (Adler et al., 2013; Eerkens et al., 2018a; Warinner et al., 2014a). Recent analyses show that nicotine, and perhaps other alkaloids, can also preserve in calculus over archaeological time scales, providing additional information on health and human behaviour (Eerkens et al., 2018b).

Recently, it has been proposed that dental calculus might also serve as a suitable substrate for isotopic investigations, including carbon and nitrogen stable isotope-based paleodietary inference (Scott et al., 2013; Scott & Poulson, 2012).

However, subsequent studies have found that interpretation of isotopic values obtained from dental calculus is far from straightforward (Eerkens et al., 2014; Price et al., 2018; Salazar-García et al., 2014a). In this chapter we review the basic biology of dental calculus, discuss sampling considerations and the suitability of dental calculus as a substrate for isotopic measurement, and explore both the potential benefits and challenges associated with utilizing dental calculus as an alternative to collagen in stable isotope-based paleodietary models. We then present a case study comparing isotopic data generated from bone collagen, bone apatite, and dental calculus from the maize farming Ancient Puebloan Basketmaker II culture of the American Southwest.

6.2   Composition of Dental Calculus

6.2.1   Formation of Dental Calculus

What exactly is dental calculus (Fig. 6.1)? In brief, dental calculus is dental plaque that has undergone a process of *in vivo* mineralization and semi-fossilization (Jepsen et al., 2011; Jin & Yip, 2002; Lieverse, 1999; Schroeder, 1969; Warinner et al., 2015a). Within the oral cavity, saliva is saturated in calcium phosphate in order to prevent the dissolution of the teeth during consumption of acidic foods. Saliva also contains a number of proteins that are capable of binding to the surface of enamel in order to form a protective barrier against dietary acids, known as the acquired enamel pellicle (AEP) (Yao et al., 2003). The AEP, however, also serves as a binding site for oral bacteria, which colonize the dentition and form a complex and structured microbial biofilm known as dental plaque (Zijnge et al., 2010).

Dead cells and complex biomolecules within dental plaque are thought to serve as nucleation centers for spontaneous mineral formation (Jin & Yip, 2002; White, 1997), and as a result the dental plaque biofilm undergoes periodic calcification events during which salivary calcium phosphate precipitates in the dental plaque matrix, eventually mineralizing the biofilm. This is then followed by the reformation of an AEP on the surface of the calcified plaque and the reestablishment of a new dental plaque biofilm. This process repeats many times during the lifetime of an individual, resulting in temporally ordered layers of calcified dental plaque, or dental calculus (Schroeder, 1969). These layers, which are typically 20–200 μm thick, are visible under both light and electron microscopy (Power et al., 2014; Warinner et al., 2014a). The rate of dental calculus formation varies by individual and is believed to depend on several factors, including host genetic variation affecting salivary content (Nancollas & Johnsson, 1994), as well as age, sex, disease state, and bacterial load of the individual (Nancollas & Johnsson, 1994; White, 1997). Diet may also influence dental calculus formation by altering oral pH either directly (e.g., dietary acids) or indirectly (e.g., acidic bacterial end products of metabolism). Among these dietary components, acidogenic sugars are thought to reduce dental
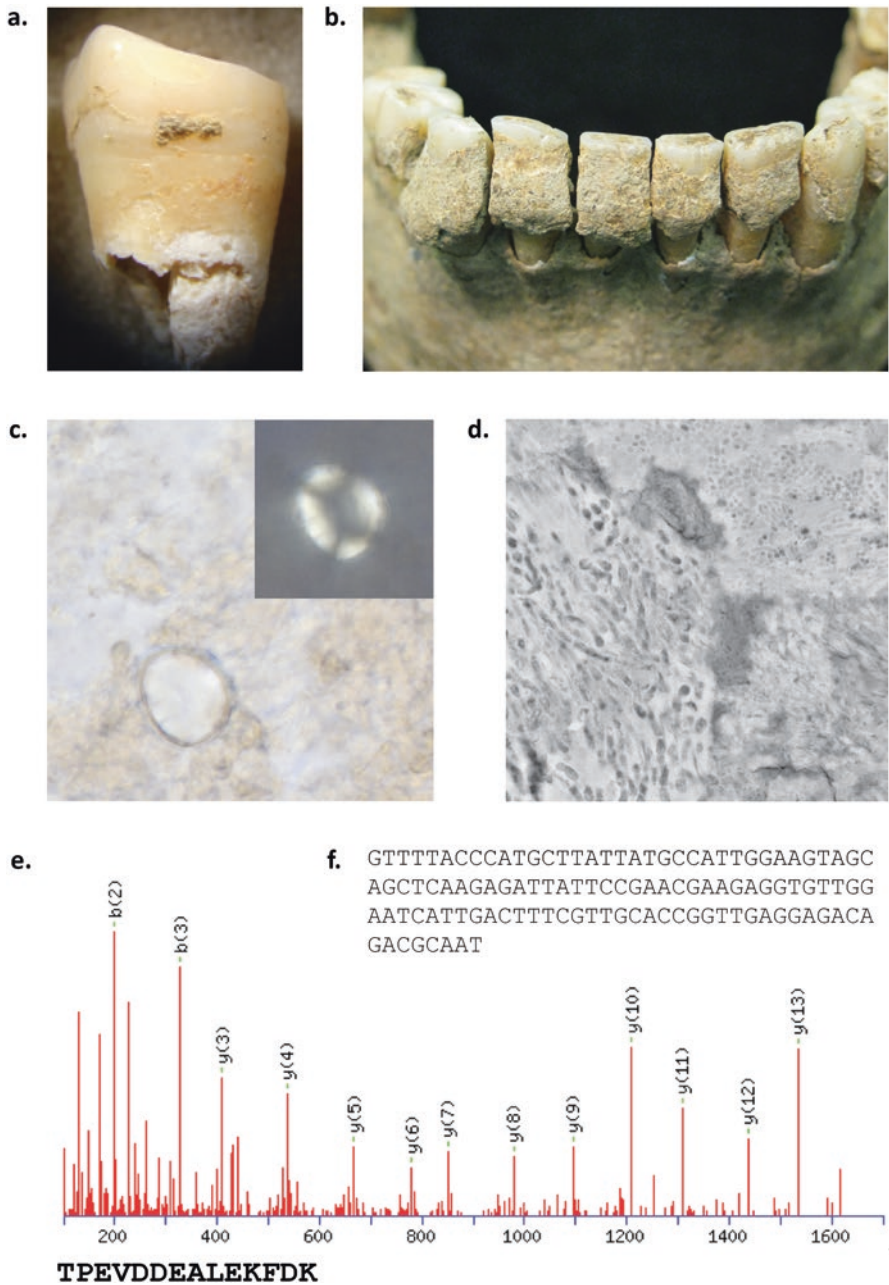
Fig. 6.1 Dental calculus deposits on archaeological human teeth. Dental calculus accumulation may be (**a**) small and localized or (**b**) massive and generalized. At a microscopic level, food particles such as (**c**) starch granules and (**d**) whole bacterial cells may be observed within dental calculus. At a molecular level, DNA and proteins (**e, f**) from a wide variety of sources have identified, including from bacteria, from the host, and from dietary sources. For example, mass spectra of peptides unique to the milk protein beta-lactoglobulin (**e**) have been identified within dental calculus from Norse Greenland, and the gingipain virulence factor produced by the periodontal pathogen *Porphyromonas gingivalis* has been identified within medieval German dental calculus on the basis of both protein and (**f**) DNA sequence evidence

calculus formation, while urea-generating animal tissues are thought to promote it (Jin & Yip, 2002; Lieverse, 1999; Scheie, 1994).

Understanding the gradual and incremental formation of dental calculus is important for archaeologists because it is during this process that food particles, bacteria, human proteins, and other minor constituents and debris become trapped in the mineralizing dental plaque matrix. It is this mineralization process that is key to the long-term preservation of both organic and inorganic microremains within dental calculus.

6.2.2 Mineral Composition of Dental Calculus

Scanning electron microscopy analysis coupled with energy-dispersive X-ray spectroscopy (SEM-EDX) of modern and ancient dental calculus (Power et al., 2014) indicates that its elemental constituents are very similar to enamel, dentine, and bone, containing a high percentage of calcium and phosphate, but also several other elements including carbon (mainly in carbonate-based minerals). Major elemental components of calculus are calcium, phosphorous and oxygen, and important minor components include carbon, sulphur, iron, silicon, sodium, aluminium, magnesium and chlorine (Fig. 6.2). Strontium is also present in trace, but measurable, quantities (M.J. Collins, personal communication).

Spatial analysis of dental calculus in cross-section using SEM-EDX has also revealed that calcium is densely and uniformly distributed throughout dental calculus, while silicon (an elemental marker of major soil components such as quartz and clays) is limited to the external surface of dental calculus and exhibits little penetration into the calculus matrix beyond minor infilling of surface cracks (Warinner et al., 2014a). One notable exception to this pattern occurs when silaceous microfossils of biological origin (e.g., phytoliths and diatoms) become embedded within dental calculus, appearing as spatially discrete inclusions of silicon within the primarily calcium phosphate matrix.

Mineralogical analysis using Raman spectroscopy has confirmed that dental calculus is highly distinct from surrounding burial soils, which are dominated in different degrees by various clay minerals (alumina-silicates), calcium carbonate ($CaCO_3$) and quartz (SiO_2) (Warinner et al., 2014a). The inorganic portion of both modern and ancient dental calculus is composed almost entirely of calcium phosphate mineral present in the mineralogical phases of hydroxyapatite (HA: $Ca_5(PO_4)_3(OH)$) and octacalcium phosphate (OCP: $Ca_8H_2(PO_4)_6 \cdot 5H_2O$) (Jepsen et al., 2011; Warinner et al., 2014a). Both HA and OCP are important in the formation and repair of mineralized biological tissues. HA is the predominant phase of calcium phosphate found in mature bone, dentine, and enamel, and OCP is primarily found in immature bone and dentine, as well as in regions of saliva-mediated enamel repair. Minor components of brushite ($CaHPO_4 \cdot 2H_2O$) and whitlockite ($Ca_9(MgFe)(PO_4)_6PO_3OH$) have also been shown to be present in both modern and ancient dental calculus using other methods (Jepsen et al., 2011; Schroeder, 1969).

Fig. 6.2 Highly abundant elements within archaeological dental calculus as measured by SEM-EDX. The most abundant elements are oxygen, phosphorous, and calcium, which is consistent with calcium phosphate mineral. (Figure adapted from Power et al., 2014)
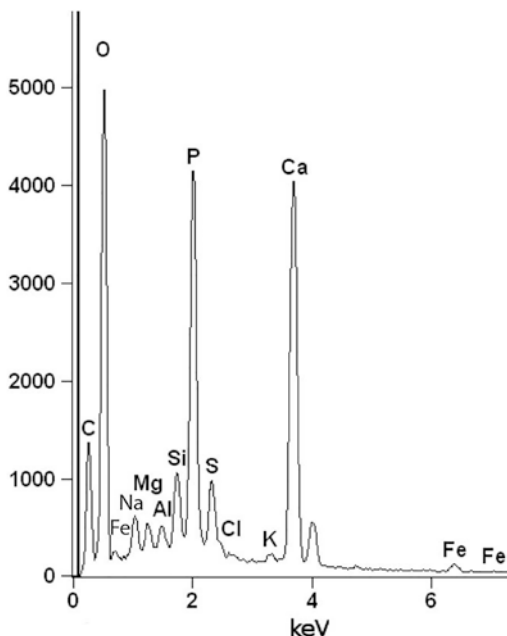
Finally, analysis of archaeological teeth in cross section using scanning electron microscopy (SEM) with backscattered electron (BSE) imaging allows taphonomic processes, such as mineral decalcification and reprecipitation, to be observed and described. In a recent investigation of a 900-year-old mandibular incisor from western Germany (Warinner et al., 2014a), it was found that taphonomic evidence for postmortem decomposition and decay was greatest in cementum and in the dentine immediately surrounding the pulp cavity. Here, zones of hypomineralization and hypermineralization were clearly visible in association with non-calcified (possibly vital) bacterial cells, microbial tunnelling, and tissue decay. Subsequent genetic testing of these tissues revealed that they were dominated by bacterial DNA of environmental origin resembling the microbial communities found in soils, leaf litter, and lakes (Warinner et al., 2014a). By contrast, dental calculus from the same tooth showed little evidence of altered mineral density or other taphonomic processes, and DNA extracted from these deposits was consistent with bacterial communities typical of the human oral microbiome (Warinner et al., 2014a).

Taken together, elemental and mineralogical analyses suggest that dental calculus preserves well in archaeological contexts and is highly distinct from the surrounding burial matrix. There is now little doubt that dental calculus represents a fourth reliably preserved mineralized tissue in the archaeological record, in addition to enamel, dentine, and bone. Moreover, the growing body of evidence that dental calculus may be more mineralogically stable than dentine offers renewed opportunities for trace element and/or strontium isotopic investigations of archaeological skeletal assemblages, something that has not been recommended for dentine or

bone because of strong diagenetic alteration (Ostwald ripening) observed in these tissues (Hinz & Kohn, 2010; Trueman et al., 2004).

Nevertheless, there are still many important aspects of dental calculus and its formation that are not well understood, including the rate of calculus formation during an individual's life and how this process varies from individual to individual. Factors contributing to this process, as mentioned previously, may include host genetic variation affecting salivary content (Nancollas & Johnsson, 1994), age, sex, health status, and bacterial load (Nancollas & Johnsson, 1994; White, 1997), as well as dietary considerations such as the relative proportion of acidogenic sugars (Scheie, 1994) versus meat and other urea-generating, high-pH foods (Jin & Yip, 2002; Lieverse, 1999).

6.2.3   Organic Composition of Dental Calculus

Similar to bone and dentine, the dry weight of dental calculus is composed of approximately 75–85% calcium phosphate mineral and 15–25% organic components (Jin & Yip, 2002; Schroeder, 1969; Warinner et al., 2015b). However, unlike bone and dentine, in which the organic fraction is dominated by a single class of proteins, collagen, the organic molecules within dental calculus are rich and diverse, including nucleic acids, proteins, carbohydrates, lipids, and even whole bacterial cells. The observation that dental calculus has been found to have no consistent C:N value (Eerkens et al., 2014; Salazar-García et al., 2014a; Scott et al., 2013) reflects the heterogeneous nature of its composition. Taken together, dental calculus is primarily a microbial substrate with several important, but variable, host contributions and dietary microfossil inclusions.

Contrary to early reports (Little et al., 1966), the quantity of nucleic acids in dental calculus is quite substantial. Even in archaeological specimens, the amount of DNA in dental calculus is typically 1–3 orders of magnitude higher than in dentine or bone from the same individual, reaching over 400 ng/mg (ng of DNA per mg of dental calculus) in well-preserved specimens from individuals with and without oral pathologies (Warinner et al., 2014a). The high DNA content of dental calculus is not entirely unexpected given that the estimated microbial cell density of dental plaque exceeds 200 million cells per mg (compared to less than 1000 osteocytes per mg, or mm³, of healthy bone) (Mullender et al., 1996; Parfitt, 2002; Socransky & Haffajee, 2005; Vashishth et al., 2000), but the abundance of well-preserved DNA in ancient specimens is nevertheless impressive, and makes dental calculus the richest known source of endogenous ancient DNA known in the archaeological record.

Proteins make up approximately 55% of the organic fraction of dental calculus by dry weight (Jin & Yip, 2002), and at least 250 human and bacterial proteins have been identified within dental calculus, ranging from digestive (alpha-amylase 1), blood (e.g., hemoglobin, albumin) and immune system (e.g., alpha-1-antitrypsin, myeloperoxidase) proteins to bacterial enzymes (e.g., dehydrogenases, kinases),

outer membrane proteins, and molecular chaperones (Warinner et al., 2014a). There appears to be great variability among the most abundant proteins found within dental calculus (Table 6.1). For example, some specimens contain high proportions of the human immunological proteins alpha-1-anti-trypsin and antithrombin, while others are dominated by blood proteins such as serum albumin, and yet others have markedly high levels of bacterial proteins (especially those of the genus *Actinomyces*). In part, these differences may reflect differences in the health status or hygiene habits of the individuals during life, with high proportions of human proteins likely indicating heightened immune response (e.g., alpha-1-antitrypsin) and host tissue destruction and bleeding (e.g., serum albumin, hemoglobin) in association with gingivitis and periodontitis. The bacterial proteins within dental calculus are likewise rich and diverse; however, they are typically underrepresented in proteomics studies due to the insufficiency of modern reference databases to adequately characterize them. Dental calculus contains an impressive range of both human and bacterial proteins that are highly distinct from those found in dentine and bone (Table 6.1).

6.3 Conventional Analysis of Collagen Carbon and Nitrogen Stable Isotopes in Paleodietary Reconstruction

6.3.1 Stable Isotope Analysis from Mineralized Tissues

Carbon and nitrogen stable isotope analysis of collagen from skeletal tissues such as bone and dentine has long been established as a reliable tool for inferring information about the diets of historic and prehistoric humans and animals (e.g. Katzenberg, 2000; Lee-Thorp, 2008; Sealy, 2001). This technique is based on the underlying rationale that the isotopic composition of food is recorded in the body tissues of a consumer (De Niro, 1985; De Niro & Epstein, 1978, 1981) after a predictable isotope fractionation (Schoeller, 1999). Well-preserved archaeological remains can retain the stable isotope ratios present during life, and although many types of human tissues are suitable for stable isotope analysis, archaeological studies typically focus on bone, dentine, and enamel. Bone and dentine are composed of both inorganic (ca. 75–80% of dry weight, mostly hydroxyapatite) and organic (ca. 20–25% of dry weight, mostly collagen) matter (Hare, 1980), while enamel is typically composed of more than 98% inorganic hydroxyapatite. Each of these components have specific stable isotope values that reflect their chemical origin and formation: the inorganic mineral derives from the end products of metabolism and reflects the carbon isotopic ratios of the whole diet, while the organic matter derives mainly from dietary amino acids (with possible modification, such as transamination) and reflects carbon and nitrogen isotope ratios linked primarily to protein consumption (Ambrose & Norr, 1993; Tieszen & Fagre, 1993).

Table 6.1 Ten most abundant proteins identified by tandem mass spectrometry in modern dental calculus, medieval dental calculus, and medieval dentine

Rank abundance	Species	Protein	% of identified protein spectra
Modern dental calculus (n = 2)			
1	*Homo sapiens*	Serum albumin	12
2	*Actinomyces sp.*	Phosphopyruvate hydratase	8
3	*Homo sapiens*	Protein S100-A9	6
4	*Corynebacterium matruchotii*	Hypothetical protein	4
5	*Corynebacterium matruchotii*	Hypothetical protein	4
6	*Homo sapiens*	Prolactin-inducible protein	4
7	*Actinomyces oris*	Glyceraldehyde-3-phosphate dehydrogenase	4
8	*Streptococcus mitis*	Glyceraldehyde-3-phosphate dehydrogenase	3
9	*Streptococcus sp.*	Elongation factor-Tu	3
10	*Actinomyces oris*	Elongation factor-Tu	3
		Total:	53.22
Medieval dental calculus (n = 4)			
1	*Homo sapiens*	Alpha-1-antitrypsin	22
2	*Actinomyces sp.*	Phosphopyruvate hydratase	7
3	*Homo sapiens*	Antithrombin-III	6
4	*Homo sapiens*	Serum albumin	5
5	*Actinomyces sp.*	L-lactate dehydrogenase	5
6	*Homo sapiens*	Myeloperoxidase	4
7	*Actinomyces oris*	ABC trasnporter-related protein	4
8	*Actinomyces sp.*	Glyceraldehyde-3-phosphate dehydrogenase	4
9	*Tannerella forsythia*	Hypothetical protein	3
10	*Homo sapiens*	Protein S100-A9	3
		Total:	62.84
Medieval dentine (n = 4)			
1	*Homo sapiens*	Collagen alpha-1(I) chain	30
2	*Homo sapiens*	Collagen alpha-2(I) chain	28
3	*Homo sapiens*	Prothrombin	6
4	*Homo sapiens*	Serum albumin	5
5	*Homo sapiens*	Biglycan	4
6	*Homo sapiens*	Fetuin-A	4
7	*Homo sapiens*	Periostin	2
8	*Homo sapiens*	Pigment epithelium-derived factor	2
9	*Homo sapiens*	Collagen alpha-2(XI)	2
10	*Homo sapiens*	Collagen alpha-2(V) chain	1
		Total:	83.97

Notes: Data from Warinner et al. (2014a)

Bulk collagen from bone or dentine is usually the preferred substrate for carbon and nitrogen stable isotope-based dietary reconstructions for three reasons: (1) its biochemistry is well known, (2) it is the major nitrogen source in skeletal remains, and (3) the atomic ratio of C to N obtained from a sample provides a robust quality indicator that can even reliably assess the isotopic integrity of material more than 100,000 years old (Britton et al., 2011; De Niro, 1985; Van Klinken, 1999). However, there are a few considerations to bear in mind when interpreting collagen isotope ratios. Due to a slow but constant collagen turnover, results obtained from adult human bone collagen represent an averaged protein diet over many years prior to death, depending on the collagen turnover rate of the bone sampled (Hedges et al., 2007; Hill, 1998; Schwarcz & Schoeninger, 1991). By contrast, dentine collagen exhibits almost no turnover, and thus the isotopic values measured from dentine primarily reflect the protein sources consumed during the short interval in which the dentine of each tooth was formed, mainly during childhood (Beaumont et al., 2012), with the added uncertainty that the values could have been influenced by breastfeeding and weaning (Eerkens et al., 2011; Fuller et al., 2006).

6.3.2 Collagen and Bioapatite Carbon Stable Isotopes

Carbon stable isotope values of collagen are used to distinguish between the consumption of marine (^{13}C enriched) or terrestrial (^{13}C depleted) foods in the diet (Chisholm et al., 1982; Peterson & Fry, 1987; Schoeninger & DeNiro, 1984), although the interpretation of $\delta^{13}C$ values becomes more complicated if fish obtained from brackish water are included in the diet (Eerkens et al., 2013; Grupe et al., 2009; Salazar-García et al., 2014b). $\delta^{13}C$ can also be used to estimate the relative dietary proportions of plants performing C_4 versus C_3 photosynthesis (O'Leary, 1981; Van der Merwe & Vogel, 1978). This is useful because all trees, shrubs and herbs, as well as temperate or shade-adapted grasses, are C_3 plants (^{13}C depleted), while C_4 plants (^{13}C enriched) include many important domesticated tropical grasses (e.g., maize, sorghum, millet, and sugar cane).

Alternatively, biological apatite from bone or enamel can be analysed as a target for carbon stable isotope ratio analysis (Lee-Thorp, 2008). Of these two skeletal tissues, enamel apatite is preferred, as several studies suggest that bone apatite is more vulnerable to the kinds of diagenesis that frequently influence isotope composition during post-depositional processes (Lee-Thorp, 2002; Wang & Cerling, 1994). One advantage of studying enamel is the fact that enamel apatite $\delta^{13}C$ reflects the composition of the overall diet, in contrast to collagen, which primarily reflects the protein portion of the diet (Lee-Thorp & Sponheimer, 2006). A second advantage is the possibility of retrieving direct dietary information from deep times when collagen is no longer present. However, one disadvantage is the fact that, in the absence of paired nitrogen data, it is not always straightforward to interpret elevated $\delta^{13}C$ values.

6.3.3 Collagen Nitrogen Stable Isotopes

Collagen nitrogen stable isotope ratios are typically used to estimate trophic level because they increase by approximately 3–5‰ with each step up an idealized food-chain (De Niro & Epstein, 1981; Minagawa & Wada, 1984; Schoeninger et al., 1983), with some acknowledgement of inherent variation among these values (Hedges & Reynard, 2007; O'Connell et al., 2012). Although this can be further complicated by special cases in which plants have higher $\delta^{15}N$ values than expected (e.g. Warinner et al., 2013) or in cases of animal tissue enrichment due to urea-recycling under water stress (see review in Pearson, 2007), $\delta^{15}N$ values can still allow diets rich in plant proteins to be distinguished from those rich in animal proteins (Bol & Pflieger, 2002; Fahy et al., 2013; Petzke et al., 2005). Additionally, because aquatic food chains tend to contain more trophic levels than terrestrial ones, individuals consuming diets rich in marine or freshwater resources typically have higher $\delta^{15}N$ stable isotope values than individuals consuming predominantly terrestrial food products (Schoeninger et al., 1983; Schoeninger & De Niro, 1984). Furthermore, when combined, nitrogen and carbon values can discriminate between the consumption of aquatic foods and C_4 terrestrial foods.

6.4 New Directions: Stable Isotope Analysis of Bulk Dental Calculus

6.4.1 Need for Alternatives to Collagen in Isotopic Analysis

Although carbon and nitrogen stable isotope analysis of collagen has been used to successfully infer diet from both recent and very ancient archaeological remains, the collagen of very old specimens is often not well preserved. In general, collagen preservation decreases over time, and well-preserved collagen is very rarely found in bones dating to earlier than the Upper Palaeolithic or late stages of the Middle Palaeolithic (ca. 40–60 kya). As a result, there has been a great deal of recent interest in investigating whether there are alternative biological substrates that could be used in isotopic analyses, such as coprolites (Ghosh et al., 2003), carbonized sherd residues (Hart et al., 2009), or the bone collagen of animals with human-like diets (Guiry & Grimes, 2013).

Given that dental calculus appears to preserve better than bone or dentine, it has been proposed as a possible surrogate substrate for stable isotope analysis of very old archaeological remains, archaeological remains whose collagen is not well preserved, and/or human remains for which destructive analysis of bone is not permitted (Scott & Poulson, 2012). Moreover, if sampling could be performed at a microscopic level, potentially each incremental layer of dental calculus could be analyzed separately, thereby providing a temporally ordered record of isotopic values spanning the period of adulthood of the individual, something that is not possible using any other conventional technique.

6.4.2 Suitability of Dental Calculus for Isotopic Measurement

Before considering issues of paleodietary reconstruction and the interpretation of dental calculus isotopic values, it is first necessary to establish that dental calculus contains sufficient quantities of carbon and nitrogen to permit accurate isotopic measurement. Until very recently, this was an open question and a highly debated topic.

Initially, many scientists viewed archaeological dental calculus as an almost exclusively inorganic substrate and expressed doubt as to whether dental calculus contained sufficient endogenous biomolecules for isotopic measurement and analysis. Such opinions stemmed in part from conflicting and, at times, out-dated and erroneous information in the published literature. For example, as recently as 2002, reviews of dental calculus in prominent oral biology journals (e.g., Jin & Yip, 2002) reported that because dental plaque undergoes "extensive degradation" *in vivo*, dental calculus is entirely devoid of nucleic acids. The data used to support this view, however, was nearly 40 years out of date (Little et al., 1966) and originated from an era when methods for the detection of nucleic acids were crude at best. Subsequent analyses of archaeological dental calculus revealed a high abundance of *in situ* DNA within calcified cells (Preus et al., 2011) and quantities of DNA equivalent to those reported for similarly sized samples of fresh animal tissues such as liver, kidney, and blood (Warinner et al., 2014a).

Likewise, similar doubts were cast upon the prospect of recovering a sufficient nitrogen signal in archaeological dental calculus. This was true despite the knowledge that proteins make up 8.6% of the total dry weight of modern dental calculus (55% of the organic fraction; Jin & Yip, 2002) and the fact that a 12,000-year old specimen of dental calculus had been shown to retain functional epitopes of the dental caries pathogen *Streptococcus mutans* (Linossier et al., 1996).

6.5 Paleodietary Inference from Dental Calculus

6.5.1 Initial Studies

In 2012, Scott and Poulson conducted the first carbon and nitrogen isotopic study of archaeological dental calculus (Scott & Poulson, 2012). Noting that bone, teeth, fingernails, and hair had all proven valid tissues for isotopic analysis and paleodietary inference, they posed the question of whether dental calculus might serve as an additional isotopic proxy. As an additional motivation, they explained that it might be easier to secure permission for destructive analysis of dental calculus compared to bone collagen, in part because dental calculus is not universally viewed as an integral or inherent part of the human body. Thus, for regions or communities where there is heightened sensitivity regarding the destructive analysis of human remains, dental calculus may offer a productive middle ground. A major focus of

this first study was simply establishing that dental calculus contained sufficient quantities of carbon and nitrogen for isotopic measurement.

Focusing on 59 dental calculus samples from medieval Spain, they reported an average C weight percent of 4.9% (range 1.7–8.5%) and an average N weight percent of 0.7% (range 0.3–4.5%) in archaeological dental calculus. They followed this study up with a similar analysis of much older samples (>4000 years old) from Chile and found that these specimens contained an even greater organic content (Poulson et al., 2013; Scott et al., 2013). Assuming the availability of a starting sample size of up to 10 mg of dental calculus, the estimated amount of available carbon and nitrogen in both the Spanish and the Chilean samples falls fully within the dynamic range of modern isotope-ratio mass spectrometer (IRMS) instruments. Thus, assuming that the measured %C and %N values are typical of archaeological specimens, dental calculus contains sufficient quantities of carbon and nitrogen for isotopic analysis.

A second goal of the study was to determine if the measured $\delta^{13}C$ and $\delta^{15}N$ values provided dietary information. Isotopic data were generated for 59 samples from northern Iberia (n = 58) and Alaska (n = 1), but unfortunately no comparative isotopic data were measured from bone collagen, bone apatite, or other tissue. The data were found to form a diffuse isotopic cluster with a mean $\delta^{13}C$ of −21.2‰ and a mean $\delta^{15}N$ of 11.8‰ (Fig. 6.3). The data were then compared to modeled food sources and interpreted within an isotopic framework developed from bone collagen models. The authors concluded that the results were consistent with the hypothesis that the isotopic composition of dental calculus tracks the isotopic composition of the diet, they followed up this study a year later with a similar experimental approach on 35 dental calculus samples from prehistoric Chile (Scott et al., 2013). As before, no samples of bone collagen or apatite were tested for comparison; however, they
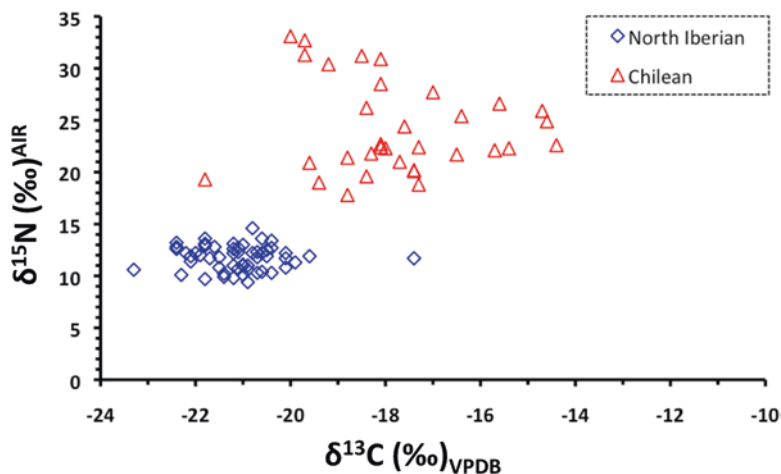

Fig. 6.3 Plot of published bulk dental calculus $\delta^{13}C$ and $\delta^{15}N$ values from Northern Iberian and Chilean populations. (Scott et al., 2013; Scott & Poulson, 2012)

did include in their analysis previously published isotopic data for South American flora and fauna, as well as unrelated samples of mummified hair and muscle. The Chilean dental calculus samples exhibited a high degree of isotopic spread and exceptionally high $\delta^{15}N$ values, ranging from 17.8‰ to 33.1‰ (Fig. 6.3), which they attributed to probable consumption of marine resources and/or consumption of isotopically heavy plants fertilized with guano. Since Peruvian and Chilean prehistoric populations routinely have $\delta^{15}N$ values of over 20‰ (e.g. Silva-Pinto & Salazar-García, 2015; Tomczak, 2003) and maize grown in fields fertilized with seabird guano can have $\delta^{15}N$ values higher than 30‰ (Szpak et al., 2012), such high $\delta^{15}N$ values in dental calculus could reflect a dietary signal.

Although both studies present plausible paleodietary scenarios, much still remains unknown about the biology of dental calculus and the degree to which it accurately reflects dietary information, if at all. Unlike collagen, apatite, fingernails, and hair, dental calculus is a complex microbial biofilm (and not a human tissue), and thus there can be no *a priori* expectation that dental calculus will function similarly to human tissues as an isotopic proxy in paleodietary studies. Both studies drew attention to the fact that basic research on the biology of dental calculus formation is needed, as are comparative studies of dental calculus and bone collagen, and – if possible – controlled feeding experiments, although the latter is likely to be challenging given the length of time required for calculus formation and the limited number of species in which it naturally forms.

6.5.2 *Recent Studies Comparing Isotopic Values from Dental Calculus and Conventional Tissues*

In response to the need for empirical comparisons, three independent isotopic studies were recently conducted on archaeological samples from medieval Spain, central California and the Sudan, and Greece (Eerkens et al., 2014; Price et al., 2018; Salazar-García et al., 2014a). In contrast to the articles by Scott and Poulson, these studies compared carbon and nitrogen isotopic values measured from paired specimens of dental calculus and bone collagen, and bone apatite (in Eerkens et al., 2014) and dentine collagen (in Salazar-García et al., 2014a), in order to test the hypothesis that dental calculus isotopic values can serve as an isotopic proxy for diet. Further, the Price study attempts to isolate different components of calculus and analyse stable isotope signatures in each. To do this, they split larger calculus samples into two, and pre-treated half with HCl and NaOH to generate an "organic" component, and the other half with NaOCl to generate a "mineral" component, though they only report $\delta^{15}N$ for the former.

Results from the Salazar-García and Eerkens studies were generally positive, but included some discrepancies in the C and N stable isotope ratios of calculus and bone collagen. Salazar-García et al. (2014a) focused on a Medieval period site in Spain. They found that $\delta^{13}C$ and $\delta^{15}N$ from calculus and bone collagen produce similar mean values among the 35 individuals included in their study. Mean values

were more divergent for $\delta^{13}C$ (calculus = $-15.5‰$ vs. bone = $-16.4‰$) than $\delta^{15}N$ (calculus = $12.18‰$ vs. bone = $12.12‰$), but when six calculus samples with C:N >12 are removed the $\delta^{13}C$ mean ($-16.5‰$) is also nearly identical to the bone mean.

At the same time, Salazar-García et al. (2014a) also show that calculus isotopic values are much more variable than bone collagen. Even removing the six samples with unusual C:N, the range of $\delta^{13}C$ values is 92% higher in the calculus samples, and 287% greater for $\delta^{15}N$. Thus, while isotopic values in calculus track site-level averages well, they do not track individual-level paleodietary indicators.

Eerkens et al. (2014) arrive at similar conclusions, especially once calculus samples with high C:N ratios are excluded. Their database includes samples from multiple prehistoric sites in California and the Sudan. They show 30–50% greater variation and higher ranges of values in calculus than bone collagen stable isotopes. At the same time, they record positive linear correlations for $\delta^{13}C$ and $\delta^{15}N$ between calculus and bone collagen across all their samples, though the strength of these correlations is lower within particular sites. This suggests, again, that stable isotope data from calculus records the same general dietary information as bone collagen at a regional level, but does not replicate individual-level dietary differences within sites as well. They also show significant intra-individual variation in calculus isotopes when multiple calculus samples are analyzed from different teeth. Correlations between $\delta^{13}C$ in calculus and bone apatite were not as apparent regionally.

The Eerkens et al. (2014) study also suggests site-specific shifts in stable isotope signatures of calculus relative to bone collagen. At some sites $\delta^{13}C$ in calculus tends to be higher, at others lower, and at others about the same, as bone collagen $\delta^{13}C$. On the other hand, calculus $\delta^{15}N$ was nearly always greater than bone collagen $\delta^{15}N$.

Finally, the Price et al. (2018) study also shows greater variation in calculus isotopes than in collagen. As well, they show little to no correlation in $\delta^{13}C$ between bone collagen and the organic component of calculus, nor in $\delta^{13}C$ between bone apatite and the mineral component of calculus. They do find a significant and positive correlation between $\delta^{15}N$ in the organic component of calculus and $\delta^{15}N$ in bone collagen. However, they reject this result as representing a consistent dietary signature between calculus and bone because of a simultaneously strong and positive correlation between $\delta^{15}N$ and C/N in calculus. They suggest that nitrogen within calculus is consumed and recycled by bacterial species causing an increase in $\delta^{15}N$ (i.e., a trophic-level effect) as more and more nitrogen is consumed by bacteria (causing C to increase relative to N). Interestingly, this relationship between $\delta^{15}N$ and C/N is not present in the Salazar-García and Eerkens studies.

In sum, while the Salazar-García and Eerkens studies suggest promise for the potential of stable isotope analysis of calculus as a means to reconstruct paleodiet, at least at a regional or population level, the Price et al. (2018) study on chemically-treated calculus samples suggests greater caution. All three studies are consistent in showing that calculus isotopes do not reproduce individual-level dietary information as determined from bone collagen. This is due to apparent site-specific differences in either the preservation or composition of calculus, or perhaps due to recycling of nitrogen by bacteria within calculus. Clearly, more analysis on calculus is warranted, and below, we outline areas where we think additional research can help resolve some of these issues.

6.5.3   Potential Problems and Pitfalls in Isotopic Analysis of Dental Calculus

One important issue that has not yet been sufficiently emphasized is the strongly heterogeneous composition of the organic fraction of dental calculus in bulk analyses. Unlike conventional tissues analyzed using stable isotope analysis, such as bone and hair, which are each characterized by a single dominant class of proteins (collagens and keratins, respectively), dental calculus is composed of a large number of whole bacterial cells, lipids and exopolysaccharides, and hundreds of diverse host and bacterial proteins. Among the more than 250 human and bacterial proteins that have been thus far identified within human dental calculus, most are bacterial in origin although several human proteins may reach relatively high abundance, especially those relating to immune function (Warinner et al., 2014a). However, the dominant classes of both microbial and human proteins vary from individual to individual. The observation that dental calculus has been found to have no consistent C:N value (Eerkens et al., 2014; Price et al., 2018; Salazar-García et al., 2014a; Scott et al., 2013) reflects the heterogeneous nature of its composition.

With respect to the extraordinary number of bacteria present within dental calculus (>200 million cells per mg), Warinner et al. (2014a) used genetic analysis to characterize more than 2600 bacterial OTUs (Operational Taxonomic Units, a classification unit of microbes approximating species) within ancient human dental calculus. Some of these bacterial taxa are known to be saccharolytic and consume dietary sugars and salivary glycoproteins (e.g., *Streptococcus mutans*), while others are proteolytic and consume host tissues (e.g., *Porphyromonas gingivalis*). Thus, dental calculus bacteria consume nutrients of different origins and at different trophic levels with potentially different isotopic values. With so many potential carbon and nitrogen sources, each with a potentially different isotopic shift relative to host diet, it is unclear what the carbon and nitrogen isotopic values from dental calculus signify.

6.6   Case Study: Human Subsistence During the Basketmaker II Period at Grand Gulch

6.6.1   Study Design and Archaeological Context

Though previous studies have suggested that stable isotope ratios of bulk calculus approximate those from collagen at a population level, only a handful of groups have yet been studied. Oral microbiota and diets vary significantly among human populations, which may influence the relationship between calculus and collagen isotope values. Only by examining a variety of groups can we begin to establish the factors that influence calculus isotope values. In order to test whether the carbon and nitrogen stable isotope values of dental calculus correlate with those of bone

collagen in an archaeological population with a known, primarily C_4 diet, we analyzed bulk dental calculus obtained from a Basketmaker II (400 BC-500 AD) population at the Grand Gulch archaeological site in southern Utah, USA. Located within the American Southwest, the Grand Gulch site was home to early maize farming communities of the Ancestral Puebloan culture (also known as the Anasazi) (Coltrain et al., 2007). The samples analyzed in this study originate from Grand Gulch Cave 7, which was originally thought to contain the victims of a single massacre (Coltrain et al., 2007). However, recent radiocarbon dating of the Cave 7 skeletal remains indicates that burials were interred over several centuries spanning ca. 205 B.C. to A.D. 536 (Coltrain & Janetski, 2013).

Previous analyses of bone collagen at the site have indicated that Basketmaker II populations subsisted primarily on C_4 resources, with maize as the staple agricultural crop. The transition to a maize-based diet among Basketmaker II populations is thought to have occurred both early and rapidly, and nitrogen isotopic analyses suggest that animal protein consumption was low (Coltrain et al., 2007). Because the Grand Gulch site has been previously well characterized and its subsistence history is well-supported by both conventional collagen-based stable isotopic analysis and archaeological evidence, it presents an ideal case study opportunity for testing whether carbon and nitrogen isotopic values measured from dental calculus can serve as a paleodietary proxy in subsistence reconstructions.

6.6.2   Methods

We collected supragingival dental calculus from the lingual or buccal aspects of 17 human teeth obtained from individuals whose bone collagen carbon and nitrogen isotope values had already been published (Coltrain & Janetski, 2013). In order to ensure that some calculus remained *in situ* for future studies, we only collected a subset of dental calculus from each individual. Samples were then transferred to the laboratory of the Plant Foods in Hominin Dietary Ecology research group at the Max-Planck Institute for Evolutionary Anthropology (Leipzig, Germany), where they were further processed prior to analysis following preparation described in Salazar-García et al. (2014a).

The carbon and nitrogen isotope ratios of homogenised bulk dental calculus were measured in duplicate at the Department of Human Evolution, Max-Planck Institute for Evolutionary Anthropology (Leipzig, Germany). The samples were combusted in an elemental analyzer Flash EA 2112 coupled to a Delta XP continuous-flow IRMS (Thermo-Finnigan©, Bremen, Germany). Stable carbon isotope ratios were measured relative to VPDB (Vienna PeeDee Belemnite) and stable nitrogen isotope ratios were analyzed relative to AIR (atmospheric N_2). The results are expressed using delta notation (δ) in parts per thousand (per mil, ‰). Repeated analysis of internal and international standards determined an analytical error better than ±0.2‰ (1σ) for both $\delta^{13}C$ and $\delta^{15}N$ values.

6.6.3   Results

The mean $\delta^{13}C$ of bulk dental calculus from the Basketmaker individuals is $-14.7 \pm 4.1‰$ (1σ; min: $-25.4‰$, max: $-10.8‰$), and the mean $\delta^{15}N$ is $9.9 \pm 1.7‰$ (1σ; min: $5.6‰$, max: $12.1‰$). If interpreted as collagen values, the calculus C and N values suggest that some individuals in this population consumed mainly C_3 terrestrial resources, some consumed mainly C_4 resources, others consumed a mixed diet of C_3–C_4 terrestrial resources, and yet others likely consumed aquatic resources. In other words, the residents of Grand Gulch appear to be practicing nearly all possible subsistence strategies. However, when plotted on a graph together with paired bone collagen carbon and nitrogen isotopic values measured from same individuals (Fig. 6.4), a very different picture emerges.

The bone collagen $\delta^{15}N$ values as well as the $\delta^{13}C$ values of both bone collagen and bone apatite plot within a narrow distribution that strongly suggests a heavy dietary reliance on C_4 terrestrial resources, such as maize (Coltrain & Janetski, 2013). Analysis of the dental calculus isotopic values in isolation, without comparative isotopic data from bone collagen and bone apatite, would have resulted in a highly inaccurate characterization of Basketmaker II diets as having been highly heterogeneous and diversified, when in fact both bone collagen and bone apatite isotopic data overwhelmingly indicate that they were exactly the opposite.

The mean $\delta^{13}C$ and $\delta^{15}N$ values of the Basketmaker II collagen, apatite, and dental calculus substrates are each quite different (Table 6.2), and there is a higher degree of inter-individual $\delta^{13}C$ and $\delta^{15}N$ variation in dental calculus compared to either bone collagen or bone apatite (Fig. 6.4). The ranges of dental calculus $\delta^{13}C$

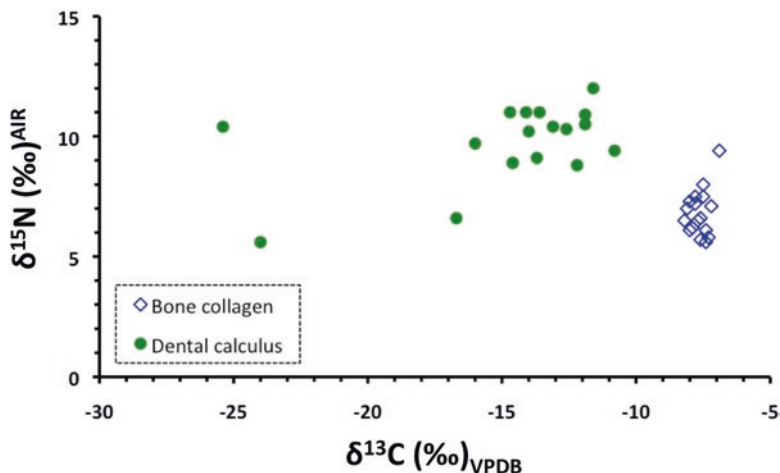**Fig. 6.4** Comparison of $\delta^{13}C$ and $\delta^{15}N$ values obtained from paired bulk dental calculus and bone collagen samples from a Basketmaker II population at the Grand Gulch site. Note the lack of concordance between bulk dental calculus and bone collagen with respect to $\delta^{13}C$ and $\delta^{15}N$ values and sample isotopic variance

Table 6.2 Basketmaker $\delta^{13}C$ and $\delta^{15}N$ adult human bulk dental calculus, bone collagen and apatite values, collagen control indicators (%C, %N, C:N), S-EVA number and curation number

S-EVA	Curation number	Teeth sampled	$\delta^{13}C_{calc}$ (‰)	$\delta^{13}C_{coll}$ (‰)	$\delta^{13}C_{apt}$ (‰)	$\delta^{15}N_{calc}$ (‰)	$\delta^{15}N_{coll}$ (‰)	C:N$_{calc}$ atomic ratio	C:N$_{coll}$ atomic ratio	%C$_{calc}$	%N$_{calc}$
25455	99/7332	Lingual upper right C + lingual lower left C	−11.9	−8.0	−4.2	10.5	7.3	6.4	3.3	6.5	1.2
25456	99/7336	Buccal upper right M2 + buccal upper left M2	−12.6	−7.8	−3.3	10.3	7.5	6.3	3.3	3.8	0.7
25457	99/7339	Labial upper left P1	−14.0	−7.7	−3.6	10.2	6.5	6.5	3.3	5.0	0.9
25458	99/7340	Lingual lower left P1 + buccal upper right M1	−14.7	−7.5	−2.7	11.0	7.5	6.4	3.3	5.7	1.0
25459	99/7342	Buccal upper left M2	−14.6	−7.4	−2.2	8.9	5.6	8.8	3.3	6.5	0.9
25460	99/7346	Lingual lower right C	−11.9	−7.5	−3.1	10.9	8.0	5.8	3.3	6.1	1.2
25461	99/7348	Distal lower left M1	−11.6	−6.9	–	12.0	9.4	8.7	3.3	5.2	0.7
25462	99/7353	Buccal upper left M2 + buccal upper right M2	−13.7	−8.0	–	9.1	6.1	5.0	3.2	5.5	1.3
25463	99/7357	Labial lower right I2 + lingual lower left P1	−16.0	−7.6	−2.6	9.7	6.6	10.3	3.2	4.8	0.5
25464	99/7358	Buccal upper left M2	−16.7	−8.1	−2.7	6.6	7.0	8.2	3.3	4.3	0.6
25465	99/7364.2	Lingual upper left P3 + mesolingual lower right P1	−24.0	−7.8	−3.1	5.6	7.2	17.0	3.3	20.7	1.4
25466	99/7365	Lingual lower left M2 + buccal upper left M1	−10.8	−7.6	−2.4	9.4	5.7	6.6	3.2	5.2	0.9
25467	99/7384	Lingual lower right P2	−13.6	−7.9	−4.6	11.0	6.2	6.8	3.2	4.5	0.8
25468	99/7420	Buccal left upper M1 + distalbuccal left lower M3	−14.1	−7.4	–	11.0	6.1	5.9	3.2	6.2	1.2
25469	99/7422	Messiobuccal upper right M2 + buccal lower right M1	−12.2	−8.2	–	8.8	6.5	8.1	3.3	8.3	1.2
25470	99/7349	Mesial lower left M1	−25.4	−7.2	−2.9	10.4	7.1	5.4	3.3	5.6	1.2
25471	99/7338	Lingual lower right P2	−13.1	−7.3	−2.8	10.4	5.8	6.7	3.2	6.0	1.1

and $\delta^{15}N$ are much wider than those observed in either bone collagen or bone apatite, and there is no consistent correlation between the isotopic values of dental calculus and either bone collagen or bone apatite (Fig. 6.5). Linear regression of bone collagen and bulk calculus $\delta^{13}C$ and $\delta^{15}N$ values show no relationship between these substrates (carbon $R^2 = 0.01$, Fig. 6.6a; nitrogen $R^2 = 0.06$, Fig. 6.6b), indicating that their isotopic values are independent from each other and must be evaluated separately. The same is true for bone apatite and bulk calculus (carbon $R^2 = 0.02$, Fig. 6.6c).

For some individuals, the lack of correlation between dental calculus and bone collagen values is quite stark. For example, the bone collagen $\delta^{13}C$ values for individuals 25,465 and 25,466 differ by only 0.2‰, a trivial difference within the range of analytical error for the instrument. However, the dental calculus $\delta^{13}C$ values of the same two individuals differ by >13‰, a substantial difference that if seen in bone collagen would indicate that the two individuals were eating radically different diets at either end of the C_3-C_4 spectrum. Similar, although less extreme, differences
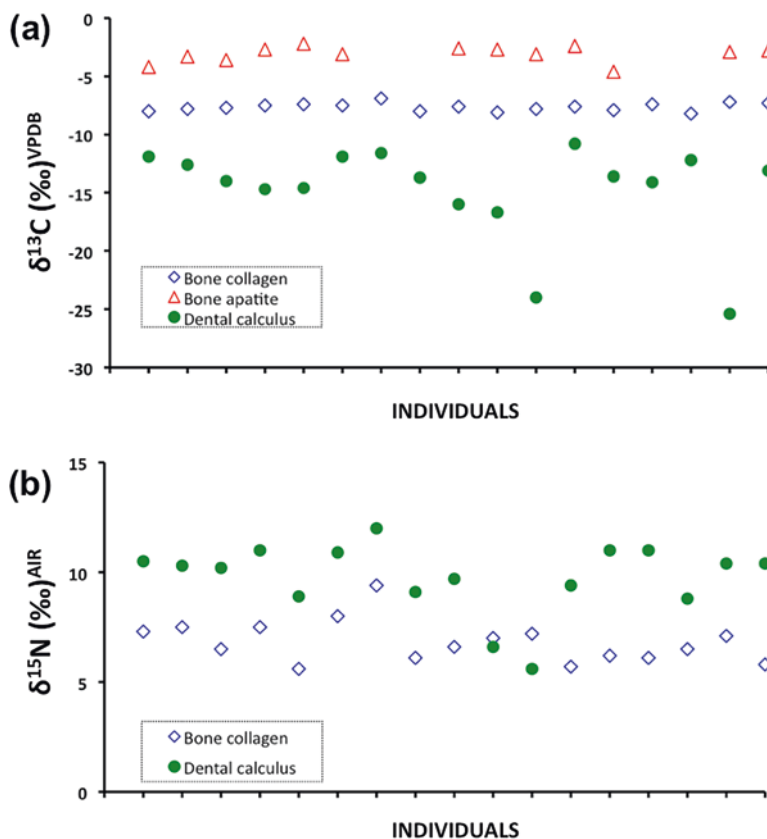
Fig. 6.5 Direct comparison of (**a**) $\delta^{13}C$ and (**b**) $\delta^{15}N$ values measured from bulk dental calculus, bone collagen, and bone apatite samples in the Basketmaker II individuals
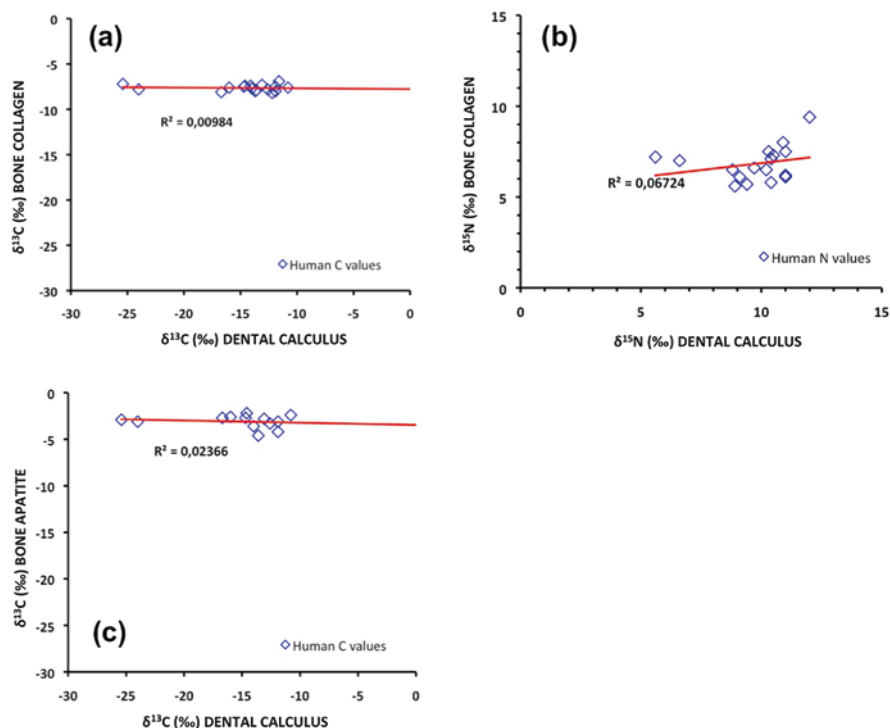
Fig. 6.6 Lack of correlation between bulk dental calculus and bone collagen isotopic values for the Grand Gulch Basketmaker II population. Linear regression of paired specimens of bulk dental calculus and either (**a, b**) bone collagen or (**c**) bone apatite yields no significant correlation with respect to (**a, c**) $\delta^{13}C$ or (**b**) $\delta^{15}N$ values

are observed with respect to nitrogen in which paired bone collagen and dental calculus $\delta^{15}N$ values differ by as little as 0.4‰ and as much as 4.9‰. Such differences span more than a trophic level.

6.6.4 Discussion

There is no ready explanation for the extreme isotopic differences observed between calculus and collagen values, except for the fact that they are compositionally distinct and dental calculus is known to be a heterogeneous substrate. On the basis of the evidence, we must conclude that bulk dental calculus does not faithfully record the isotopic signal derived from the averaged diets of individuals. There is no statistical correlation between the carbon and nitrogen stable isotope values of bulk dental calculus and either bone collagen or bone apatite, and thus bulk dental calculus

cannot serve as a simple collagen or apatite substitute in stable isotope-based paleo-dietary models for studying human behaviour.

Concern that some of the high isotopic variance observed in dental calculus samples may be attributable to taphonomy has raised the issue of how to distinguish well preserved and poorly preserved dental calculus. The metrics used to identify well preserved archaeological collagen (%C, >35%; %N, >10%; C:N, 2.9–3.6) (De Niro, 1985) are not relevant to understanding the preservation quality of calculus because these values are specific to the protein sequence of collagen. Other proteins, such as serum albumin and alpha-1 anti-trypsin, two proteins found to be abundant within modern and ancient dental calculus (Table 6.1), have higher C:N than collagen (3.7 and 3.9, respectively), and lipids, which make up >10% of the organic fraction of dental calculus (Jin & Yip, 2002), typically contain no nitrogen at all. There are no alternative quality markers for dental calculus yet published, although most of the calculus samples reported in Eerkens et al. (2014) and Salazar-García et al. (2014a) show C:N ratios between 6.5 and 10, which led Eerkens et al. (2014) to suggest removing dental calculus with a C:N ratio higher than 12. The observed %C, %N, and C:N for dental calculus are quite different from what is expected for bone/dentine collagen (Table 6.2). In particular, the C:N of dental calculus is extremely variable between samples, ranging from 5 to 17, as is expected for a heterogenous substrate not dominated by a single protein, nor necessarily even a single macromolecule type. Because biological variation alone can account for the wide variance observed in dental calculus C:N, it is not necessary to invoke taphonomy as an explanation for these values, nor is it justified to exclude C:N outliers without further evidence. In sum, the problem of how to quickly and inexpensively assess preservation in dental calculus remains unresolved.

While we acknowledge that, at a population level, average bulk dental calculus isotopic values exhibit a coarse correlation with those of bone collagen, it is clear that the biology underlying any such correlation is complex and poorly understood. We feel strongly that both the theoretical and empirical uncertainties observed at the level of individuals are sufficient to suggest that the application of this new approach must be taken with extreme caution when attempting to reconstruct ancient human behaviour.

6.7 Conclusion and Future Perspectives

The observed differences in the $\delta^{13}C$ and $\delta^{15}N$ values and the associated control parameters (%C, %N, C:N) between dental calculus, bone collagen, and bone apatite highlights the fact that bulk dental calculus is a highly heterogeneous substrate. Although the source of the inorganic portion of dental calculus is primarily dissolved salivary calcium and phosphate ions, the organic portion is a highly complex mixture of whole bacterial cells (>200 million cells/mg), microbial extracellular

matrix (e.g., exopolysaccharides, proteins, extracellular DNA), blood and other host-derived substances (e.g., immune proteins), and foreign debris (e.g., dietary microfossils). Moreover, the organic composition of dental calculus varies by individual with respect to the frequencies of bacterial taxa present, the proportions of human to bacterial proteins, and the presence or absence of foreign debris. As such, it is unsurprising that dental calculus has a more variable C:N ratio and that there is no significant correlation between the carbon and nitrogen stable isotope values of bulk calculus and human tissues such as bone collagen or bone apatite.

Rather than investigating dental calculus as a bone collagen substitute or analogue, a more productive approach would be to focus on the unique properties of dental calculus and its specific strengths, namely the fact that is a rich and diverse source of many ancient biomolecules. By its nature, bulk dental calculus is too biologically complex to ever be adequately described by an isotopic model as simple as that used for bone collagen. However, through the isolation of specific biomolecules from dental calculus, such as specific carbohydrates, lipids, or proteins, more targeted questions about ancient human behavior can be asked. Advances in mass spectrometry will continue to facilitate the analysis of smaller and smaller samples, allowing us to focus individually on these different biomolecular components.

At present, the preponderance of evidence indicates that bulk dental calculus should not be treated in the same manner as that of collagen or apatite when performing stable isotope-based dietary interpretations. This does not mean that there is no future for dental calculus in stable isotope studies, but rather that an evidence-based biological model must first be established before any reasonable interpretation of dental calculus isotopic values can proceed. We do not recommend that bulk calculus be used for stable isotope analysis when any other skeletal material with well-known biochemistry is available, and we caution against the application of dental calculus-based stable isotope analysis even for the interpretation of low-resolution, general dietary trends at a population level. Moreover, we strongly encourage researchers to consider alternative methods for paleodietary reconstruction from dental calculus, such as microfossil, ancient DNA, and ancient protein analysis, that draw on the strengths of dental calculus rather than its weaknesses.

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