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Title: Plant Microremains in Dental Calculus as a Record of Plant Consumption: A Test with Twe Forager-Horticulturalists

1. Introduction:

Starch granules and phytoliths in dental calculus are increasingly used as dietary markers in archaeological investigations. Direct signatures of ancient plant consumption are rare in archaeological contexts, but plant microremains in dental calculus have helped to elucidate diets in many contexts, ranging from the early consumption of domesticates in the Holocene (cf. Henry and Piperno, 2008; Mickleburgh and Pagan Jiminez, 2012; Li et al., 2010; Piperno and Dillehay, 2008) to early hominin plant consumption (Henry et al., 2011, Henry et al., 2012; Henry, 2014; Salazar Garcia et al., 2013). Other direct measures of plant consumption such as carbon stable isotopes or tooth microwear analysis provide only general information on categories of plants consumed or the physical properties of those plants. Plant microremains like starches and phytoliths can be taxonomically distinct, and their presence in dental calculus sometimes reveals the consumption of specific plant families or genera.

Despite promising results in many time periods and geographic regions, we have yet to determine exactly what type of dietary signal plant microremains in dental calculus record. For example, some authors suggest that a high incidence of starches and phytoliths from certain plants indicates that those plants were consumed at high frequency (Henry and Piperno, 2008; Middleton and Rovner, 1994; Piperno and Dillehay, 2008). Others draw comparisons between individuals or groups based on the numbers of plants represented by microfossils (Dudgeon and Tromp, 2012; Henry et al., 2014; Mickleburgh and Pagan-Jiminez, 2012). While such comparisons are logically appealing, we do not yet understand the mechanism for preservation of microremains in dental calculus. It is recognized that calculus formation rates vary among individuals (Jin and Yip, 2002; White, 1997), and some researchers have acknowledged this as a potential source of variation in the preservation of microremains (Henry et al., 2014). However, it is unclear the extent to which individual differences in calculus formation might create individual variation in the microremain record. Starches appear to be more plentiful than phytoliths in modern human dental calculus (Boyadjian et al., 2007; Fox et al., 1994; Fox et al., 1996; Henry and Piperno, 2008; Juan-Tresserras et al., 1997; Scott Cummings and Margennis, 1997), possibly because humans preferentially eat starchy foods, but we do not know what other biases may exist in the dental calculus record.

Here we present the first comparison of diet and plant microremains in dental calculus from a living population with a well-documented diet. We report on the relationship between plant consumption and plant microremains in dental calculus from Tve forager-horticulturalists in order to characterize the preservation of plant microremains in human dental calculus. Our analyses address the following questions: 1. Is diet consistently recorded across all individuals in the same population, given their similar diet? 2. Do plant microremains in Tve dental calculus reflect the range of plants consumed? 3. Is starch quantity in dental calculus proportional to dietary concentration? Initial results suggest that

starches and phytoliths do record diet, but that the relationship between diet and microremains preserved in calculus is not as straightforward as previously assumed.

1.1. Background: Plant Microremains and Dental Calculus

Plant microremains are microscopic plant residues with taxonomically specific diagnostic features. Microremains include but are not limited to starch granules, phytoliths, diatoms, spores, and pollen granules. This paper discusses only starch granules and phytoliths. Starch granules are comprised of complex carbohydrates and are formed in plant tissues for energy storage. Starches are formed in specialized plant organelles called amyloplasts. Starch granule formation begins at a central point called the hilum, and continues with alternating layers of amylose and amylopectin. The alternation of amylose and amylopectin results in a semi-crystalline structure which gives starch some unique properties, such as a polarization cross under cross-polarized light (Barton and Fullagar, 2006; Field, 2006; Gott et al., 2006). Plants produce two types of starch, transient and reserve starch. Transient starches are formed for short term energy storage in photosynthetic tissues like leaves, while reserve starch is formed for long term energy storage in plant storage organs, fruits, and seeds (Gott et al., 2006; Henry, 2012; Sivak and Preiss, 1988). Transient starch morphologies are simple and of limited use in dental calculus studies (Shannon et al., 2009). Reserve starch morphologies may vary along taxonomic lines (Reichert, 1913; Torrence, 2006), but also within species and within individual plants. Surface features like the presence and placement of the hilum, striations called lamellae, cracks, and fissures, as well as the shape and symmetry of the polarization cross are used to distinguish between starches from different taxa (Torrence et al., 2004; Torrence and Barton, 2006).

While starch can survive for thousands of years in certain conditions, heat and moisture cause starches to gelatinize, and acidic conditions and enzymatic activity also damage starches. Dental calculus provides a protective environment that facilitates starch survival (Henry, 2012).

Phytoliths are microscopic noncrystalline silica bodies that are formed in and between plant cells when soluble silica from the ground water precipitates into plant tissues (Henry, 2012; Pearsall, 2000; Piperno, 2006). Phytoliths provide structural support and defense against herbivory (Weiner, 2010). Many plants produce phytoliths, and phytolith production is largely under genetic control, such that phytolith-producing plants tend to occur in the same families, genera, and species, regardless of region of origin (Bamford et al., 2006). Environmental conditions including the soil temperature and water content, concentration of monosilicic acid in the soil, soil pH, and climate can also affect phytolith production (Madella et al., 2002; Piperno, 1988). Phytolith concentration is highest in leaves, husks, rinds, bark, and fruits (Piperno, 2006; Rovner, 1983). Phytolith morphologies are often taxonomically distinct, and may also reflect the specific plant tissue in which they form (Tsartsidou et al., 2007). Diagnostic features include size, shape, texture, and ornamentation (Madella et al., 2005; Piperno, 2006). Phytoliths are soluble in basic conditions (Rovner, 1983), but may persist for millions of years (see Prasad et al., 2005). The oldest phytoliths recovered from dental calculus date to at least 2ma (Henry et al., 2012).

Dental calculus provides a protective environment where starches and phytoliths can survive for thousands of years (Henry et al., 2011). Dental calculus is mineralized plaque which forms both above and below the gingival margin. In this project we consider only supragingival calculus deposits, as calculus was recovered from living people. Supragingival calculus deposits form preferentially near salivary glands in the mouth, on the lingual sides of mandibular incisors and the buccal sides of maxillary molars and premolars (Jin and Yip, 2002; Bergstrom, 1999). Supragingival calculus forms when plaque on tooth surfaces is bathed in calcium and phosphate rich saliva (Jin and Yip, 2002; Lieveise, 1999). The rate of mineralization varies among individuals according to age, oral hygiene, and possibly diet (Bergstrom, 1999; Lieveise, 1999). Smoking increases the rate of calculus formation (Bergstrom, 1999). Dental calculus is 80% inorganic, comprised of calcium phosphate in various phases, including hydroxyapatite, brushite, whitelockite, and octacalcium phosphate (Abraham et al., 2005; Lieveise, 1999). Older deposits tend to be richer in hydroxyapatite, while both young deposits and supragingival calculus are richer in brushite (Schroeder and Bambauer, 1966). The organic portion of calculus includes bacteria, DNA, lipids, proteins, pollen, phytoliths, and starch granules (Hillison, 1996; Lieveise, 1999; Warinner et al., 2014).

Due to individual variability in the amount and rate of calculus formation, we cannot assume a simple relationship between plant consumption and plant representation in dental calculus deposits. We know that starch granules are more common than phytoliths in human dental calculus (Boyadjian et al., 2007; Fox et al., 1994; Fox et al., 1996; Henry and Piperno, 2008; Juan-Tresserras et al., 1997; Scott Cummings and Margennis, 1997), but we do not know what factors bias starch preservation in dental calculus. For example, population-level or individual differences in salivary amylase copy number may correspond to differences in amylase activity (Perry et al., 2007). This in turn could affect the rate of starch digestion in the mouth, which combined with variable calculus formation rates complicates our understanding of starch incorporation. Below we assess how reliably Twa plant consumption is recorded in Twa dental calculus.

1.2. Background: The Twa:

The Twa are a group of forager-horticulturalists who live in Northwestern Namibia and Southwestern Angola. Culturally they resemble the well-studied Himba pastoralists, but most Twa do not own animals and make a living by foraging and gardening (Vashro, 2014). The Twa live in an arid, mountainous environment with marked seasonality. During the rainy season, many Twa grow maize (*Zea mays* L.) which they dry and grind into a coarse meal, as well as pearl millet (*Pennisetum glaucum* (L.)), squash (*Cucurbita* sp.), melons (*Cucurbitaceae*, several species), and sugarcane (*Saccharum* sp.). Twa also collect several wild foods during the rainy season, most notably *Berchemia discolor* (Klotzsch) Hemst. (bird plum) berries which are eaten in large quantities. Gardening is not possible during the dry season, and Twa rely on dried maize meal and foraged foods including fruit from *Hypheane petersiana* Klotzsch ex Mart. (makalani palm) and *Diospyros mespiliformis* L. (jackalberry), as well as various underground storage organs. Since the end of 2007 the Twa have received subsidies of maize meal from the Namibian government, as well as small herds of goats, which produce a limited amount of sour milk. Today the Twa are heavily reliant on the government maize meal subsidies, but still garden and regularly collect a wide range of wild plant foods.

The Twe are semi-mobile. Most people have a 'home' where they spend much of their time, but they also move to different compounds around the region and occasionally visit friends and families in distant locations where different foods may be available. This work focuses on Twe living at a government camp called Otjomoru in the Zebra Mountains and the nearby traditional settlement Okau, and Twe living at a government camp near Epupa Falls called Ohayuua. These camps are considered 'home' locations by many people due to the availability of government maize subsidies. The Twe do not have any access to dental care. Many people occasionally chew on a specific type of stick (called 'omundumise' in Herero), but no one uses toothbrushes, toothpaste, or dental floss, and there is no access to dental care and very limited access to medical care.

2. Methods:

One of us (CL) stayed with the Twe in July-October 2012 (dry season) and April-May 2013 (late rainy season) in order to collect dental calculus samples, dietary information, and samples of plant foods. This project was approved by the University of Utah Institutional Review Board, the Namibian Ministry of Health and Social Services, the Namibian Ministry of Environment and Tourism, and was conducted with a Namibian Research Visa. All work was conducted using an interpreter who is a native Otjiherero speaker. Participants were selected based on presence of calculus deposits, targeting equal numbers of men and women in age groups from 20-29, 30-39, 40-49, 50-59, 60-69, and 70+ years. Participants were informed about the nature of the study and were compensated for their time.

2.1. Diet:

Information on diet was collected using a variety of methods. 1) We interviewed each participant at the time of dental calculus sampling. They were asked about the range of gardened foods in the diet, the range of foraged foods in the diet, the range of commercial foods in the diet, seasonal changes in diet, the frequency of meat consumption, how diet responds to drought, differences in wild plant availability in different locations, the frequency and duration of trips away from the 'home' location, and whether or not the participant smokes (see supplement 1 for a list of questions). 2) We conducted 24-hour diet recalls opportunistically with each participant. The participant listed the number of meals and foods consumed during the preceding 24-hour period. 3) We conducted camp scans where we walked past each compound at meal times and noted what people were eating and approximate quantities.

The following analyses rely on information collected during interviews. 24-hour diet recalls and camp scans were used to gain a general idea of the frequency and relative proportions of common foods in the diet.

2.2. Dental Calculus Collection:

We collected dental calculus from the bucco-mesial surface of the bottom left canine (the Twe remove their mandibular incisors, and often have thick calculus deposits on their canines that extend toward the midline) of 74 participants between the age of 25 and 83, using a new autoclaved dental scaler for each person. The sample consists of roughly equal numbers of men and women of each age

(see supplement 2 for data spreadsheet, which includes participant information). Participants were asked to clean their teeth with a sterile single-use toothbrush, and we cleaned the teeth further by removing plaque before sampling calculus. We removed as much calculus as possible, but in some cases were not able to remove an entire deposit. Samples were stored in aluminum foil inside of plastic micro-centrifuge tubes. At the time of sampling, small bowls of water were placed in the workspace and saved in order to control for airborne contaminants. Calculus samples were weighed to the nearest tenth milligram using a Denver Instruments APX-260 scale.

2.3. Plant Reference Collection:

When possible, specimens of each of the plant foods mentioned in interviews were collected with the help of Twe guides. Samples of all but three of the foraged foods were collected, but many gardened foods were not available because we worked with the Twe during a poor rainy season when gardening was not feasible for most people. However, we did collect the 'staple' gardened foods, which are maize (*Zea mays*), pearl millet (*Pennisetum glaucum*), pumpkin (*Cucurbita sp.*), and sugarcane (*Saccharum sp.*). All plants were cleaned with water and dried with silica gel beads in plastic sample bags. Voucher specimens were dried in a plant press and identified at the Namibian Botanical Research Institute (NBRI). Not all specimens were identifiable at NBRI. We refer to unidentified plant specimens by the Herero names used by the Twe.

Slides for starch description were prepared by directly mounting ground dry plant material on slides with 15 microliters of distilled water and 15 microliters 25% glycerol. Starches were observed at 400x magnification using a Zeiss Axioscope microscope. At least fifty starches were photographed and measured, and a written description was generated for each plant following the terminology of the ICSN (ICSN, 2011). Most plants were collected and observed for starches in both 2012 and 2013 from different locations within the Twe range. We calculated the number of starch granules per milligram dry weight for each plant by mounting 1mg dried, gently ground plant material suspended in water, then counting the number of starches in 9 randomly chosen fields of view on each slide, using an in-house random number generator designed to identify one random field of view within each of nine concentric squares on a 22x22mm microscope cover glass slip. This provided a random sample of all of the starches present on 68 percent of the slide, from which we could then calculate the number of starches in the total sample.

Phytoliths were extracted by digesting between 100 and 500 mg dried plant material in nitric acid. The remaining material was weighed to calculate phytolith content as a percent of dry weight. A heating block was used to speed the reaction, and potassium chlorate was added to further accelerate the process. Phytoliths were weighed and mounted on slides using Permout. Phytoliths were photographed, measured, and described following the ICPN (Madella et al., 2005).

2.4. Dental Calculus analysis:

Each dental calculus sample was dissolved in 10% HCl for up to 2.5 hours. We first tested this concentration of HCl on raw maize and millet starch for up to 24 hours to ensure that starches would not be damaged. Each sample was centrifuged in a Thermo Scientific Heraeus Megafuge 16 centrifuge at

3500rpm for seven minutes, and rinsed twice with distilled water. Supernatant was removed until only 30 micrometers remained. This sample was then mounted on slides with 25% glycerol and observed at 400x magnification. Each microremain was photographed, measured, and compared with the plant reference collection. Starch identifications were made based on size, 2D and 3D shape, the placement of the hilum, presence or absence of lamellae, cracks, fissures, and the placement and shape of the polarization cross. Many starches were not identified because they were nondiagnostic or because they were damaged to an extent that no diagnostic features remained. Phytoliths were identified based on size, shape, and surface features.

2.5. Statistical analyses:

All analyses were conducted using R 3.1.0 (R Core Team, 2014) with MASS package (Venables and Ripley, 2002). Figures were also generated using R 3.1.0. All models are described below. Missing data were excluded from all analyses (see Supplement 2 for data spreadsheet).

3. Results:

3.1. Tve plant foods:

The Tve regularly eat 31 cultivated and foraged plant foods (Table 1). This includes the foods that were mentioned in the majority of interviews. Other plants are included in the diet, but are less common. Maize porridge is consumed the most frequently, and in the largest quantities, followed by various foraged foods depending on the season and location. In the Zebra Mountains, *Berchemia discolor* fruits are eaten in large quantities in the rainy season and *Diospyros mespiliformis* fruits in the dry season. At Ohayuua, *D. mespiliformis* fruits are not available, but palm nuts (*Hyphaene petersiana*) are eaten frequently during the dry season. Palm nuts are not consumed in the Zebra Mountains, because participants say they are not sweet enough. Gardened foods like melons and squash are frequently consumed during the rainy season in years with sufficient rainfall. Of the 31 commonly consumed plant foods, 11 contain starch and 8 contain phytoliths. Table 2 shows the number of starch granules per milligram dry weight. Table 3 shows the phytolith content as a percentage of total dry weight. Figure 1 shows the most common starch types in starch producing foods, and Figure 2 shows the most common phytolith types.

Table 1. Most commonly consumed Tve plant foods

Plant Name	Plant Part Consumed	Preparation
<i>Gardened</i>		
<i>Zea mays</i> L. [maize]	kernels	dried, ground, boiled with water to create porridge
<i>Pennisetum glaucum</i> L. [pearl millet]	grains	dried, ground, boiled with water to create porridge
<i>Arachis hypogaea</i> L. [peanut]	nut	unknown
<i>Citrullus lanatus</i> (Thunb.) Mastumura & Nakai	fruit	raw
<i>Cucurbita</i> sp. [pumpkin, several species]	fruit, leaves	boiled
<i>Cucurbitaceae</i> , other species [melon]	fruit	raw
<i>Daucus</i> sp. [carrot]	taproot	unknown
<i>Fabaceae</i> sp. [beans, species unknown]	bean	boiled
<i>Ipomoea</i> sp. [sweet potato]	tuber	boiled
<i>Opuntia</i> sp. [prickly pear cactus]	fruit	raw
<i>Saccharum</i> sp. [sugarcane]	stalk	raw
<i>Solanum</i> sp. [tomato]	fruit	raw
<i>Foraged</i>		
<i>Berchemia discolor</i> (Klotzsch) Hemst.	berries	raw, often dried
<i>Diospyros mespiliformis</i> L.	fruit	raw, often dried
<i>Ficus sycomorus</i> L. [fig]	fruits	raw or dried and ground, cooked as porridge
<i>Fockea angustifolia</i> K. Schum	tuber	raw
<i>Grewia</i> sp. [ozohamati]	berries	raw
<i>Grewia</i> sp. [ozombapu]	berries	raw
<i>Grewia tenax</i> (Forssk) Fiori [ozonjenjere]	berries	raw
<i>Hyphaene petersiana</i> Klotzsch ex Mart. [Makal]	nut	raw or ground and cooked as porridge
<i>Lapeirousia</i> sp. [ozondungua]	corms	roasted for approximately 5 minutes to remove tunic
unknown species [ombowa/garden weed]	leaves	boiled
unknown species [omundumise/toothbrush stick]	stick	chewed raw to clean teeth
unknown species [ozonduvi]	corms	roasted for approximately 5 minutes
unknown species [otjihakariwa]	tuber	raw
<i>Tylosema esculentum</i> A. Schreib	bean and rhizome	bean preparation unknown, rhizome roasted for an hour or more
<i>Ximenia americana</i> L.	fruit	raw

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Caption: Plant species are listed alphabetically, with the exception of *Zea mays* L. and *Pennisetum glaucum* L. These are highlighted to indicate that they are the most important contributors to the plant portion of the Twe diet.

Table 2. Starch Content of Starch Containing Foods in the Twe Diet

plant	starches/mg
<i>Adansonia digitata</i> (baobab)	5116.77
<i>Cucurbita</i> sp.	8433.5
<i>Fockea angustifolia</i> K. Schum	294.45
<i>Lapeirousia</i> sp.	4988.99
otjihakariwa	616.68
ozonduvi	negligable
<i>Pennisetum glaucum</i> L. (pearl millet)	922.13
<i>Tylosema esculentum</i> A. Schreib	300.01
<i>Ximenia Americana</i> L.	27.78
<i>Zea mays</i> L. (maize)	4366.23

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Caption: Starch contents listed are per milligram dry plant matter.

226 Table 3. Phytolith Content of the Phytolith Producing Foods in the Twe Diet

<i>Cucurbitaceae</i> , unidentified melon fruit	8.19
<i>Ficus sycomorus</i> L.	4.02
<i>G. tenax</i> (Forssk.) Fiori	negligible
<i>H. petersiana</i> Klotzsch ex Mart. fruit	5.14
<i>H. petersiana</i> leaf	9.41
omundumise	0.109
ozombapu	negligible
ozonjenjere	negligible

227 Caption: Phytolith content is listed as the percent of total plant dry weight.

228 Figure 1. Most Common Starch Types in Starch Producing Domesticates in the Twe Diet

229 Caption: All images are shown at the same scale. Each box is 50µm on a side. Images are paired; the
230 first of each pair is shown under brightfield light and the second under cross-polarized light.

231 A-B. *Cucurbita* sp. flesh, small granules, both simple and compound, tend to form in aggregates (a) or
232 occasional compounds, with distinct crosses with thin, curved arms (b); C-D. *Pennisetum glaucum* L.
233 seeds, polygonal granule with a deep stellate fissure, textured surface with radial cracks (c), and
234 characteristic cross with wide, straight arms; E-F. *Zea mays* L. kernels, characteristic polygonal granules
235 with marked hilum, single or three-armed fissures and visible radial cracks (e), distinct crosses with
236 bending arms (f).

237 Figure 2. Most Common Starch Types in Starch Producing Wild Plants in the Twe Diet

238 Caption: All images are shown at the same scale. Each box is 50micrometers on a side. Images are
239 paired; the first of each pair is shown under brightfield light and the second under cross-polarized light.

240 A-B *Adansonia digitata* L. fruit, small spherical granules (a) with centric, straight-armed crosses (b); C-F
241 *Fockea angustifolia* K. Schum tuber, spherical granules with deep radial fissures and lamellae (c) and
242 interrupted straight-armed cross (d) and plano-convex granules (e) with radial fissure and interrupted
243 cross with curved arms (f); G-H *Lapeirousia* sp. corms, tripartite compound granules with eccentric long
244 hilum and radial cracks (g) with indistinct, asymmetric crosses (h); I-L otjihakariwa tuber, large polygonal
245 granule (i) with thin-armed, curved cross (j) and rounded polygonal granule (k) with wide armed cross (l);
246 M-N ozonduvi corms, elongate hemispherical granule with refractive hilum (m) with wavy-armed cross
247 (n); O-R *Tylosema esculentum* A. Schreib tuber, pyriform granule (o) with interrupted cross (p), and large
248 ovoid polygonal granule with distinct lamellae and radial cracks (q) with curved-armed cross (r); S-T.
249 *Ximenia Americana* L. fruit, small unequal compound granules (s) with symmetric straight-armed crosses
250 (t).

251 Figure 3. Most Common Phytolith Types in Phytolith Producing Twe Foods

Caption: All images are shown under brightfield light at the same scale, and boxes are 100µm on the side unless otherwise noted.

A. *Cucurbita* sp. leaf, elongate lanceolate phytolith (possible hair cell); B. *Citrullus lanatus* (Thunb.) Matsumura & Nakai fruit, oblong laminate phytolith ; C. *Ficus sycomorus* L. fruit, lanceolate hair cell; D. *Grewia* sp. (ozombapu) fruit, cylindrical elongate phytolith; E-F. *Grewia tenax* (Forssk.) Fiori fruit, (e) bilobate short cell, (f) acicular hair cell (box is 50µm); G. *Hyphaene petersiana* Klotzsch ex Mart fruit, globular echinate phytolith; H. *Hyphaene petersiana* leaf, spherical granulate phytoliths; I-J. omundumise/toothbrush stick, twig (i) elongate dendritic phytolith, (j) lanceolate dendritic phytolith; K. *Sacharrum* sp. pith, elongate phytolith with three equally-spaced longitudinal ridges, forming a pointy triangle in cross-section.

3.2. Is diet consistently recorded across all individuals?

In general, older people and men had larger calculus deposits than young people (under 30/35) and women. This meant that it was not possible to collect samples from several young people and women, leading to a bias in our sample collection. It is possible that the factors controlling calculus formation in people with larger deposits also affect preservation of microremains within those deposits.

Calculus samples vary in both the number of starches and the number of plants represented by starches. This variation is summarized in Tables 4 and 5. Many of the following analyses use the ‘number of plants represented’ as a variable. This variable includes only starch granules and phytoliths that were assigned to a taxon (“identified” starches and phytoliths). Unidentified starches and phytoiths were excluded from this analysis because we were interested in knowing how well the microfossils could record known diet. Most samples with identified starches or phytoliths contain only one identified plant (40%). In samples with only one plant represented, maize is the most common (42%). Figure 4 shows examples of identifiable starches recovered from dental calculus.

Many starches (71%) were not assigned to a taxon, either because they were nondiagnostic or because they were unidentifiable due to damage. In our sample, damage to starches was not consistent with cooking (eg. gelatinization of boiled maize starches), nor did it resemble damage from processing calculus samples in HCl. We suspect that some granules were damaged by salivary amylase in the mouth, but have not assessed this experimentally. All recovered phytoliths were identified to a taxon.

Table 4. Summary of Plant Microremains Recovered from Twe Dental Calculus

	Mean	Standard deviation	range	% samples present
Starch	8.27	8.50	0 to 48	98.64
Phytoliths	0.24	0.57	0 to 3	18.92
Number of plants represented by starch	0.77	0.97	0 to 4	n/a
Total number of plants represented	1.01	1.16	0 to 6	n/a

Table 5. Summary of Starch Granules Recovered from Twe Dental Calculus

	% samples	% total starch
Damaged starch	81.08	38.24
Unidentified (includes damaged and nondiagnostic starch)	93.24	71.24
Maize starch	31.10	9.64
Contain at least one plant	63.51	n/a
Contain more than one plant	21.62	n/a
Contain more than two plants	8.11	n/a
Contain more than three plants	4.10	n/a

Figure 4. Examples of Identifiable Starches Recovered from Twe Dental Calculus.

Caption: All images are shown at the same scale. Each box is 50µm on a side. Images are paired; the first of each pair is shown under brightfield light and the second under cross-polarized light.

A-B. *Adansonia digitata* L.; B-C. *Fockea angustifolia* K. Schum; D-E. *Pennisetum glaucum* L.; F-G. *Tylosema esculentum* A. Schreib; H-I. *Zea mays* L.

3.2.a. Individual differences based on age and sex:

We estimated the effects of age and sex on the total number of starches per calculus sample. These analyses use negative binomial regression because the dependent variable is overdispersed count-data. Calculus collected from both men and older individuals contained more starches, but when we controlled for sample weight, the effects of age and sex disappeared (see Table 6). Because we are ultimately interested in the number of plants represented in each sample, we repeated this analysis with the total number of plants per sample and the total number of starchy plants as variables. In each case, neither age nor sex had strong or significant affects, but sample weight is a strong predictor of the total number of starches (see Table 6).

Table 6. Regression models for dental calculus starch content

Dependent Variable	<u>Intercept</u>	Age		Sex		Sample Weight		Total starches	
		<u>B</u>	<u>SE B</u>	<u>B</u>	<u>SE B</u>	<u>SE</u>	<u>SE B</u>	<u>SE</u>	<u>SE B</u>
Total starches	1.445	0.015*	0.006						
Total starches	1.792			0.564**	0.204				
Total starches	1.346	0.011	0.006	0.436*	0.203				
Total starches	2.030	-0.004	0.007	0.241	0.214	189.590*	69.949		
Total plants	0.078	0.002	0.010	-0.227	0.300	165.101*	65.005		
# starchy plants	-0.356	0.001	0.011	-0.156	0.348	179.600*	73.355		
# starchy plants	-0.862							0.054***	0.009

*p<0.05 **p<0.0 ***p<0.001

Caption: The dependent variable for each model is listed in the column on the left, with the independent variables following. The beta value (B) and standard error (SE B) for each model show the strength of each independent variable's effect. A missing value means that independent variable was not included in the model.

We also expected a sex difference in the preservation of palm leaf (*H. petersiana*) phytoliths in Twe calculus. Women make palm leaf baskets and first chew the leaves to soften the fibers. Palm leaf phytoliths were only observed in three samples, all collected from women. This sample size is too small for statistical analysis, but this datum highlights the potential of the method to capture sex differences in behavior, as well as non-dietary processing of plants in the mouth.

3.2.b. Individual differences based on 'home' location:

We also compared samples collected at Otjomuro/Okau with samples collected at Ohayuua. Dry season staple foods are different in these locations. Twe eat palm nuts often at Ohayuua, but not at all at Otjomuro/Okau. *Diospyros mespiliformis* fruits are a dry season staple at Otjomuro/Okau, but do not grow at Ohayuua. *Diospyros mespiliformis* fruits do not contain starch or phytoliths, but palm fruits contain a high concentration of diagnostic phytoliths (refer to Table 3 for % dry weight). We found that dental calculus from people who live at Ohayuua is no more likely to preserve palm fruit phytoliths than calculus from people living at Otjomuro/Okau. Palm nut phytoliths were only found in seven of 74 samples (2 men, 4 women), and only one of these was collected from an individual living at Ohayuua. This finding likely reflects the level of mobility among the Twe, since they often leave their "home" camp and visit camps with different foods.

3.2.c. Intra-Individual comparison of microfossil record

In order to assess how consistently microremains in dental calculus record diet across the population, we used a resampling approach to determine how similar any two randomly drawn individuals are in terms of the types of plants represented. Each person's plant representation was compared to each other person in the population to determine how many plants they share. We then calculated the mean number of plants shared across all individuals. We did this for subsets of the population with at least one plant represented, more than one, and more than two plants represented. Table 7 shows the mean number of plants shared by individuals in each subset.

Table 7. Similarity of Plant Representation in Twe Calculus Based on Resampling from Data

Number of Plants in Calculus Sample	Mean number of shared plants
1+	0.3996
2+	1.0583
3+	2.1333

Most samples do not share even a single plant. Looking only at the subset with two or more plants, there is still slightly less than one shared plant between samples. This lack of shared plants does not improve looking only within region or within sex. We would expect that most of these starches

should come from the same plant, given the similarity in diet, but this was not the case. This large amount of individual variation in plant representation suggests differences in plant representation do not necessarily reflect differences in plant consumption.

3.3. Are starches and phytoliths in Twe dental calculus a strong predictor of the range of plants consumed?

Microremain findings per individual are a poor predictor of the range of starch and phytolith producing plants consumed. Although nineteen Twe plant foods contain starch and phytoliths, the maximum number observed in an individual calculus sample is six. On a population level, starch grains and phytoliths are a better predictor of diet breadth. Across all 74 samples, starches and phytoliths from eleven different plant parts of ten plants (including two palm parts) are observed. Of these, six plants are represented by starches, and five plant parts of four plants are represented by phytoliths. We calculated the odds of finding any given starch producing plant in a given number of individual dental calculus samples by resampling from our data (iterated 50,000 times). Table 8 lists the number of individual samples needed to have 95% confidence of finding a given plant. Figure 4 shows the same data in graphical form. In our simulation, some plants are visible at much smaller sample sizes than others, and for 95% confidence of viewing the full range of starchy plants represented in Twe calculus, our sample of 74 individuals is insufficient. We excluded phytolith producing plants from this analysis because very few calculus samples contained diagnostic phytoliths (18%).

Table 8. Number of individuals needed for 95% confidence of viewing each plant

Plant	Required number of individuals
<i>Adansonia digitata</i> L.	Did not converge- 86% odds of finding with 75 people
<i>Cucurbita</i> sp.	Did not converge- 63% odds of finding with 75 people
<i>Fockea angustifolia</i> K. Schum	21
<i>Pennisetum glaucum</i> L.	16
<i>Triticum</i> sp.	55
<i>Tylosema esculentum</i> K. Schum	36
<i>Zea mays</i> L.	8

Figure 4. Number of individuals needed for 95% confidence of viewing each plant

3.4. Is starch quantity in dental calculus samples proportional to dietary concentration?

We estimated the expected relative contribution of starches from each plant by multiplying the number of starches per gram dry weight by the average amount of each plant food consumed. On a population level, the relative dietary contribution of starch from each plant does predict the amount of observed starch in dental calculus ($B=0.57$, $SE=0.12$, $p=0.0024$). However, the overwhelming majority of observed starch and expected starch comes from maize. When maize is excluded from the analysis, the relationship between expected and observed starch disappears ($B= -1.51$, $SE=1.28$, $p=0.28$), suggesting that the relative representation of starch granules in dental calculus is not a good predictor of the dietary importance of plants with low starch content, or plants that are not intensively exploited.

4. Discussion:

The identification of starch granules and phytoliths in Twe dental calculus gives an incomplete picture of Twe diet. This is in part because not all plants consumed by the Twe produce starches and phytoliths, but also because many of the starch and phytolith producing plants are not represented in calculus samples. There is a large amount in individual variation in the number of plants represented per calculus sample: from zero to a maximum of six. This maximum represents fewer than one quarter of the

plants regularly consumed by the Twe, and fewer than one third of the starch and phytolith producing plants in the Twe diet. These results suggest that dental calculus studies sampling a very small number of individuals are not representative of diet in larger populations.

On a population level, starches and phytoliths in Twe calculus are a better predictor of diet, with eleven plants represented, or one third of the plants regularly consumed. However, the most commonly observed plant is maize (represented by starches), while other plants are represented at much lower frequencies. This indicates that the probability of observing many commonly eaten foods in dental calculus is very low. Resampling of our data from a probability perspective shows that a sample of upwards of 50 individuals is necessary to have 95% confidence in observing several plants. This has important implications for archaeological studies trying to identify the consumption of specific plants. Depending on the importance of the plant in the diet and its starch/phytolith content, a very large sample may be necessary.

The amount of variation between individuals suggests that comparisons of individuals' diets based on starches and phytoliths in dental calculus are problematic. Two randomly selected Twe with nearly identical diets may have a very different number and range of plants represented in their dental calculus (Table 6). Population level comparisons of diet using large sample sizes are probably more appropriate for assessing differences in plant consumption between groups.

Our results indicate that the proportion of microremains from different plants is not correlated with the exploitation intensity of those plants. However, analysis of microfossils in calculus still speaks to interesting behavioral questions. For example, the presence of certain plants may point to changes in diet breadth. We observed several starch granules from *Tylosema esculentum* A. Schreib rhizomes in Twe calculus. This rhizome grows more than 0.5 meters below hard packed, rocky soil, and takes considerable time and effort to extract. Once collected, the rhizome must be roasted for several hours, then pounded and peeled. Pending nutritional analysis, we suspect that this rhizome has a low caloric yield relative to the energetic and time costs of procurement and processing. This indicates that the Twe diet sometimes broadens to include resources with a low rate of energetic gain.

Though the majority of taxonomically identified starches in Twe calculus came from maize, we expected that maize starch would be more prevalent. Each participant reported eating maize at least once a day, every day, but maize starch was found only in 30% of the calculus samples. We expect this may be in part due to processing. Maize is always ground and cooked, which causes significant damage and gelatinization to the starches, removing them from the calculus record.

The prevalence of maize starch in the Twe sample suggests a bias towards starch from domesticates in populations with mixed economies. Many plant domesticates are selected for high starch content. Maize has more starches per gram dry weight than all but two of the starchy wild foods in the Twe diet. These two starchy foods, baobab (*A. digitata*) and *Lapeirousia* sp. (grass) corms are consumed regularly when in season, but are underrepresented in Twe calculus samples. Baobab starches were recovered from only two samples, and *Lapeirousia* starches were absent from all samples. Although these foods are not consumed at the same frequency year-round as maize, given their high

starch content we expected to observe them more frequently in Twe calculus samples. It is unlikely that processing or cooking removed these starches from the calculus record. Baobab is eaten raw and *Lapeirousia* corms are cooked on hot coals only long enough to loosen the tunics (~2-5 minutes), which does not damage the starch. The size and features of these starches may have affected their preservation in the record or our ability to recognize them. Baobab starches are very small and difficult to confidently identify. However *Lapeirousia* starches are larger and more distinctive, so it is unlikely that we failed to recognize them. It may be that *Lapeirousia* starches are more affected by salivary enzymes or more easily damaged during HCl processing, but we have not assessed this.

The starchy wild plants that are most regularly consumed are *Fockea angustifolia* K. Schum and *Ximenia Americana* L., *F. angustifolia* tubers have a moderate starch content and are probably an important part of the Twe diet. We suspect that *F. angustifolia* consumption is underreported due to a stigma against digging for food, but all Twe in our sample admit eating it whenever food stores run low. *F. angustifolia* is relatively common in Twe calculus (identified in 14% of Twe calculi), but compared with maize is probably underrepresented (23% of samples). Seasonally, *X. Americana* fruits are eaten as regularly as maize flour, but with only 27 starches per gram dry weight, they are much less likely to contribute starches to the calculus record, and we did not observe any *X. Americana* starches in our sample. Like baobab starches, *X. Americana* starches are very small, and this may make them more difficult to identify than starches from domesticates.

Perry (2002) shows that starches from manioc and sweet potato are systematically larger than wild predecessors, and Piperno et al. (2009) show that maize has a higher starch content and larger starch granules than wild teosinte varieties. Due to a high starch content, many domesticates are probably more likely to contribute to the dental calculus microfossil record. Additionally, large diagnostic starch granules may mean that domesticates are more easily identified in dental calculus. There are several wild foods in the Twe diet that are as important as maize flour in different seasons (eg. *Berchimia discolor* berries in the rainy season and *Diopyros mespiliformis* fruits in the dry season), but these plants contain neither starches nor phytoliths, and are invisible in dental calculus.

5. Conclusions:

We suggest that analysis of starch granules and phytoliths in dental calculus is best suited to questions about the presence or absence of specific plants in the diet, keeping in mind sample size. This method probably does not address 'bigger picture' questions about diet such as exploitation intensity, or inter-individual differences in the range of plants consumed. Starches and phytoliths in dental calculus have the potential to identify specific plant taxa consumed. In this way, this method provides a complement to other methods of plant-diet reconstruction such as stable isotope analysis and tooth macro-and micro-wear studies that give information about the categories of plants consumed or mechanical properties of those plants. The ability to identify the consumption of specific plants is both important and exciting.

This work represents a first attempt to understand how diet is recorded in dental calculus. However, we do not address dental calculus formation and the mechanism of starch and phytolith

incorporation. The dental science literature highlights individual variation in calculus formation, and our results suggest that the factors controlling this variation also contribute to individual variation in starch and phytolith preservation. We also suspect that other analytical methods may complement microscopic analysis of dental calculus, leading to a fuller understanding of ancient diet and lifeways. These include pyrolysis of dental calculus and proteomic analysis (cf. Buckley et al., 2014; Hardy et al., 2012; Warinner et al., 2014).

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Figure 1

Figure 2

Figure 3

Figure 4