Nutritional contribution of plant foods to human diet in evolution

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PREFACE

Diet and food are an indisputably core facet in all human societies. In the not too distant past, before industrialization, rise of the middle class, and market economies, procuring and preparing enough food to feed oneself and one's family occupied a significant portion of daily labor. In modern unindustrialized small-scale societies, this is still largely the case. Appropriately, anthropologists take great interest in the role that food and diet have had in the genesis of human biological and social variation (Messer, 1984). The old proverbs, "you are what you eat" and "tell me what you eat and I'll tell you what you are" speak about food not only as a physical embodiment of the environment, but also as a means of social identity and symbolic construction of community culture (Messer, 1984). Naturally, these are anthropological dialogues, but to have substance for discussion, they require the collective conversations between historical, social, biomedical, and nutritional researchers. Each field certainly has more to say than what can feasibly enter cross-discipline communication, but it is worth the effort of maintaining a comprehensive approach that recognizes how humans are uniquely cultivated by their social culture and are yet still bound by the biological consequences of millions of years of natural selection. What follows is a summarized history of the field of human nutrition, which has its formal origins with the "chemical revolution" and quantitative science, in France at the end of the eighteenth century (Carpenter, 2003). This background is facile but relevant to understand how once non-overlapping domains of science – anthropology and clinical nutrition – are united through the common foci of human diet and physiology under the umbrella of evolution. Since the subject material of my thesis research embodies the shared nature of these two disciplines, both how foods contribute to the maintenance of human health and how this is dictated by and informative of human evolution, this historical perspective is important contextual information for why this work is meaningful to anthropology, and to me.

CHAPTER 1

INTRODUCTION

The study of nutrition and the research on human diets have historically blazed remarkably different trails on the subject of food and nutrition in human health. The former most notably deals with the relationships between food composition, physiology, and disease while the latter may be considered a more anthropological perspective on the role of food in socio-cultural, biological, and evolutionary aspects of human life. However, their paths have obvious overlap, especially when it comes to human biological adaptation to nutrition, but this has not always been fully appreciated. Nutritionists are largely concerned with applications in public health whereas anthropologists may find controlled dietary studies too restrictive or specific to modern Western society to have evolutionary significance. Diet is one of the most profound factors of influence on an organism's physiology and behavior. In human evolution specifically, whole genera of archaic hominins are identified on the basis of morphological variations to the masticatory apparatus that may be suggestive of divergent dietary patterns. Indeed, diet is considered one of the main vectors for early hominid differentiation and dispersal across the Plio-Pleistocene. Colossal effort is dedicated to elucidating the diets of these early groups because we believe this information holds vital clues to how and why the human lineage evolved, what led to its successes, and what led to its dead ends. Eventually, highly derived groups within Homo emerged such as H. erectus, H. heidelbergensis, Neanderthals, Denisovans, and modern humans. These highly intelligent, social, and geographically widespread groups show further reduction in masticatory robusticity coincident with cranial expansion (excluding Denisovans, for whom we lack cranio-dental remains), traits highly suggestive of a significant biological response to dietary changes sometime after the emergence of Homo (Organ et al., 2011).

But how do we investigate ephemeral events in the past and, more importantly, how do we quantify the influence of these events? The combined efforts of anthropological sub-disciplines – archaeology, archaeological science, ethnography, and morphology – construct our knowledge base surrounding the history and evolution of human diet, while nutritional science provides the necessary quantitative or mechanistic information about food and digestion. Together, both fields stand to gain tremendous collective insight about human nutrition and adaptive physiology. But reaching this idealized goal of cross-disciplinary cooperation towards a realized mutual outcome has taken considerable time and evolution in both fields. It is perhaps fortuitous that the present zeitgeist is a strong public interest in evolutionary health, effectively forcing a cross-disciplinary response to health research and policy. The explosion of interest in evolutionary medicine, evidenced by the current popularity of "Paleo-diets" or alternative medicine, may (in the most optimistic view) speak to a progressive undercurrent of realization that the factors affecting human health are immutably dictated by millions of years of human evolutionary history. Even our distinctly human characteristic within Primates, a big brain, reaches far back in time, built upon foundations laid out in the Cambrian explosion when intracellular complexity, driven by membrane lipid synthesis, arose and established

the precursors for the nervous system (Crawford et al., 2008). As a result, we have likely crossed a cognitive threshold in awareness of who we are as humans and irrevocably formalized that anthropology is the necessary partner to all endeavors in human biology. Correspondingly, as anthropological studies continue to dig deeper within an evolutionary framework, their topics become unambiguously intertwined with the relevant sub-disciplines of biology. However, this was not always the case as both fields experienced a long, non-overlapping, parallel evolution since their inception roughly 200 years ago. The precedence for this is understandable given the age and origin of both fields.

1.1 Chemical revolution and nutritional science history

The setting for the formal beginnings of food and nutritional science is late eighteenth century France, in which the "Chemical Revolution" spawned an era of investigation and elucidation of [periodic] elements in organic matter (Carpenter, 2003). Prior to this, the pioneering work in nutrition amounted to systematic trials of different therapies recommended for scurvy. Progress towards understanding the composition of animal tissue jumped forward when ammonia emanating from decomposing animal matter was determined as three parts hydrogen and one part nitrogen. Nitrogen, it was soon found, resided in abundance in other nutritional plant foods such as wheat and legumes as well as meat, so that these foods were thought to be especially "animalizing" (Carpenter, 2003). People were interested in the paradox of how grazing animals could convert plant food, particularly plant food considered to be deplete of nitrogen, into nitrogen-rich animal tissue. Such knowledge had relevance even at the political level of society, particularly because it could inform about how best to sustain rapid population growth in expanding nations and colonies with a cheap mass-produced staple crop. And so it was that by trying to understand the "animalizing" property of food that the study of how animals internalize and utilize different nutrients (i.e. nutritional composition and digestion physiology) became an empirical scientific endeavor. These efforts were not limited to food composition and intake, but also looked at how heat production and respiration (metabolism) figured into the mass balancing equation (Carpenter, 2003).

The early strides in chemical and nutritional research by many European physicians, chemists, and scholars throughout the mid to late nineteenth century provoked enough action from the medical establishment so as to set off a wave of medical science devoted to the study of the dietary etiology of disease (Taubes, 2007, pp ix-xxv). By the late nineteenth century, nutrition scientists and physicians were keen to establish a cause, and thus a therapy, for obesity and its associated diseases at that time-

diabetes, gout, and hypertension. Claude Bernard, the famous French physiologist, made especially pivotal revelations about liver function that yielded an initial understanding of the mechanisms of obesity and adult onset diabetes. More modern iterations on this theme began nearly a century later in the U.S. after WWII with clinical investigations into the relationship between infectious disease and malnutrition (Keusch, 2003), and the treatment of progressive manifestations of 'diseases of civilizations'. The latter was pursued ardently by clinical investigators, with six major conferences and symposia devoted to obesity research by 1973. The medical and by this point U.S. government regulated nutritional policies, however, insisted on a different agenda that aligned with corporate economics. This brought the focus away from discovering the dietary factors deemed essential to health, a pursuit that naturally appeals to evolutionary principles, to instead isolating dietary components arbitrarily considered to be unhealthy. The result was the promotion of certain foods and food groups over others. These contrasting research strategies essentially codified the formal distinction between 'biological science' and 'dietary health research'. The notion of diet, or specifically foods, as a factor in the holistic cultivation of health or etiology of disease fell quite out of favor. By the time mainstream medical establishments were entrenched in the diet-heart hypothesis of the last 50 years, demonstrating their own appalling attempts at empirical science, Bernard's "milieu intérieur" or stability and equilibration of the internal environment was antiquated and long forgotten. Certainly there has been a pressing need for the re-establishment of an evolutionary-based perspective in nutritional science.

1.2 Anthropological study of human diet

Leaving behind this brief history of clinical and medical diet research, we can turn to the seemingly unrelated field of anthropology at the end of the nineteenth century. Although formative exploratory work with native communities had already captured the interests of nineteenth century evolutionists such as Herbert Spencer, Edward B. Tylor, and Lewis H. Morgan 50 years earlier, this era of sociological work focused on religion was still the relative dark ages for anthropology (Tylor, 1871; Orlove, 1980). The end of the nineteenth century was a turning point for the field from the murky, century-old, ethnological societies stuck on reifying long-held racist convictions about European superiority, to more comprehensive approaches towards documenting social institutions of so-called primitive societies (Boas, 1911; McGee and Warms, 2008).

The rebirth of ethnography was mostly dominated by early British-trained social anthropologists working in unindustrialized colonial Africa and the South Pacific (Messer, 1984). As both researchers and explorers, these people incidentaly gave some of the first dedicated accounts of food culture in traditional societies, out of the broader goal to understand the origins of "modern" (the

industrialized West) cultural institutions. When the first fossil discoveries of Neanderthals and ancient hominins put western civilization face to face with the enigmatic traces of human prehistoric ancestry, suddenly isolated traditional cultures became a convenient analogy and easy point of reference to bridge the gap between modern and savage "man". Ethnography, it was then thought, was an opportunity to witness "living fossils" and define a kind of socio-behavioral spectrum (Orlove, 1980). At the bottom was primitivism, exemplified by indigenous tribes and attributable to earlier hominins, with radiating linear stages of cognitive progression towards Western civilized society, thought to mirror the biological stages of human evolution (Orlove, 1980). Though still deeply flawed and mired in the institutionally presiding dualism of savagery and barbarism versus civilization and enlightenment, for the first time there was an emerging systematic process to the field of ethnography (Firth, 1934; Orlove, 1980; Messer, 1984).

The A. C. Haddon expedition to the Torres Straits in 1898 was a pinnacle event for anthropology thanks to Haddon's penchant for meticulous detail and scholarship. Haddon's published accounts of the expedition demonstrated the importance of attending to cultural edifices as a whole rather than observing aspects such as religion and ritual in isolation (Richards, 1932). This ideology was soon pushed into the doctrine of social anthropology as "participant observation" through the work of Bronisław Malinowski, who asserted that cultures are to be wholly understood through contact and observation of daily life (Malinowski, 1920). Soon, American anthropology, led by Franz Boas, began to unravel social evolution from biological evolution, in which Boas stated that all humans had the same intellectual capacity and that this was the basis for all human culture (Boas, 1911). Ethnography could finally come to understand cultural systems as the suite of cognitive and behavioral adaptations to the myriad of social and environmental pressures, including subsistence, rather than how these were emblematic of cognitive deficiencies (Firth, 1934).

The work of F.L.S. Bell (1931) and Audrey I. Richards (1932) independently demonstrated that diet is not merely biological, but that the social structure and bonds of family and community are tied to food (Bell, 1931; Richards, 1932). Thus, anthropology rounded another pivotal theoretical premise in the study of diet, that understanding society required an understanding of its subsistence. Soon, the recognition of diet as a powerful evolutionary force necessarily penetrated the interpretive strategies of paleoanthropology. In order to describe and differentiate rapidly emerging hominid fossils, especially on the basis of cranial morphological features in a single specimen, dietary behavior must be invoked (Pilbeam and Gould, 1974). Therefore, dietary ecology research is also ancillary to the needs of paleoanthropologists (Teaford and Ungar, 2000). In the landmark "Man the Hunter" symposium at the 1966 Conference on Hunting and Gathering Societies, influential researchers of that time brought forward a comprehensive dialog about how ethnographic research can inform other areas of anthropology (Devore and Lee, 1968). In both the title and content of the meeting, diet ecology was recognized as the most salient arbiter in the relationship between known and unknown modes of human existence. It was one of the first attempts to bring together anthropology, ethnography, biology, and

archaeology under the auspice of establishing a formative theory of subsistence for past humans and societies. Similarly, the sub-discipline of primatology improves our understanding of the niche participation patterns among extant hominoids, and helps us to infer where we might draw the line among extinct hominids for inclusion, or not, in the human family lineage (Isaac, 1978). At the cross-roads of classical social anthropology and a new ecologically motivated mindset, the anthropological and archaeological study of diet in and of itself was thus formally established.

1.3 Why the synthesis of nutrition and anthropology is important

Presently, diet specific studies in anthropology are quite common. Ethnological data and dietary specimens from modern human populations are assembled and assayed to understand the strategic factors that enable a particular mode of human subsistence in a particular region. Archaeology is also a crucial voice towards understanding past dietary behaviors because it provides direct evidence for human activity from ancient remains or deposits that are in anthropogenic association. We live in a time when our Western industrialized modes of subsistence have probably never been more dissimilar to those of our past, the Neolithic farmers or Paleolithic hunter-gatherers, prompting a thrum of academic and public curiosity about the prospects of revisiting these older, more enduring, lifeways. Subsistence impacts diet and health by determining the type foods we eat, how we procure them, and how this affects our overall well-being. Longevity is at a relative peak, but life expectancy for U.S. adults past the age of 60 has only marginally increased in the last 100 years (Arias, 2014). But although we are living longer lives, most people over the age of 60 live with pervasive chronic illness in spite of enormous advances in medicine in the last century (CDC, 2011). It stands in direct contradiction to the fact that we attribute our health and longevity to modern medicine that populations without access to medical care were and are still capable of similarly long lifespans (Hawkes, 2003; Gurven and Kaplan, 2007). One main difference, however, is that while the industrialized West succumbs to noncommunicable diseases and chronic conditions, those in more traditional non-industrial societies are more prone to parasitic or infectious disease. In fact, for these individuals, avoiding infectious disease and physical trauma almost guarantees a life unencumbered by obesity, diabetes, cancer, cardiovascular disease, stroke, or Alzheimer's disease. Instead, a natural senescence eventually concludes their life, something only experienced by less than a quarter of U.S. adults over the age of 65 (WHO, 2011).

The fact that advance in modern medicine parallels an increasing burden of chronic illness in Western society has not escaped public notice, and whether by fad or genuine concern, people are increasingly turning to diet and lifestyle interventions. One of the more popular and increasingly pervasive trends is to emulate ancestral human diets, presumably and arbitrarily targeting the

Paleolithic era, which is supposedly informed by the fertile fields of anthropological research. Though the accuracy of scientific research that is distilled into the public arena certainly leaves much to be desired, there is no doubt now that the world is listening, and anthropology stands to become a potent informant of human health policy for years to come. Therefore, dietary research in anthropology is necessarily an umbrella for nutritional science. Anthropologists should respond by developing and executing research that can unravel the cultural, ecological, and physiological factors that impinge our understanding of how food affects growth, development, and reproduction. We can reduce the complexity by eschewing major technological advances to food production and processing and focus on the technologically simpler subsistence practices of the few remaining hunter-gatherers, subsistence farmers, horticulturists, and pastoralists. Even further, we can develop models of ancient human and hominin ecology by retracing the developmental underpinnings of our own modern physiology and disease etiology.

Throughout my research that is described in the following chapters, I was mindful of the historical trajectory of anthropology and nutritional science, if only to help make my contribution more broadly applicable to both fields. While my work has been largely proof-of-principle, it does open theoretical avenues to future research that can build a more deductive knowledge base for the complex story of what human ancestors ate, how they ate it, and whether this related to the events that made us human.

1.4 The importance and contribution of plant foods to the human diet in evolution

Understanding the dietary ecology of ancient hominins, which accounts for both behavioral and physiological parameters, is a difficult prospect. The main hurdle is the need for preserved material that unambiguously associates with anthropogenic activity and that also informs about dietary or ecological conditions. However, the circumstance of these two requirements, namely preservation, unfortunately leads to a bias in the type information provided by recovered fossil material and artifacts. Specifically, mineralized and inorganic materials from bones and stone tools are found in abundance, whereas organic matter from plants is usually completely degraded or decomposed long before fossilization or imprinting can occur. As a result, the evidence for animal consumption is much better represented than evidence for plant consumption. Therefore, it has been the objective of the Plant Foods Research Group at Max Planck Institute for Evolutionary Anthropology (MPI-EVA) to develop novel investigations specifically aimed at reconstructing the dietary habits relating to plant food consumption and ecology of early humans and human ancestors. In this endeavor, the group has undertaken three main

investigative strategies that work from opposite but complementary angles towards a central goal of understanding the role played by plant foods in hominin evolution: 1. Recovery of plant microfossils and artifacts that give direct evidence of anthropogenic plant use and ancient environments; 2. Modeling technology, behavior, and physiology using extant hunter-gatherers as a proxy for Paleolithic human lifestyles; and 3. Exploring new methods or novel application of existing techniques that are sensitive to "invisible" evidence such as organic residues, DNA, and isotopic signatures. As a member of the Plant Foods Group, my research falls mainly in line with the second strategy, which is to model the digestive physiology and plant cooking techniques of the Hadza hunter-gatherers of Tanzania. However, as part of studying digestive physiology, my work bisects the third of these strategies with a study of the composition and function of the Hadza gut microbiota, effectively incorporating the new field of microbiome research within the anthropological study of the relationship between human physiology and diet. In the following paragraphs, I give an overview of what we currently know about the study of plant foods in the human diet throughout evolution. The first sections focus on the plant food ecology of modern human hunter-gatherers in different parts of the world, as well as that of extant great apes. In the following section, I incorporate information about archaeological evidence for ancient hominin plant food ecology, focusing especially on the East African fossil hominins and ecological evolutionary context. The final section describes what we know about the nutritional value of plant foods as it relates to human nutritional requirements. These requirements are driven by physiological constraints on brain growth and energy allocation, especially in more derived members of *Homo*.

1.4.1 Plants in the diets of extant great apes

Humans are a derived ape whose history extends far back in time with the origin of Hominoidea, roughly 35 million years ago. Much of what we can discern about the original ape diet comes from tooth-wear analysis, and is highly suggestive of frugivory (Teaford et al., 1996). Furthermore, the folivorous and frugivorous diets of extant great apes and the considerable evidence for various plant-based diet regimes among the australopiths (Teaford and Ungar, 2000; Hohmann, 2009; Ungar and Sponheimer, 2011) makes it fairly assured that humans are an omnivorous ape derived from a deeply herbivorous ancestral past (Milton, 1999).

Most modern non-human primates (herein referred to simply as 'primates') are technically omnivores, taking advantage of food resources from more than one trophic level, but within that classification are a wide-range of strategies to meet a similar suite of nutritional needs (Milton, 1987). The difference in feeding strategy among primates mainly depends on digestive capacity of the gut, niche selection, body size, and sociality (Hohmann, 2009). However, extant primates are neither pure carnivores nor strict herbivores. Instead, primates have diversified to exploit mostly arboreal forest

resources such as fruits, nuts, seeds, young leaves, flowers, and small mammals or invertebrates, but also occasionally terrestrial resources such as tubers or honey. Some small-bodied primates (members of *Loris*, *Tarsius* and *Galagos*) are an exception and feed almost exclusively on insects. Perhaps this insectivorous adaptation is dimly reminiscent of the ancestral mammalian precursor to all primates, but regardless, it serves as a good reminder that the diet composition and niche radiation of primates is surprisingly diverse (Milton, 1987; Hohmann, 2009).

As forest feeders that almost universally target fruits and leaves of dicotyledonous plants, most extant primates, including the Anthropoideae great apes – gorillas, chimpanzees, and orangutans – have a C_3 isotopic signature. The C_3 designation refers to the process of single carbon fixation during photosynthesis from CO_2 into 3-phosphoglycerate in most dicot herbs and trees, while C_4 plants fix atmospheric carbon initially into 4-carbon organic acids before continuing on with the Calvin cycle (Grine et al., 2012). As expected, $\delta^{13}C$ values (the difference of the $^{13}C/^{12}C$ ratio from an international standard) for both New World monkeys and Old World apes are highly depleted, falling in range with environmental canopy or forest flora (~ between -31‰ and -23‰) (Schoeninger et al., 1997, 1999; Oelze et al., 2011; Fahy et al., 2013). Early hominins however deviate from the fruit and leaf-eater pattern and consistently present a stronger C_4 signature, indicating both a mixed C_3/C_4 diet and one that increasingly specialized in C_4 resources (Ungar and Sponheimer, 2011; Sponheimer et al., 2013). As a result, the earlier and especially robust hominins appear curiously similar to a grazer isotopic profile (Schoeninger et al., 1999; Cerling et al., 2013). This evidence certainly indicates a resource shift from the tropical forest foods, favored by extant African apes, to those of mixed grassland or semideciduous woodlands. It is unknown to what extent these C_4 signatures signify exploitation of grasses, sedges, or underground storage organs (USOs) by early hominins, but dietary and isotopic information from the gelada baboon (*Papio spp.*), savanna chimpanzee, and African mole rat provide support for diets consisting of candidate C_4 roots, grass seeds, gums, flowers, leaves, fruit, or even termites (Schoeninger et al., 1999; Peters and Vogel, 2005; Sponheimer et al., 2005; Yeakel et al., 2007; Cerling et al., 2013). Yet, only one of the robust australopithecine forms, *Paranthropus boisei*, seems to have even approached a near 100% C_4 based diet (Ungar and Sponheimer, 2011; Sponheimer et al., 2013), owing in large part to extreme masticatory adaptations, perhaps for grinding sedges, grasses, and seeds.

1.4.2 Plants in the diets of contemporary hunter-gatherers

Human diets today are mostly sustained by plant food staple crops and their derivatives (Fuller, 2007; Khoury et al., 2014), though these are primarily from monocot grass seeds that require extensive processing to be edible. As Richard B. Lee concluded in, ironically, "Man the Hunter" (1968), the cumulative picture of the human diet among extant hunter-gatherers based on a variety of ethnographic accounts is thus; over the long-term, gathered plant and marine resources are far more contributive to daily calories than hunted game animals (Lee, 1968; Kelly, 1995). When considered in broad view, a

logical pattern comes to light in which hunter-gatherer diets tend to be centered on the routinely available and reliable resources. Such resources typically exclude hunted game meat, for which return rates can vary seasonally, geographically, or in other unpredictable ways (O'Connell et al., 2002). Despite the title, one of the major initiatives that sprung to life as a result of the preeminent 'Man the Hunter' conference was to codify women's contributions in a foraging society by analyzing the collection and distribution of plant foods. However, swapping one narrow ideology for another is missing the point that an inclusive rather than hard-lined interpretive approach is necessary to accurately characterize human dietary ecologies. Some work has since made in-roads towards improving our resolution of hunter-gatherer subsistence strategies, but we are still a long way from making sound predictions about diet and social organization of Pleistocene human ancestors across their expanse of occupied ecological zones (Kelly, 1995).

Contemporary full-time subsistence by way of hunting and gathering has almost completely vanished. Aside from a handful of isolated villages in the impenetrable Amazon rain forest, aboriginal inhabitants of the Andaman Islands, and a few ephemeral foraging camps who self-identify as part of larger, well-known, mixed-subsistence, ethnic groups (meta-populations), most hunter-gatherer societies have had to shed their traditional "aboriginal affluence" (Sahlins, 1968). These groups have thus adopted a more cosmopolitan mode of existence, often relying on government rations, trade, and tourism to offset displacement from their original habitats and economies. The Hadza of Tanzania, the group with whom I worked, are likely one of the only remaining full-time hunter-gatherer societies known today (Marlowe, 2010). The last handful of primarily foraging groups profiled in Man the Hunter (Lee and DeVore, 1969) and in the Ethnographic Atlas (Murdock, 1967), namely the Ache, Agta, Efe, Hiwi, !Kung, Mikea, Tsimane, and others listed in Table 9 of Lee (1968), eventually succumbed to a final spurt of encroachment that removed them from the already marginal territories they occupied. Displacement from their traditional territories effectively terminated their traditional means of subsistence. The lasting result has meant a compression of world-wide foraging populations to essentially two or three groups that still supply the majority of their diet from wild foods. In fact, the ethnographic lists accumulated by Lee (1968) and Murdock (1967) were already more of a historical archive rather than a modern reference at their time of publication. Indeed, many of the entries were societies who no longer engaged in full-time hunting and gathering (having been long ago relocated, settled, and provisioned), or who had already vanished (Kelly, 1995). Consideration of this discrepancy in the primary ethnographic collation of hunter-gatherer societies leads to the realization that post-hoc and observer bias is impossible to escape. Nevertheless, these records are the sole legacy of original first-hand accounts of contemporary traditional communities and their subsistence strategies that at one time existed across all major occupied continental regions (Europe excluded): North and South America, Africa, Siberia, continental and oceanic Asia, and Australia.

The stereotyped hunter-gatherer society, inspired by R.B. Lee's description of the Kalahari Bushmen (Lee, 1968), rests on the image of a highly mobile, non-territorial, materially indifferent,

egalitarian group, which is banded together in a harmonious male-female partnership driven by the relative parity in the perceived value of men's and women's labor. Although this sounds endearingly "noble savage", it is accurate to the extent that most hunter-gatherer societies are usually organized by a split between the activities of men and women, utilizing sexual division of labor for the procurement and handling of food, with some notable exceptions (Estioko-Griffin and Griffin, 1981). While there is a large degree of variability, the division of labor matches a biological constraint in which, for women, the burden of reproduction is greater than for men (Trivers, 1972; Hawkes, 1996; Bird, 1999). Therefore, women's foraging activities to acquire plants and occasionally small game are more accommodating to the constraints imposed by pregnancy and infant care. Thus, tracing the labor and activity of women in hunter-gatherer societies is usually a good proxy for the overall group reliance on plant food resources (Isaac, 1978; Hurtado et al., 1985; Hawkes et al., 1989).

The environment is highly predictive of gross foraging strategy and general trends become apparent based on temperature, biomass, rainfall, and territory size patterns. As the effective temperature (a predictive measure of solar radiation and its distribution) increases, so too does the percent of food derived from plants (Kelly, 1995; Binford, 2001), however, a discrepancy exists depending on whether measurements were made based on kilograms of wet weight brought to camp or on kilocalories consumed. Put another way, male contribution to the diet decreases linearly as effective temperature increases. Following this pattern, several well documented African hunter-gatherer groups, including Hadza, Efe, G/wi, Ju/'hoansi, Baka, Mbuti, Gwi, and Dorobo, are cited as having obtained their primary subsistence from gathering [plants – but this is not necessarily independent of small animal resources], often upwards of 70 or 80% of total subsistence (Lee, 1968; Woodburn, 1968; Hart and Hart, 1986; Kelly, 1995; Dounias, 2001). The distribution of solar radiation is in part related to the amount of primary biomass that an environment is capable of producing, and is a significant factor in the energy investment strategy of the vegetation, and by extension, the foraging strategy of resident humans. In high biomass production zones (a function of the effective temperature and total rainfall), plants invest in producing and maintaining structural tissues such as leaves and stems, which are low quality foods. However, in areas of low primary biomass, plants divert solar energy to the production of reproductive tissues such as nuts and seeds and to storage organs such as tubers (Andersen, 1987). Therefore, primary biomass production is counter-intuitively inversely related to the abundance of human consumable plant resources (Kelly, 1995).

The "wild yam question" (Headland, 1987), which asked whether human populations living in a tropical rainforest ecosystem were sustainable prior to agriculture, encapsulates one of the major dilemmas of human subsistence in high primary biomass zones; namely that the edible vegetation may be too low-quality and energy-poor to sustain a large-bodied and large-brained hominin (Leonard et al., 2003). The viability of high-biomass zones for an encephalized hominin may have only been possible after a certain technological threshold was reached, or through intensive exploitation of higher trophic-level resources. This is seemingly consistent with the ethnographic accounts of the traditional

subsistence patterns of tropical-forest hunter-gatherers. These groups reportedly relied, to varying degrees, on agricultural products, horticulture, or intensive hunting and fishing as a supplement to pure foraging of wild plant foods, as many groups still do today. Examples span world-wide equatorial tropical forests, including groups such as the Ache [high hunting return rates (Hill et al., 1987)], Mbuti [heavy reliance on agriculture (Hart and Hart, 1986)], Baka [paracultivation (Dounias, 2001)], Aka [heavy reliance on hunting (Bahuchet, 1999)], Hiwi [stable reliance on hunting but with extreme seasonality of net incoming energy (Hurtado and Hill, 1990)], Yanomamo [use of gardens and agriculture (Lizot, 1977)], Matses [intensive hunting and horticulture (Morelli, 2013)], and Agta [male and female hunting (Estioko-Griffin and Griffin, 1981)]. If the implications of the wild yam question can be trusted, as the evidence implies, then niche diversification from tropical forests to more open woodland or savanna-mosaic ecosystems may have offered an immediate upgrade in the diet quality for our early herbivorous ape ancestors. Therefore, arid environments with high effective temperatures, such as the East African savanna-mosaic Rift Valley ecosystem and the more southern Kalahari Desert, may have paradoxically been better suited for hungry large-brained hominins (and modern humans) subsisting chiefly on plant foods. Curiously, the known geographical distributions of early australopiths and members of *Homo* encompass these key areas in Eastern and Southern Africa. Therefore, the human lineage likely experienced a long evolutionary gestation in relatively arid, high-temperature, and low primary-biomass zones where plants are ideally suited to provide for the majority of subsistence. The coalescence of archaeological as well as ethnographic evidence presents an extremely compelling argument that plants have long occupied a central place in the human diet and were implicit within the circumstances that promoted hominin encephalization.

1.4.3 Archaeological evidence for plant food consumption by humans

The archaeological evidence for plant food consumption in prehistory has improved substantially just in the last decade of research. Prior to the more recently established interrogation of dental calculus from archaeological remains, Paleolithic plant consumption was difficult to determine because most plant organic material simply does not preserve well. Occasionally, an archaeological golden ticket appears, treating our investigative palates to the barest taste of information about ancient plant use, such as rock art drawings of plants (Welch, 2003). Some sites have, in fact, contained preserved macrobotanical remains such as seeds and legumes, which give direct evidence of the types of plants targeted by early humans (Lev et al., 2005). These tiny glimpses portend an unimaginably deep well of botanical cultural knowledge hidden beneath our scant superficial awareness (Hardy et al., 2012). Yet the evidence offers little means for an empirically or quantitatively driven experimental approach. Plant microfossils and microremains such as pollen, phytoliths, and starches, however, are more resistant to degradation, preserve over very long times (Prasad et al. 2005), and can provide reliable

indicators about ancient environment and diet (Piperno, 1995, 2006; Henry and Piperno, 2008; Henry et al., 2014). I focus the following discussion on starch and phytolith recovery since these two microremains more directly inform about plant use for consumption. Starches are semi-crystalline structures composed of amylose and amylopectin, which are long-chain polymers of glucose, and are used by plants to store energy (Hoover, 2001). Starches and starch containing plant parts, such as USOs, are direct sources of food for humans, and their presence in archaeological remains is usually attributed to food. Phytoliths are the silica bodied metabolic by-products found in plant structural tissue, produced from monosilicic acid-rich water drawn from the soil by plant root systems, and are useful for paleoenvironment reconstructions (Piperno, 1988; Hunt et al., 2008). However, information from plant microfossils about the diet and environment is still biased by the fact that only some plants are starch or phytolith producing, and the presence of these microremains would lead to very different inferences about plant use: phytoliths are only found in the structural plant tissues, which are not typically consumed, whereas starches are found in the non-structural reproductive or storage tissues most highly prized for food. Starches from ancient wild plants have been found on grinding stones recovered in a number of Mid-Upper Paleolithic sites, evidencing that plant processing technology was widespread across Northeastern Europe and the Mediterranean (Revedin et al., 2010). These findings suggest that grinding, such as for making flour, was already common practice in the Paleolithic, and hints at a much earlier origin for the technological refinement of plant foods for human consumption, perhaps even prior to the first wave of human migrations out of Africa (Stahl, 1984; Wrangham and Conklin-Brittain, 2003; Organ et al., 2011).

Improving analytical techniques to detect plant microremains (including starches, phytoliths, and pollen) and residues (fatty acids and aromatic compounds) has successfully brought archaeology into the next research frontier. What began as a histological basis for identification of microremains embedded in dental calculus (Henry et al., 2011, 2014; Power et al., 2014), has transformed into a technically complex workflow designed to maximize the capture of dietary and environmental information that can be inferred both from foreign (trapped food particles, residue, DNA and microorganisms) and endemic (digestive and immunological proteins) artifacts (Salazar-García et al., 2013; Warinner et al., 2014, 2015; Hardy et al., 2015). The prospects of what we will learn from these new techniques are tantalizing. In essence, we finally have the ability to detect "invisible" preservation that may inform about plant food ecology to a degree that is eventually commensurate with our knowledge about ancient animal resource use.

Aside from the rare fortuitous preservation of macrobotanical remains and the more recent exploration of plant microremains, tooth microwear analysis has been one of the most fruitful investigative endeavors for inferring about the diets of early hominins. Tooth microwear analysis is the morphological observation and quantitation of microscopic marks left by food or use wear on the teeth (and the shape of the teeth themselves), such as pits, abrasions, scratches, and striations (Teaford and Walker, 1984; Grine and Kay, 1988). The type of mark and patterning is directly related to oral

processing, either from diet, tool-making, or some other type of abrasive wearing imposed on the dentition. Material properties of plant foods inflict pronounced and often diagnostic macro- and microwear on teeth, which along with tooth size, shape, and enamel thickness, signal important dietary adaptations to particular food properties (Daegling and Grine, 1999; Teaford and Ungar, 2000). To briefly summarize this immense body of work, it is understood that Australopithecines had fairly selective diets, unlike the late Miocene apes, but with a suite of dental characteristics that were well suited to gallery forest and grassland resources (Teaford and Ungar, 2000). These species most likely focused on soft fruits, seeds, some abrasive foods requiring incisoral stripping (possibly tubers with bark-like peridermal tissue), and hard but brittle foods such as nuts and corms (Dominy et al., 2008; Grine et al., 2012). The especially powerful combination of stable isotope and dental microwear analysis further supports the notion that C_4 resources, particularly USOs, were a major factor in the divergence of early hominins from late Miocene apes (Grine et al., 2012; Lee-Thorp et al., 2012). Previous work depicts the East African Pleistocene setting and constituent hominins with the following conclusions: an expansion of arid savanna-mosaic environments gave rise to prolific USO producing habitats (Vincent, 1985; Cerling, 1992), while contemporaneous but divergent mammals acquired comparable C_4 enrichment signatures by presumably targeting mixed C_3/C_4 resources, including USOs (Yeakel et al., 2007), and finally, the complex microwear patterns for Australopithecines fall between the profile of a hard-food and tough-food specialist, implicating a diet requiring some grinding and notably with more brittle components than pure foliage (Ungar and Sponheimer, 2011). Furthermore, since USOs are a universally important component of early and modern human diets, the most parsimonious explanation is that this behavior is a relic of the continued pattern of USO exploitation by later *Homo* in savanna-mosaic habitats, including wetlands. This also happens to be supported by mounting evidence that arid and lacustrine habitats were the key areas that could nutritionally support hominin brain growth, owing to the abundance of hydrophilic sedge and small fauna associated with these environments (Laden and Wrangham, 2005; Braun et al., 2010; Organ et al., 2011; Dominy, 2012; Zink et al., 2014). In sum, the evidence weighs heavily in favor of the significant role that plant foods have played, particularly USOs, throughout human evolution.

1.4.4 Nutritional value of plants, processing techniques, and human physiology

The expansion of nutritional and immunological research in the wake of the infectious disease/malnutrition synthesis 60 years ago (Keusch, 2003) is mirrored by a similar advancement in genomics and computing of the last decade, both of which worked to reinvigorate research on mammalian physiology. With specific regard to digestive physiology, we can look at effects from the biochemical, to cellular, to multi-cellular and microbiological levels of interaction, which offer exciting venues to test for yet unseen dietary adaptations.

Early work in the 1980's by Katherine Milton on digestive physiology and kinematics in primates (Milton, 1987; Milton and Demment, 1988) was prescient in its recognition that not only is the gut a bottleneck to nutritional acquisition for all mammals, but also that enteric microbiota enhance the nutritional value of resistant foods, even in the relatively unspecialized anthropoid gut. This work set the stage for Aiello and Wheeler's enduring paradigm on gut-brain energetic tradeoffs, termed the expensive tissue hypothesis (Aiello and Wheeler, 1995). Notably, Milton and Demment (1988) found that humans have a similar digestive response to high concentration (10-15%) neutral detergent fiber (NDF) as that of chimpanzees and other extant hominoids, and the ability to degrade cellulose and hemicellulose via fermentation. Several factors affect this process, including rate of passage through the gut, particle size, lignification, food source, and the level of fiber purification from the native food matrix (Cummings, 1984). Humans have experienced a severe reduction in colon volume relative to the other great apes and therefore a greatly reduced capacity to ferment insoluble fiber (Popovich et al., 1997; Milton, 1999). Chimpanzee diets include on average 33.6% fiber (Conklin-Brittain et al., 2002), whereas the minimum recommendations for fiber consumption in modern human diets are only 20-30g, or about 6% of kcals consumed for a 2000 kcal diet (Papazian, 1997). Humans are incapable of rapid passage of ingesta typical of a carnivore-like digestion pattern (Milton, 2000), but do have marginally shorter mean gut transit times than chimpanzees (Milton and Demment, 1988). Digestive rate along with differences in the gut proportions for humans relative to other great apes (high volume small intestine and low volume colon) clearly indicate that the human dietary strategy at some point shifted away from processing copious amounts of low-quality fibrous plant foods, and instead focused on high quality, easily digestible foods. Presumably, much like today, early humans and hominins were able to utilize sophisticated food processing techniques to initiate an "externalized" digestive phase, as well as target high energy fruits, USOs, nuts, meat, and possibly honey (Crittenden, 2011). Humans and great apes collectively are inefficient degraders of highly lignified foods that have a greater cellulose-to-hemicellulose ratio such as monocot cereal fibers or plant fibers. Instead, relatively unlignified fiber from dicot vegetables, such as from cabbage or carrots, are more compatible with hominoid digestive physiology and gut microbiota (Milton, 1999). Even the fecal microbiota from the ape grazer specialist, *Papio spp.* (baboons), seems to be no better than human gut microbiota at fermenting grass-derived fibers, suggesting that other characteristics such as energetic trade-offs and social structure may enable *Papio* to exploit grassland ecosystems rather than an increased capacity for grass fermentation (Frost et al., 2014). Therefore, a high-breadth plant-based diet must have posed insurmountable barriers to encephalization until sufficient technological innovations for external plant food processing, such as mechanical processing, thermal processing, fermentation, and germination, could enhance the nutritional value of these foods. In fact, use-wear analysis and experiments determined that, of a subsample of 2.0 Ma Oldowan lithics from the Kanjera South site in Kenya, 70% are attributable to plant tissue processing (Lemorini et al., 2014). These results push back our previous estimations of conclusive plant-related tool use by another 1.5 million years, closing in on the putative origin of Oldowan stone

tools (Semaw et al., 2003). A decrease in cranial and mandibular robusticity, as well as reductions in the masticatory structures such as mandible size, zygomatic flare, molar size, prognathism, and occlusal relief in early *Homo* all indicate a gracilization process, presumably from reduced reliance on prolonged chewing of tough or hard foods (Teaford et al., 2002). Reduced chewing stress could be the result of targeting softer foods, developing tools and processing technologies, or both. Either way, naturally high-energy yielding plant foods such as nuts, fruits, and USOs are undoubtedly rooted in hominin diet evolutionary history, extending as far back as two million years and likely beyond.

On account of having an extremely large brain relative to body size, humans have notably rigid dietary requirements that are not experienced by the rest of the extant hominoids. In addition to requiring a consistent supply of energy for the brain (either from glucose, ketones, or lactate) (Zilberter et al., 2010), brain growth and maintenance for anatomically modern humans (AMH) is predicated on the availability of several [periodic] elements, such as iron, iodine, selenium, and magnesium, and other complex molecules, such as folate, antioxidants, and omega-3 fatty acids, that are necessary for structural and functional maintenance (Chen and Berry, 2003). Many of these substrates are found exclusively or in sufficient quantities in plants. Wild plants in particular, such as tree nuts and USOs, harbor high quantities of plant oils, starch, protein, and vital micronutrients (Bradbury and Holloway, 1988; Alasalvar and Shahidi, 2008). Furthermore, as a vestige of our deep herbivorous and frugivorous ancestry, humans, like all primates, are incapable of producing vitamin C, and must instead obtain it from plant foods such as fruits or leaves (Milton, 1999).

There is a curious pattern in the relationship between the potential for vitamin toxicity and the primary source of that vitamin in the diet. Vitamins that are found in high abundance in most vegetables typically lack an upper limit for ingestion (which is not necessarily the same as absorption) (Hathcock, 1997). Vitamins that come from primarily animal foods or those that are synthesized into an active form from precursor provitamins, such as vitamins A, D, niacin, B6, folate, selenium, and choline, however, can induce toxicity symptoms from overconsumption (Hathcock, 1997). A logical explanation is that for millions of years, human ancestors consumed large amounts of wild plants for a significant portion of their daily caloric needs, and in the process ingested extremely high quantities of plant-derived vitamins and minerals. Most of these vitamins function as antioxidants and are deficient in today's diet of highly processed and domesticated foods (Milton, 2000). As one of the thirteen essential vitamins, vitamin A is necessary for maintenance of epithelial cells, visual acuity, immune system function, and brain development (Goodman, 1984). Too much vitamin A, though, can induce a toxic and fatal state of hypervitaminosis, as well as cause profound developmental malformations by disrupting patterns of retinoid-induced gene expression in-utero (Lammer et al., 2010). Evidence of chronic hypervitaminosis A is seen in a *Homo erectus* individual known as KNM-ER 1808, and it is believed that acute toxicity eventually resulted in its death (Walker et al., 1982). Preformed vitamin A, called retinol, is physiologically active, potentially toxic, and found in high concentration in animal liver, especially that of carnivores. However, provitamin A, which is the carotenoid precursor to retinol (primarily beta-

carotenes), is found instead in plant foods and can be used by the body to manufacture the active vitamin. There is no designated upper limit to the consumption of carotenoids, while an excess of 100,000 IU of retinol can induce acute toxicity (Hathcock et al., 1990). Therefore, plant foods have probably adequately supplied the human need for precursor provitamin A over millions of years of evolution, and did so without the danger of hypervitaminosis due to the physiological control over retinol conversion.

Plants resist consumption through a variety of mechanical and chemical means, such as with protective peridermal tissue, resistant fibrous parenchymatous tissue, or through secondary compounds called "antinutrients" (Johns, 1996). Curiously, extant human foragers tend to eschew above-ground, tannin rich, leafy green plant parts in favor of the plant root systems that are vital energy and micronutrient reservoirs. Not only are roots a nutritionally rich resource, but their defense against consumers is often purely physical (residing deep underground or covered in thick peridermal tissue). In fact, a survey of edible USO-bearing plants concluded that at least 65% of species from savanna areas are reportedly consumed raw, compared with only 9% of tropical rainforest plants (Laden and Wrangham, 2005). When chemical toxins or anti-nutrients do appear in edible roots, such as alkaloids, saponins, raphides, glycosides, and enzyme inhibitors (cholinesterase inhibitors and protease inhibitors), they are easily deactivated by simple thermal or mechanical processing (Stahl, 1984; Johns, 1996).

Low nutritional bioavailability, an abundance of antinutrients, and an imbalance in the concentration of certain essential nutrients makes purely vegetarian diets sometimes problematic for modern humans. This is especially so when the diet is dominated by intensively farmed staple monocrops such as maize, wheat, rice, soy, cassava, potatoes, sorghum, and millet, and less inclusive of diverse plant species or wild foods (Eaton and Konner, 1985). In some cases, simply eating a wide variety of plant foods enables the consumer to overcome the burden of anti-nutrients or meet nutritional needs, and is the typical strategy of most non-human primates (Hohmann, 2009). However, humans in particular have adopted advanced processing technologies, considered by some to be biologically essential (Wrangham and Conklin-Brittain, 2003), to mitigate or deactivate antinutrients, while at the same time enhancing nutritional absorption and energy acquisition. Control of fire for use in cooking is undoubtedly a significant consumer advantage because it enables dietary niche expansion, and is proposed to be the catalyst for phylogenetic rate shifts in feeding time between early *Homo* (*H. habilis* and *H. rudolfensis*) and *H. erectus* (Organ et al., 2011). The advantage is three-fold: 1) humans are spared the physiological burden required of detoxification by the liver and kidneys and can thus funnel more resources to other metabolically expensive tissues like the brain, 2) dietary breadth and flexibility is increased by technology, which promotes niche diversification, and 3) environmental buffering using technology unshackles adaptation from the meandering processes of natural selection, thus sparing the need for genetic changes to occur. Additionally, technologies, unlike genetic traits, are highly transmissible, broadly applicable, and do not constrain an organism to genetic specializations or

fixations that may be maladaptive in a different environment. Regardless of the importance of animal foods in the human diet, the near endless iterations of culturally and environmentally specific processing techniques for plant foods is a testament to the rich and vital history that plants have played throughout human evolution and migration. Therefore, plant processing technology was likely a great ecological leveler in the adaptive radiation of humans across highly variable environments, quite possibly unlocking the gateway to global colonization.

1.4.5 Concluding remarks

The material presented in this cross-sectional overview of scholarly work in the arena of diet in human evolution has hopefully provided ample evidence for the importance of plant foods in past and present human diets. The results of research from wide ranging fields such as ethnography, primatology, archaeology, and human biology, are surprisingly concordant in reinforcing the notion that plant foods made a significant contribution to human evolution. The research described within this thesis has thus endeavored to open and contribute to the well-established yet under-appreciated discourse about whether plant foods stand in parity with meat as catalysts in the genesis of the human lineage. Importantly, this work attempts to bridge past and present research by focusing on the biological relevance and limitations involved with plant food nutrition and human physiology.

1.5 Theoretical background to the central thesis

The following chapters entail three discreet project papers united by a common theme to understand the nutritional contribution of plant foods in the human diet. The core subject matter revolves around the dietary ecology of Hadza hunter-gatherers from Tanzania. The Hadza are powerful informants for the adaptive strategies of a foraging human population in the East African savanna-mosaic environment, however they are just one example amongst the myriad of past and present human populations. The conclusions we can draw from work with the Hadza are still no less valuable. We gain insight about the necessary depth of behavioral or physiological specializations that must coincide with a human hunter-gatherer subsistence on wild foods using a basic level of technology – our best present-day representation of what human life may have been like for African Paleolithic hunter-gatherers (Marlowe, 2010).

The Hadza are a tribe of people living around Lake Eyasi in northwestern Tanzania, of which approximately 200-300 members still live exclusively by hunting and gathering (Marlowe, 2010). Bush camps comprise usually between five to 30 people throughout the year, and camp membership and

location is fluid. Labor is divided amongst men and women where the men hunt game meat and obtain wild honeycomb while the women mainly dig for tubers or collect baobab fruit. When in season, berries are collected by all members of the camps. Hunters use bow and arrows with poison tips or axes to obtain the foods they target, and the women fashion a digging stick out of long brush, which is tapered to a fire-hardened point. The dominant foods in their diet fall into five main categories: meat, berries, honey, baobab, and tubers. Of these foods, tubers stand out as the most consistently available and consumed food resource, with several edible species known and targeted.

Image 1. Hadza women, from young mother to elder grandmother, dig for tubers in the morning while younger children look on.

Tubers, or more generally, underground storage organs (USOs) are central to key theories about hominin evolution and brain growth (Laden and Wrangham, 2005); most notably the grandmothering hypothesis posited by Hawkes and O'Connell (Hawkes et al., 1998) and the cooking hypothesis championed by Richard Wrangham (Wrangham et al., 1999). USOs are also staple foods for many modern populations throughout the world (Oke, 1990), and it stands to reason that these resources have been exploited by human groups for a very long time. Many favorable qualities of USOs lend great support to this concept. Such qualities include: a high nutritional content in the form of vitamins, minerals, and carbohydrates (Finglas and Faulks, 1984; Bradbury and Holloway, 1988); the hidden and protected nature of their below-ground location, thus reducing competition with other species for access (Coursey, 1976; Vincent, 1985); a widespread growth across diverse ecosystems (Vincent, 1985);

an often high moisture value (Schoeninger et al., 2001; Schoeninger and Bunn, 2009); and finally, that their acquisition by women, unlike game meat, is possible even with the burden of pregnancy and lactation (O'Dea, 1991; Hawkes, 1996). The USOs targeted by Hadza are all technically root tubers, which are lateral enlargements of the root that store nutrients during plant inactivity, and so I will refer to Hadza USO's as tubers throughout this text.

Image 2. Collection of three different species of tuber by Hadza women in one morning's forage.

Under the premise of the cooking hypothesis, which says that cooking foods made nutrients more readily available to human ancestors and fostered the rapid brain growth seen at a transitional period approximately 2.5 million years ago coincident with emergence of *Homo erectus* (Carmody and Wrangham, 2009; Organ et al., 2011), cooked tubers should be especially more nutritious than uncooked tubers. Heat reduces the physical structure of complex nutritional and structural components of plants, including starch, inulin, cellulose, and arabinoxylans (Kakade and Liener, 1973; Stahl, 1984). Of note is the effect of heat and moisture on semi-crystalline starch grains, which are polymers of amylose and amylopectin. Heating beyond a certain temperature, typically ≥50-60° C, in the presence of moisture reduces the integrity of the semi-crystalline structure and allows water molecules to enter the starch structure, eventually dissociating its polymers and bursting the granule in a process called gelatinization (Hoover and Vasanthan, 1994). Since gelatinized starch has exposed amylose, this makes it much more susceptible to digestion by amylase enzymes from human saliva or pancreatic fluid (Butterworth et al., 2011). However, critically, whether the gelatinization of starch in tuber foods is the goal of cooking has not previously been tested, it is merely assumed, in the context of various cooking

techniques used by modern populations.

1.6 Summary of projects

1.6.1 Brief roasting on starch gelatinization

In my first study I look at the relationship between roasting time and starch gelatinization using an experimental model based on Hadza roasting behavior. Hadza briefly roast tubers directly over a high-flame surface fire, which has been likened to "light roasting" (Tomita, 1966). In this work, I aim to understand the effect of heat application on starch gelatinization in a generalized starchy tuberous food. I model the high-flame fires using an outdoor fire-pit and use this to roast a variety of whole, unmodified, starchy plant organs and record the percent of gelatinized starch in cross-sectioned specimens. The data demonstrate that brief roasting is not adequate for starch gelatinization, implying that this type of cooking serves an alternative purpose. Additionally, my work calls attention to the fact that there are many broad untested assumptions about the roles of cooking across human culture that deserve much more focused examination. Awareness of the simple fact that "cooking" is a blanket term for an incalculable number of methods should also encourage stricter delineations in future studies to specify crucial details such as time, temperature, and technique. Otherwise, results can have no bearing or interpretive power on the topic of the effect of cooking in changing food properties and how this modulates human biology.

1.6.2 Bioaccessibility of Hadza tubers

Pursuing this further, the next logical step towards understanding the contribution of plant foods, and specifically Hadza tubers, to human nutritional requirements is to quantify exactly how much of these foods are absorbed by the gut. This is termed the bioactive, bioavailable or bioaccessible fraction of a food, and is an important metric for ranking the actual biological value of a food resource. While nutritional composition work can tell us the potential contribution, it is not able to account for the resistant, refractory, and inaccessible components. Therefore, bioaccessibility is an especially important evaluation for plant foods, particularly tubers, which can contain a high proportion of fibers and anti-nutrients. The second paper chapter depicts a study to measure the bioaccessibility of glucose in four species of Hadza tuber, collected by the author in cooperation with Hadza women, using a dynamic *in-vitro* model of the stomach and small intestine. The results show that tubers are especially resistant to digestion, and that brief roasting does not appreciably affect absorption. The conclusions

offer insight not only about the importance of forager strategy in finding the highest quality resources, but also suggest that low bioaccessibility might be overcome by the sheer abundance of tuber resources across the foraging landscape.

1.6.3 Contributions of Hadza gut microbiota to nutritional acquisition

The last paper chapter in this manuscript entails a slightly different topic and approach, but with the same end goal of understanding how plant foods can contribute to human nutritional needs. In the course of digestion, food particles that are not absorbed in the small intestine enter the colon, and once there, digestion is continued by the trillions of co-resident microorganisms, collectively referred to as the gut microbiota. Earlier work established that commensal bacteria in the human gut can specialize in the breakdown of resistant polysaccharides, and that the metabolic products of this fermentation (namely short chain fatty acid but also other essential compounds) can be a source of energy used by the host (den Besten et al., 2013). Therefore, the gut microbiota contribute, in a secondary fashion, to host metabolism. The findings of Hehemann et al. (2010) showed that horizontal gene transfer from environmental bacteria to enteric bacteria provided the gut microbiota of some Japanese the ability to efficiently breakdown raw seaweed regularly consumed as a part of the traditional diet (Hehemann et al., 2010). This proof of concept study was formative to my initial interest about the potency of the gut microbiota to assist in human digestion of refractory foods and facilitate nutritional acquisition throughout human evolution. These are not new ideas, and with the inception of the National Institutes of Health (NIH) funded Human Microbiome Project in 2008, many scientists have recognized that humans and their resident microbiota form a kind of supraorganism with a collective hologenome (Turnbaugh et al., 2007; Zilber-Rosenberg and Rosenberg, 2008). When comparing the number of human cells to the number of microbial cells, the difference is an order of magnitude in favor of the microbiota. However, when comparing genomic information, the disparity propels beyond our comprehension at a factor of 150 times more unique bacterial genes than human genes (~3,300,000 versus 22,000) (Gill et al., 2006; Warinner et al., 2015). Therefore, our microbial partners are an extra-somatic means of both functional activity and evolutionary selection. In order to begin to understand the microbial landscape, the first task is to assess "who" is present, or in other words, analyze the phylogenetic diversity and taxonomic profile of the microbiome. I hypothesized that the gut microbiota have a major role in the digestive physiology of the Hadza, especially with their unique diet and status as one of the last remaining truly foraging populations. As a result, I applied for the permission to conduct fecal sampling among the groups I would stay with in Tanzania so that for the first time, the gut microbiome of a human hunter-gatherer population could be identified. The final paper chapter is thus the end product of my original, slightly non-traditional, specimen sampling proposal to study the diet related effects on the gut microbiota of the Hadza hunter-gatherers. With this work, we can begin to understand the plant

polysaccharide degradation potential of this enteric ecosystem and demonstrate that colonic activity is also substantially contributive to human health, nutrition, and evolution.

1.7 Description of scholarly contributions to research projects

1.7.1 Brief Roasting on Starch Gelatinization

My role as author of this paper was inclusive at all stages of the work. I developed the main hypothesis and designed the experimental processes with input from my two co-author advisors, A.G. Henry and A.N. Crittenden. I conducted the experimental roasting trials with help and support from the Plant Foods lab group, and carried out all subsequent processing, staining, and histological analysis for the count-based methods. A tailored random coordinate generator was generously created by A. Strauss for this project. For the semi-quantitative analysis of gelatinization kinetics, I developed and executed the methods with initial discussions and training with C. Debono-Spiteri. I performed the data analysis with initial help from R. Mundry to design an appropriate mixed effects model. All subsequent model improvements and additional analyses were conducted independently. The paper was researched and written by me with input and revisions from A.N. Crittenden and A.G. Henry.

1.7.2 Bioaccessibility of Hadza Tubers

Successful completion of this study required two major phases; first a field work component and then a lab component. I helped design the study, which was initially conceptualized by A.G. Henry with significant input from K. Venema and A.N. Crittenden. A.N. Crittenden organized the field work itinerary and logistics and I carried out the acquisition, handling, and transportation of field samples from Tanzania while working with the Hadza. I conducted all sample pre-processing at the MPI Plant Foods lab in Leipzig, Germany independently. Plant Foods group members provided help and support for the roasting trials. I conducted the *in-vitro* digestion trials under the supervision of K. Venema at the TNO Department of Pharmacokinetics and Human Studies in Zeist, NL. The sample aliquots were analyzed for glucose and amino acids by an external specialist. I compiled all results of the trials with help from K. Venema and analyzed the data independently. I wrote and researched the paper with extensive feedback and revisions from A.G. Henry and A.N. Crittenden.

1.7.3 Hadza Gut Microbiota

This project and paper was a multi-disciplinary collaboration with a specialist group in human microbiome studies from the University of Bologna Department of Pharmacy and Biotechnology (Unibo) in Bologna, Italy. I conceived of and designed the project with input from A.G. Henry. M. Candela provided expert oversight and revision of the proposed research design and methods. I conducted fecal sample collections from the Hadza cohort in Tanzania, Africa with oversight and organization by A.N. Crittenden. Italian cohort samples were collected by Unibo and DNA extraction, amplification, and sequencing were carried out by Unibo and members of the Institute of Biomedical Technologies at the Italian National Research Council in Milan, Italy. 16S rDNA reads were curated by S. Rampelli, who also conducted analysis and statistical reporting of results for the beta-diversity and cross-study comparative analyses. I conducted taxonomic assignment and alpha-diversity analysis, as well as analysis of dietary data. Metabolomic analysis was conducted by J. Fiori with Unibo. I co-wrote the paper with M. Candela with extensive discussions, revising, and editing contributed by A.G. Henry and A.N. Crittenden.

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CHAPTER 2

IMPACT OF BRIEF ROASTING ON STARCH GELATINIZATION AND IMPLICATIONS FOR PLANT FOOD NUTRITIONAL ECOLOGY IN HUMAN EVOLUTION

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ABSTRACT I

Cooking is an important technology for its capacity to increase food digestibility, potentially playing a significant role in human evolution as a means to increase nutritional density of food. However, the contextual impetus for cooking is less well understood. One mode of cooking used by modern humans is to roast food directly in an open flame fire for a wide range of times. This technique may be a behavioral analogue to that of early human ancestors. Here, we provide the first experimental data on starch gelatinization patterns of USOs and starchy fruit as a result of brief fire roasting. We employed a count-based method using light microscopy and a semi-quantitative photometric strategy to observe the kinetic process of gelatinization. We find that brief, five-minute roasting over an open fire results in low levels of starch gelatinization in the parenchymal tissue, which persists even after longer roasting durations, exceeding ten minutes. However, at fifteen and twenty minutes, the majority of starch throughout each specimen is sufficiently gelatinized to enable alpha-amylase activity. These findings suggest that instances of very brief roasting, up to ten minutes, may not necessarily benefit a consumer through changes to starch structure and susceptibility to alpha-amylase activity alone.

2.1 Introduction

Cooking is widely recognized as a highly significant step in human evolution, in part because it can increase the nutritional value of food, especially for starch-rich foods (Boback et al., 2007; Carmody et al., 2011). Cooking causes mechanical and chemical changes to food, which increases digestibility through a reduction in molecular structure, softening of tissues, or facilitating exposure of nutrients to digestive enzymes (Englyst and Cummings, 1986; Holm et al., 1988; Kataria and Chauhan, 1988; Bornet et al., 1989; Zink et al., 2014). Cooking also helps deactivate anti-nutrients that often inhibit nutrient uptake (Greenhouse, 1981; Johns, 1996; Wandsnider, 1997). The extra energy derived from cooking could be used to build other "expensive tissues" in the human body (Aiello and Wheeler, 1995), and it has been proposed that the increased brain size seen in *Homo erectus* was a direct result of the calories gained from cooking (Wrangham and Conklin-Brittain, 2003; Carmody and Wrangham, 2009; Carmody et al., 2011; Organ et al., 2011). While earlier work has explored patterns of starch gelatinization and detoxification as a result of differential degrees of cooking, the actual effect of direct brief fire roasting on starch gelatinization in whole, intact foods has not, to our knowledge, been thoroughly investigated, particularly within the context of studying human evolution (Holm et al., 1988; Wollstonecroft et al., 2008; Henry et al., 2009; Messner and Schindler, 2010; Crowther, 2012). Furthermore, previous research that looks at the effect of cooking on caloric gain by irradiating food in a microwave does not account for the differential degree of change to food properties as a result of variation in intensity, duration, and type of thermal treatment (Carmody et al., 2011). Here, we aim to address this lacuna by investigating the pattern of starch gelatinization as a result of brief roasting on a high flame open fire.

Cooking facilitates caloric acquisition for most foods, yet recent evolutionary models have focused on the contribution of starchy underground storage organs (USOs) as a major source of calories that potentially led to hominin brain growth (Wrangham et al., 1999). Several lines of evidence support the importance of USOs, including observations of modern hunter-gatherers, research on thermal processing and nutrition, studies on wild ape feeding ecology, microfossil analysis, amylase gene copy variation, and even isotope analysis (Perry et al., 2007; Marlowe and Berbesque, 2009; Carmody et al., 2011; Henry et al., 2011).

Though we expect cooked USOs and other plants were important in human history, there is very little archaeological evidence for their consumption or early use of fire for cooking. Fire definitively enters the archaeological record around 300-400 thousand years ago in Europe, although earlier dates are proposed, they remain less well supported and a consistent presence over time is notably absent (Roebroeks and Villa, 2011). African sites contain evidence of fire presence between 1.0-1.5 million years ago, however these are open-air sites that lack anthropogenic association and only in Wonderwerk Cave do we find unambiguous evidence of fire in the Acheulean strata 1.0 million years ago, though the source of the fire is debatable (Berna et al., 2012). Plants, and the tools used to harvest

and process them, are rarely preserved in the archaeological record (Marean, 1997; O'Connell et al., 1999; Richards, 2002; Lemorini et al., 2014). Only in the last two decades has the ability to find and identify microscopic plant artifacts such as starches and phytoliths been rigorously applied to archaeological materials (Cortella and Pochettino, 1994; Lamb and Loy, 2005; Piperno et al., 2009; Henry et al., 2011; Hardy et al., 2012). Due to this lack of information from the archaeological record, we must heavily rely on modern experimental simulations to test the significance of plant consumption and associated processing techniques (Zink et al., 2014).

One of the most important effects of cooking plant foods is the breakdown of structural components, such as cellulose, and nutritional components, such as starch, which helps to soften the food and expose nutritional elements to digestive enzymes (Lee et al., 1985; Holm et al., 1988; Kataria and Chauhan, 1988; Bornet et al., 1989; Muir et al., 1995; Wandsnider, 1997; Dona et al., 2010; Carmody et al., 2011). Cooking practices vary by ecological and ethnographic environment depending on a variety of factors such as food type, resource availability, cultural precedence, and consumer preference. Cooking of a single food type can also vary both in frequency and in duration. In the context of cooking techniques that may be attributable to early hominins, we focus on "brief" roasting characterized by short duration roasting (five to 20 minutes) on an open-flame surface fire. This technique represents a basic method of cooking that is employed by extant hunter-gatherers, particularly in arid tropical environments (see Table 1) (Silberbauer, 1981; O'Connell et al., 1983; Malaisse and Parent, 1985; Youngblood, 2004; Marlowe, 2010; Leonard et al., 2015), and may fall within the behavioral and cognitive capacity of early hominins. Therefore, this body of research helps fill a gap in our knowledge about the effect of a specific type of cooking, namely open fire roasting, on starch gelatinization in various whole, intact, starchy plant foods.

Table 1. Summary of USOs and preparation technique for extant foragers from arid tropical regions; NS not specified.

			Preparation technique								
Family	**Plant**	**Type**	**Raw**	**Roast**	**Boil**	**Bake**	**NS**	**Notes**	**Region**	**Group**	**Reference**
Agavaceae	*Sansevieria scabrifolia*	bulb	X					chewed raw for fluid, high use	Central Kalahari	G/wi	Silberbauer 1981
Apiaceae	*Chamarea capensis*	tuber					X	aromatic, like fennel	Cape, SA	?	Youngblood 2004
Apocynaceae	*Leichhardtia australis* R. Br.	taproot	X	X				emergency food	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Apocynaceae	*Ceropegia sp.*	tuber	X	X				food and fluid, favored, rare	Central Kalahari	G/wi	Silberbauer 1981
Apocynaceae	*Brachystelma thunbergii*	tuber	X				X	juicy	Zeekoe Valley, SA	?	Youngblood 2004
Apocynaceae	*Fockea angustifolia* K. Schum	tuber	X						NW Namibia	Twe	Leonard et al. 2015
Apocynaceae	*Brachystelma barberiae*	tuber	X	X				food and fluid, favored, rare	Central Kalahari	G/wi	Silberbauer 1981
Apocynaceae	*Caralluma lutea*	root	X					pounded and eaten raw	Central Kalahari	G/wi	Silberbauer 1981
Apocynaceae	*Raphionacme burkei*	tuber	X					fluid source, shredded pulp	Central Kalahari	G/wi	Silberbauer 1981
Asparagaceae	*Albuca sp.*	bulb					X	eaten by children	Zeekoe Valley, SA	?	Youngblood 2004
Asparagaceae	*Scilla sp.*	bulb		X				peeled and eaten, high use	Central Kalahari	G/wi	Silberbauer 1981
Burseraceae	*Commiphora pyracanthoides*	root	X					fluid, moderate use	Central Kalahari	G/wi	Silberbauer 1981
Convolvulaceae	*Ipomoea transvaalensis*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Convolvulaceae	*Ipomoea costata* F. Muell. ex Benth.	tuber				X		pit hearth; staple	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Convolvulaceae	*Ipomoea transvaalensis*	tuber	X	X				food and fluid, moderate use	Central Kalahari	G/wi	Silberbauer 1981
Cucurbitaceae	*Coccinea surantiaca*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Cucurbitaceae	*Coccinia rehmanni, C. sessifolia*	tuber		X				food and fluid, staple, poisonous	Central Kalahari	G/wi	Silberbauer 1981
Cyperaceae	*Cyanella hyacinthoides*	bulb/corm		X					Zeekoe Valley, SA	?	Youngblood 2004
Cyperaceae	*Cyperus esculentus*	sedge					X		Zeekoe Valley, SA	?	Youngblood 2004
Cyperaceae	*Cyperus usitatus/fulgens*	sedge		X				slightly roasted	Zeekoe Valley, SA	Bushmen	Youngblood 2004
Cyperaceae	*Cyperus bulbosus* Vahl	sedge	X	X				raw or lightly roasted; staple	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Fabaceae	*Eminia entennulifa*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Fabaceae	*Rhynchosia comosa*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Fabaceae	*Vatovaea pseudolablab*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Fabaceae	*Vigna frutescens*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010

Fabaceae	*Vigna macrorhyncha*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Fabaceae	*Vigna sp.*	tuber	X	X				briefly roasted ~5-15min	NW Tanzania	Hadza	Marlowe 2010
Fabaceae	*Tylosema esculentum* A. Schreib	rhizome		X				roasted >1hr	NW Namibia	Twe	Leonard et al. 2015
Fabaceae	*Erythrina vespertilio* Benth.	roots		X				emergency food	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Fabaceae	*Vigna lanceolata* Benth.	taproot	X	X				raw or lightly roasted; staple	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Fabaceae	*Bauhinia esculenta*	tuber	X					fluid source, moderate use	Central Kalahari	G/wi	Silberbauer 1981
Fabaceae	*Bauhinia macrantha*	root	X					fluid source, chewed, slight use	Central Kalahari	G/wi	Silberbauer 1981
Fabaceae	*Vigna dinteri*	root	X	X				food and fluid, favored, high use	Central Kalahari	G/wi	Silberbauer 1981
Fabaceae	*Vigna sp. (triloba?)*	root		X				food and fluid, moderate use	Central Kalahari	G/wi	Silberbauer 1981
Geraniaceae	*Pelargonium antidysentericum*	tuber	X					eaten by children	Zeekoe Valley, SA	?	Youngblood 2004
Hyacinthaceae	*Dipcadi sp.*	bulb		X				food and fluid, slight use	Central Kalahari	G/wi	Silberbauer 1981
Iridaceae	*Babiana dregei, B. nana*	corm					X		Zeekoe Valley, SA	San	Youngblood 2004
Iridaceae	*Babiana hypogea*	bulb/corm				X			Zeekoe Valley, SA	Bushmen	Youngblood 2004
Iridaceae	*Lapeirousia sp.*	corm		X				roasted ~5min to remove tunic	NW Namibia	Twe	Leonard et al. 2015
Iridaceae	*Lapeirousia*	corm		X				food, slight use	Central Kalahari	G/wi	Silberbauer 1981
Lamiaceae	*Clerodendrum floribundum* R. Br.	roots		X				emergency food	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Lobeliaceae	*Cyphia sp.*	tuber	X			X		watery, sweet	Zeekoe Valley, SA	Bushmen	Youngblood 2004
Lobeliaceae	*Cyphia stenopetala*	tuber		X				fluid, slight use, small tuber	Central Kalahari	G/wi	Silberbauer 1981
Nyctaginaceae	*Commicarpus pentandrus*	tuber					X	overcollected and rare	South Africa	?	Youngblood 2004
Nyctaginaceae	*Boerhavia diffusa* L.	taproot		X				emergency food	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Oxalidaceae	*Oxalis sp.*	bulb/corm	X						Zeekoe Valley, SA	Bushmen	Youngblood 2004
Pedaliaceae	*Harpagophytum procumbens*	root	X					fluid, chewed	Central Kalahari	G/wi	Silberbauer 1981
Pezizaceae	*Elderia arenivaga* (Cooke) McLennon	desert truffle					X	rare but favored	Northern Territory, AUS	Alyawara	O'Connell et al. 1983
Portulacaceae	*Talinum caffrum*	taproot stock	X					medicine, food, moisture	Kalahari Desert	Bushmen	Youngblood 2004
Tecophilaeaceae	*Walleria nutans*	bulb	X	X				food and fluid, slight use	Central Kalahari	G/wi	Silberbauer 1981
Unknown	*Unknown [ozonduvi]*	corm		X				roasted ~5min	NW Namibia	Twe	Leonard et al. 2015
Unknown	*Unknown [otjihakariwa]*	tuber	X						NW Namibia	Twe	Leonard et al. 2015

The diets of contemporary hunting and gathering populations are important informants about the nutritional ecology of early human ancestors (Eaton and Konner, 1985). Hunter-gatherers should not be mistaken as Paleolithic emulations, but rather referents to the behavioral and nutritional adaptations necessary for foraging subsistence regimes common throughout human evolution (Marlowe, 2005). USOs and starchy plants are an important resource for many extant forager groups, and their exploitation in evolutionary history is tentatively evidenced by comparative C_4 isotope signatures in early robust hominins (Yeakel et al., 2007; Ungar and Sponheimer, 2011), sedge phytoliths found in *Australopithecus sediba* (Henry et al., 2012), and a climate induced shift to more USO producing arid and grassland ecosystems in the Pleistocene (Andersen, 1987; Cerling, 1992; deMenocal, 2004). Most wild USOs are rarely targeted by other primates unless they can be found close to the ground surface, which would have made them a relatively uncontested food resource for early hominins (Peters and O'Brien, 1981; Hernandez-Aguilar et al., 2007). More recent human adaptations to starch consumption highlight the extent to which starch is also a valuable component of the modern human diet (Perry et al., 2007). Wild and domestic starchy plant foods, which normally include USOs, nuts, seeds, sedges, and fruits, are widely exploited by many different hunter-gatherers ranging from tropical to temperate climates in both humid and arid environments around the world including: rainforest dwelling African hunter-gatherers (Coursey, 1976; Milton, 1985; Hladik et al., 1993; Bahuchet, 1999); inhabitants of southern Shaba and tropical African grasslands (Malaisse and Parent, 1985; Marean, 1997); Hadza of Tanzania (Vincent, 1985); Twe of Namibia (Leonard et al., 2015); Batek of Malaysia (Endicott and Bellwood, 1991); Ainu of Hokkaido (Watanabe, 1972); Australian Aborigines (Gott, 1982, 2008; O'Connell et al., 1983; O'Dea, 1991); Hiwi of Venezuela (Hurtado and Hill, 1990); prehistoric groups from Northern Chile (Holden, 1991); and indigenous peoples of northwestern North America (Couture et al., 1986; Thoms, 1989; Turner and Davis, 1993).

Modern human foragers, especially groups in tropical regions such as sub-Saharan Africa and the Northwest Territories of Australia, which we summarize in Table 1, consume starchy plant foods often for major portions of their diet and may constitute a representative model for plant food exploitation by ancient hunter-gatherers. Some well-known and studied forager groups include (but are certainly not limited to): the Hadza of Northern Tanzania (Marlowe, 2010), the Mbuti pygmies of Eastern Zaire (Hart and Hart, 1986), the Baka pygmies of Southern Cameroon (Dounias, 2001), Twe of Namibia (Vashro, 2014; Leonard et al., 2015), the G/wi, San and ǃKung of the Kalahari Desert (Lee, 1979; Silberbauer, 1981; Youngblood, 2004), ancient and contemporary Native Americans (Couture et al., 1986; Thoms, 1989), and Australian Aborigines (O'Connell et al., 1983; O'Dea, 1991). Common resources are typically non-cultivated wild species that come from larger plant families such as Fabaceae (legumes), Dioscoreaceae (yams), Typhaceae (wetland rhizomes), Apiaceae (aromatic herbs and taproots), Liliaceae (flowering bulbs), Apocynaceae ('dogbane' family, or milkweeds), Convolvulaceae (morning glories and herbaceous vines), and Cucurbitaceae (squashes, melons, and gourds). Hadza women in particular roast wild tubers over a quick-burning open-flame fire, lasting

approximately 15 minutes, with roasting times that range between three to 30 minutes – with most accounts settling on an average of five minutes (Tomita, 1966; Woodburn, 1966; Schoeninger et al., 2001; Mallol et al., 2007; Dominy et al., 2008; Marlowe and Berbesque, 2009; Schnorr et al., 2015). Woodburn (1966) described Hadza tubers as "not really cooked" and "lightly charred" as a result of this brief roasting (Woodburn, 1966). Species of *Vigna* that the Hadza consume are very fibrous and are chewed and wadged in the cheek to expel the starchy intercellular matrix. A similar starchy fibrous parenchymatous matrix is described for tubers consumed raw and roasted in the central Kalahari Desert (Silberbauer, 1981; Malaisse and Parent, 1985; Youngblood, 2004). It must be noted, however, that not all human consumed USOs contain starch, and a great many contain fructose polymers called fructan or inulin (especially members of Cyperaceae, Liliaceae and the amaryllids such as onions), which cannot be digested by mammalian enzymes and are instead fermented by the microbiota in the colon. For many such fructan containing USOs, long-duration roasting in hot coals or in earth ovens is more commonly utilized in order to hydrolyze fructans into their constituent simple sugars (Thoms, 1989; Wandsnider, 1997; Kubiak-Martens, 2002).

Plant cooking technique and duration varies widely across culture and geography: brief fire-roasting may last three to five minutes for some high moisture, small, or non-toxic species (Tomita, 1966; Woodburn, 1966; Youngblood, 2004; Marlowe and Berbesque, 2009; Leonard et al., 2015), whereas wild varieties of Dioscoreaceae can require extensive preparation, such as boiling and leaching over many days, for detoxification (Couture et al., 1986; Hart and Hart, 1986; Headland, 1987; Raj Bhandari and Kawabata, 2006). The ethnographic literature on tropical foragers that we summarized in Table 1 is dominated by depictions of USO consumption in either raw or roasted forms, compared to boiled or baked (Table 2). Roasting, as opposed to boiling or baking, may be a more valuable cooking method in arid regions where water is scarce or seasonal (Silberbauer, 1981), and when the plant carbohydrates are more easily digested simple sugars and starch rather than fructose polymers (Wandsnider, 1997). From the food science literature, we know that different cooking techniques, such as frying, boiling, or dry roasting, can greatly affect digestibility and glycemic load in isolated starch and starch-containing foods (Lee et al., 1985; Wandsnider, 1997). However, if experimental models are to have explanatory power as to how adoption of cooking accommodated significant physiological adaptations in human evolution, then we must attempt to replicate putative early cooking technologies (Wollstonecroft et al., 2008; Messner and Schindler, 2010; Zink et al., 2014). In this way, we can begin to clarify the varying effects that different cooking techniques inevitably have on the physical and chemical properties of food that may affect the overall caloric accessibility to a consumer.

Table 2. Tabulations of all preparation techniques by plant family from Table 1; NS not specified[1]

Family	Species	Raw	Roast	Boil	Bake	NS
Agavaceae	1	1	0	0	0	0
Apiaceae	1	0	0	0	0	1
Apocynaceae	7	7	3	0	0	1
Asparagaceae	2	0	1	0	0	1
Burseraceae	1	1	0	0	0	0
Convolvulaceae	3	2	2	0	1	0
Cucurbitaceae	2	1	2	0	0	0
Cyperaceae	4	1	3	0	0	1
Fabaceae	13	10	11	0	0	0
Geraniaceae	1	1	0	0	0	0
Hyacinthaceae	1	0	1	0	0	0
Iridaceae	4	0	2	0	1	1
Lamiaceae	1	0	1	0	0	0
Lobeliaceae	2	1	1	0	1	0
Nyctaginaceae	2	0	1	0	0	1
Oxalidaceae	1	1	0	0	0	0
Pedaliaceae	1	1	0	0	0	0
Pezizaceae	1	0	0	0	0	1
Portulacaceae	1	1	0	0	0	0
Tecophilaeaceae	1	1	1	0	0	0
Unknown	2	1	1	0	0	0
Total	**50**	**30**	**30**	**0**	**3**	**7**

[1]Note that there are multiple entries for single specimens from Table 1 when more than one preparation technique is listed.

In the current experiments, we processed four varieties of domestic tubers, one tap root, and one starchy gourd to quantify the extent of starch gelatinization resulting from different durations of direct fire-roasting between five and 20 minutes. We focus primarily on the five minute duration, which represents the briefest fire-roasting duration that has been regularly observed primarily among the Hadza of Tanzania, but is also recorded for the Twe of Namibia (Tomita, 1966; Woodburn, 1968; Mallol et al., 2007; Dominy et al., 2008; Leonard et al., 2015). Additionally, "light roasting" has been used to describe the cooking practice for some resources frequently exploited by the San, !Kung, and G/wi Kalahari desert, and the Alyawara Aborigines from Australia (Table 1) (Lee, 1979; Silberbauer, 1981; O'Connell et al., 1983; Youngblood, 2004), and accounts of raw and roasted USO consumption far exceed that of boiled or baked modes of cookery (Table 2). We use these results to make inferences about starch gelatinization kinematics *in-situ* in whole, unprocessed, starchy plant foods submitted to brief open fire roasting. We can then answer whether brief roasting acts to improve digestibility by breaking down the semi-crystalline starch structure, or whether brief thermal treatment serves an alternative purpose that otherwise facilitates consumption, such as softening cell-wall structure.

Domestic cultivars, while highly divergent from wild species in their nutritional composition (O'Dea, 1991), can still inform us as to the propensity for heat transfer across a high-moisture starchy organ and address the likelihood that the starch molecular structure is affected. Wild plants are highly

variable in their nutritional content and often contain pronounced resistances against consumers by either mechanical or chemical means (Johns, 1996; Wandsnider, 1997). Intensification of plant food exploitation is evidenced by the expansion of dietary breadth and concomitant plant processing techniques in the Epipaleolithic (Hillman et al., 1989; Wollstonecroft et al., 2008), and by the gradual cultivation and domestication of more compliant taxa (Fuller, 2007). The effect of long-term plant domestication leads to morphological and physiological changes that reduce refractory structures, increase palatability, increase caloric density and digestibility, and reduce plant chemical defenses (which includes phytohormones, phytates, phenols, tannins, vitamins and minerals) (O'Dea, 1991; Johns, 1996; Purugganan and Fuller, 2009). Domesticated species, such as those we analyzed in the current study, would therefore be expected to overestimate the effect of thermal treatment on starch gelatinization. This would, therefore, bias our conclusions to determine that gelatinization happens more rapidly and at lower temperatures than what might actually occur in wild species. We provide some images and comparisons from preliminary work with roasting wild tubers to expand on this important topic in our discussion. Overall, our results suggest that starch gelatinization is not a driving factor for brief roasting as a cooking strategy, particularly at the briefest durations lasting approximately five to ten minutes (Schnorr et al., 2015). Alternatively, other factors such as improved taste or ease of peeling and chewing may better explain why brief roasting is practiced. Future work to interpret processing techniques in specific contexts should endeavor to use the wild foods in question when they are available.

2.2 Methods

2.2.1 Experimental setup

To characterize tuber starch gelatinization, we used domestic cultivars taxonomically related to wild tuber species commonly consumed by extant foragers as a simple proxy. All specimens, time trials, and fire measurements are listed in Table 3. In particular, *Cucurbita pepo* (summer squash) and *Ipomoea batatas* (sweet potato) were selected for this study because of their close taxonomic relation to the wild East African tubers, *Coccinea surantiaca* and *Ipomoea transvaalensis*, at the family and genus level respectively. In addition, *Dioscorea rotundata* (yam) and *Manihot esculenta* (manioc) are prominent cultivars in Africa and South America, the former related to important wild-types found in rain forest habitats, and the latter of which has sweet varieties that can sometimes be consumed raw. *Daucus carota* (carrot), the only tap root, was chosen due to its smaller size and cylindrical shape, which we expect may change heat distribution and rate of gelatinization. Finally, *Solanum tuberosum* (potato) was included in this study because it represents one of the universal starch standard specimens (Finglas

and Faulks, 1984; Zeeman et al., 2010; Nguyen Do Trong et al., 2011; Parada and Aguilera, 2011; Crowther, 2012). Together, these plant species share in common the production of insoluble starch in above and below ground fruit and storage organs (Reichert, 1913).

We conducted fire-roasting cooking trials at five minutes in triplicate (a, b, and c specimens) for each species and compared the outcome against a "d" specimen, which was roasted for 20 minutes. We then selected *I. batatas* (sweet potato) and *S. tuberosum* (potato), the two tuber species that can be reliably found year-round in local markets, for a second roasting trial at 10 and 15 minutes on single specimens. This second trial fills the time gap between five and 20 minutes, serving as a proxy assessment of gelatinization kinematics across four total time points. Gelatinization was measured quantitatively from the center (C), middle (M), and edge (E) of cross-sectioned specimens using light microscopy to spot count gelatinized starches with Congo Red (CR) staining (Fig.1). We opted to use non-enzymatic methods because the extra processing required by enzyme-based assays (freeze-drying and grinding) can affect enzyme susceptibility of starch and confound the effects from thermal treatment alone.

Fig. 1 Example of a cross-section of a recently roasted *M. esculenta* and the location of the three sampling points used throughout the study: center (C), middle (M), and edge (E).

CR is a water soluble chemical dye and is routinely used as a contrast stain for cellulose, amyloid fibrils, and starch (Carroll and Van Dyk, 1953; Carroll and Cheung, 1960; Collison and Chilton, 1974; Valetudie et al., 1995; Lamb and Loy, 2005; Weston, 2005). CR is a good indicator of gelatinization due to its affinity for damaged rather than undamaged starch and characteristic red staining under brightfield illumination and polarized light (Adler et al., 1994). Starch granules are semicrystalline structures made from layers of amylose and amylopectin chains (Jane et al., 1994; Wang et al., 1998;

Mcpherson and Jane, 1999; Hoover, 2001; Yoo and Jane, 2002; Stevenson et al., 2005; Zeeman et al., 2010; Parada and Aguilera, 2011). When starches are heated or damaged, the crystalline structure breaks down and absorbs water, allowing CR to react with the amylose. Therefore, CR staining can only occur when starch damage is sufficient to expose amylose within the confines of the highly branched amylopectin structure (Carroll and Cheung, 1960). The reduction in the granule integrity and crystallinity similarly helps increase human salivary α-amylase activity on the starch by increasing the potential binding sites for the enzyme (Muir et al., 1995; Butterworth et al., 2011). Therefore, the same starch modifications that enable CR staining also greatly increase α-amylase activity such that a positive stain can infer digestibility of differentially gelatinized starch. Studies showing an increase in starch digestibility after cooking indicate the reliance of amylase activity on initial starch damage, lending further support to the importance of interrupting the starch structural integrity to increase binding sites on the amylose chain (Carroll and Van Dyk, 1953; Englyst and Cummings, 1986; Muir et al., 1995).

Finally, we conducted a third roasting trial using only *I. batatas* tubers to witness the kinematics of starch gelatinization across four time points from five to 20 minutes of fire roasting. We fire-roasted whole *I. batatas* tubers in triplicate (n = 12 tubers), and took cross-sectional samples from the C, M and E of each specimen. Gelatinization was inferred from photometric measurement of amylose-iodine complex formation with iodine potassium iodide solution (IKI) and amylose chains dispersed using a potassium hydroxide solution (KOH). This final trial, termed the kinetics of gelatinization, provided a secondary, semi-quantitative, and objective approach to help validate the results derived from the previous, slightly more subjective, histological methods. Therefore, the results represent an initial assessment of the kinetic properties of gelatinization in whole tubers as a result of fire roasting. The gelatinization kinetics method was also advantageous for the ability to rapidly analyze a high number of samples and technical replicates, which was not possible using the manual histological count-based method.

Table 3. Specimen, cooking time, diameter, fire temperatures (before [i] and after [o] roasting), and specimen surface temperature (after [o] roasting) for all experimental trials

Trial: Sample ID	Common name	Species name	Family	Time (min)	Diam (cm)	Fire Temp °C (i)	Fire Temp °C (o)	Surface Temp °C (o)
1:1a	Potato	*Solanum tuberosum*	Solanaceae	5	5.0	550	600	88
1:1b	Potato	*Solanum tuberosum*	Solanaceae	5	5.5	550	600	100
1:1c	Potato	*Solanum tuberosum*	Solanaceae	5	5.5	550	600	90
1:1d	Potato	*Solanum tuberosum*	Solanaceae	20	4.5	500	750	112
1:5a	Cassava	*Manihot esculenta*	Euphorbiaceae	5	4.5	550	600	75
1:5b	Cassava	*Manihot esculenta*	Euphorbiaceae	5	5.0	550	600	71
1:5c	Cassava	*Manihot esculenta*	Euphorbiaceae	5	5.0	550	600	66
1:5d	Cassava	*Manihot esculenta*	Euphorbiaceae	20	5.0	500	750	120
1:6a	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	5	4.5	500	600	71
1:6b	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	5	5.5	500	600	70
1:6c	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	5	5.5	500	600	66
1:6d	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	20	5.0	500	750	113
1:7a	Yam	*Dioscorea rotundata*	Dioscoreaceae	5	5.0	500	590	71
1:7b	Yam	*Dioscorea rotundata*	Dioscoreaceae	20	5.0	500	750	97
1:8a	Carrot	*Daucus carota*	Apiaceae	5	2.0	500	590	97
1:8b	Carrot	*Daucus carota*	Apiaceae	5	2.5	500	590	88
1:8c	Carrot	*Daucus carota*	Apiaceae	5	2.0	500	590	46
1:8d	Carrot	*Daucus carota*	Apiaceae	20	1.5	600	800	165
1:9a	Zucchini	*Cucurbita pepo*	Cucurbitaceae	5	3.5	600	700	94
1:9b	Zucchini	*Cucurbita pepo*	Cucurbitaceae	5	4.0	500	700	75
1:9c	Zucchini	*Cucurbita pepo*	Cucurbitaceae	5	3.0	550	700	99
1:9d	Zucchini	*Cucurbita pepo*	Cucurbitaceae	20	3.5	550	750	79
2:sp10	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	10	5	200	312	105
2:sp15	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	15	5	450	560	106
2:pot10	Potato	*Solanum tuberosum*	Solanaceae	10	7	200	312	100
2:pot15	Potato	*Solanum tuberosum*	Solanaceae	15	6.5	450	560	104
3:5A	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	5	6.7	200	300	69
3:5B	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	5	8.4	200	300	72
3:5C	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	5	8.0	200	300	74
3:10A	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	10	4.9	400	400	94
3:10B	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	10	7.5	400	400	78
3:10C	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	10	9.7	400	400	84
3:15A	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	15	6.2	350	400	87
3:15B	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	15	8.6	350	400	98
3:15C	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	15	9.2	350	400	106
3:20A	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	20	6.2	350	580	121
3:20B	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	20	9.2	350	580	81
3:20C	Sweet Potato	*Ipomoea batatas*	Convolvulaceae	20	9.7	350	580	276

2.2.2 Preparation and controls

S. tuberosum, *M. esculenta*, *D. rotundata*, *I. batatas*, *D. carota,* and *C. pepo* were purchased from various markets in Leipzig, Germany. Each species was tested in triplicate (a, b, c) for the five minute roasting time, and singly (d) for the 20 minute roasting time, except for *D. rotundata*, for which only two specimens could be obtained. Raw control samples were taken from each of the test species before cooking in order to confirm presence of starch and ensure that the CR dye did not stain any completely raw starches. To do this, we removed a small slice containing about one cubic centimeter of material from the distal end of each specimen and then mounted starch samples onto a standard glass microscope slide as stained and unstained raw controls (following methods of Matthews, Robertson and Loy (Torrence and Barton, 2006)). Slides were observed under brightfield illumination and cross polarization to confirm starch presence and non-activity of CR.

2.2.3 Fire and cooking

We modeled our fires on Hadza tuber cooking fires, as they represent a basic and well documented cooking fire used by extant foragers. Hadza cooking fires are described in detail by Mallol and colleagues (2007) and tuber roasting fires, in particular, have been observed and anecdotally reported on in many additional studies (Tomita, 1966; Woodburn, 1968; Vincent, 1985; Hawkes, 1996; Schoeninger et al., 2001; Marlowe and Berbesque, 2009; Marlowe, 2010). These sources consistently describe the tuber roasting fire as an above ground, high-flame, and short-lived fire, using only small to medium sized sticks from surrounding brush. Tubers are positioned in the middle of the brush pile and turned regularly during roasting. Using this as a model, we created our own replica roasting fire using a low-standing metal fire pit comprising a shallow basin to contain the flames. This basin was filled with sand to about three centimeters and pine firewood was layered to form a small platform on which the tubers could be placed (Fig. 2).

Fig. 2 Representative tuber-roasting fires documented from Hadza bush camps (**a**) and the experimental fire pit used for roasting trials to emulate a short duration open-fire suitable for roasting USOs (**b**). Photos by authors S.L.S. and A.N.C.

Once fire was established, the temperature was measured with a Voltcraft IR 800-20D infrared thermometer on the top of the platform, and then again after cooked tubers were removed (Table 3). Average fire temperatures for each trial fall in range of moderate to high intensity fires (>350° C), similar to what has been reported for Hadza tuber roasting fires (Mallol et al., 2007; Dominy et al., 2008). Wood was added if needed between cooking trials to maintain a high flame. Tubers were roasted by species for five minutes for specimens a, b, and c, and were turned 90° every 75 seconds to ensure even roasting on all sides. D specimens for each species were roasted together for 20 minutes to obtain fully gelatinized reference specimens. We elected not to snap freeze or cool specimens on ice after roasting because this can affect the starch structure, often causing cracks or breaks in the starch as a result of freezing. We felt these changes could potentially damage native (intact) starches and increase the likelihood of false positives when staining with CR. We recognize that when starch is cooled slowly, recrystallization, or retrogradation, occurs, causing the amylose and amylopectin chains to realign, forming a gel of even higher crystallinity than the native starch. To rule out that this would negatively impact CR staining of actual gelatinized starch, we conducted preliminary tests with whole potatoes cooked in a variety of ways (boiling, baking, and roasting) at different times (5, 10, and 20 minutes) and allowed to cool to room temperature over a period of two to 24 hours. We spot sampled these specimens at two, five, 12, and 24 hours with our CR staining protocol, and found that retrogradation did not impact the efficacy of CR to stain gelatinized starch.

2.2.4 Histological methods

After cooking, a transverse slice was made at the midpoint of each tuber and photographed. Starch samples were taken twice from the C, M, and E positions of each specimen: one for the CR stain and one for the NS sample slide. Slides were prepared following the methods of Torrence and Barton (2006) for starch isolation, and Lamb and Loy (2005) for CR staining. A small amount of specimen was finely diced with a scalpel on a dish and allowed to dry. A pipette with 40 µl of water was used to agitate the sample and distribute the starch granules and then 20 µl was drawn off and ejected onto a slide and allowed to dry. To stain, 40 µl of CR was applied to the dried sample on the slide and the cover slip placed on top. After 15 minutes of staining, 20 µl of 1 M NaCl solution was applied at one edge of the slide and drawn across using filter paper to wash the stain. Clear nail polish was applied at each corner of the cover slip to hold it in place. Slides were rehydrated for microscope analysis and observed under brightfield and cross polarized light to quantify the starch concentration. All observations were conducted using the Zeiss Axio A.1 compound light microscope under brightfield illumination at x200 and x400 magnification with cross polarization capability. Images were captured using the AxioCam MRm and accessed with AxioVision release version 4.8.2.0. Visual fields were selected using a randomized coordinate generator that produced ten points along ten concentric squares (Supplementary

Figure 1), totaling ten observation points per slide. This provided a balanced representation of the entire 22 mm^2 slide cover-slip area containing the specimen. For stained slides, the number of stained starches and the number of total starches within the entire visual field were counted and recorded per observation point. For view of fully gelatinized starches where the starch grain boundary is fully degraded, we used Paint.net v3.4.9 to detect color contiguity across a stained area in order to overcome the inability to count individual starches. However this is less relevant as our model uses only the percent of stained to total starch.

2.2.5 Gelatinization kinetics methods

To measure the starch gelatinization kinetics in roasted *I. batatas* (sweet potato), we adapted a previous method developed by Birch and Priestley (1973), and validated against newer techniques by Baks *et al.* (2006), which involves measuring the amylose-iodine complex formation in alkali dispersed starch (Birch and Priestley, 1973; Baks et al., 2007). In this, we were able to take advantage of a unique property of *I. batatas* in which once the tuber starch is sufficiently heated (approximately 60° C), it is susceptible to endogenous diastase such that most of the starch is rapidly hydrolyzed into dextrin and maltose (Gore, 1920, 1923; Losh et al., 1981). Therefore, we can expect that gelatinized sweet potato no longer contains amylose, but instead, oligosaccharides, which cannot complex with iodine to form the distinctive violet-blue color change. Our assay thus follows the reduction of amylose from hydrolysis, signaling gelatinization, by comparing cross sectioned samples of roasted tuber against individual raw and gelatinized controls. The process of starch hydrolysis from endogenous diastase does not occur above boiling point, presumably because this temperature deactivates the enzymatic activity, but starch gelatinization still proceeds rapidly (Gore, 1923). Therefore, the starches in the "edge" position, just underneath the peel, did not undergo complete hydrolysis due to extreme temperature of the fire, though in all specimens this starch was fully gelatinized, and is therefore not included in subsequent analysis.

We roasted *I. batatas* tubers in triplicate for four separate time trials: 5, 10, 15, and 20 minutes, totaling 12 individual tuber specimens. Just prior to the roasting trial, we peeled and removed a small slice, ~4 cm^3, from one distal end of each tuber to serve as a raw and gelatinized reference. The raw references were frozen immediately at -20° C in individual 15 ml tubes. The gelatinized reference samples were each finely diced and gelatinized in a 10 w/w % tuber-water ratio in a water bath held constant at 90° C for 60 minutes. After 60 minutes in the water bath, gelatinized references were placed immediately on dry ice for 30 minutes, and then frozen for 24 hours at -20° C. The roasting fire was set up and established in exactly the same way as for the previous two roasting trials. Temperatures of the fire before and after roasting as well as the surface temperature of each tuber after roasting are likewise reported in Table 3. Upon removal from the fire pit, tubers were immediately put on dry ice to stop further gelatinization and enzymatic activity. After approximately 10 minutes in dry ice, tubers were

cross-sectioned in the middle and approximately 3-5 grams of fresh weight samples were removed from the center, middle and edge of each cross-sectioned half. These samples were put in individual 15 ml tubes and frozen at -20° C for 24 hours. Upon freezing, all samples, including the references, were lyophilized and then ground to a fine powder using an IKA tube mill (IKA®-Werke GmbH & CO. KG, Staufen, Germany) at 2000 rpm for six cycles of 20 second intervals.

To disperse and isolate the amylose in each sample, triplicate measures of each powdered sample (0.012 g) were dissolved in 15 ml of 0.60 M KOH and shaken vigorously for 20 minutes. The resulting solution was centrifuged (3000 rpm, 10 min, acc. 3, dec. 9) to separate the insoluble part of the sample. After centrifugation, 1 ml of the supernatant was removed and neutralized with 9 ml 0.066 M HCl. Afterwards, 100 µl IKI reagent (1 g iodine, 4 g potassium iodide in 100 ml water) was added to each neutralized sample to form the blue amylose-iodine complex. Samples were pipetted in triplicate for technical replicates in a 96 well plate and the absorbance measured at 620 nm. Degree of gelatinization is expressed as a fraction of the raw reference measurement after subtracting out the fully gelatinized reference measurement, which is depicted in the following equation:

$$\text{Degree of Gelatinization} = 1 - \frac{(A_e - A_g)}{(A_r - A_g)}$$

where A_e stands for absorbance measurement of the experimental sample, A_r is the absorbance measurement of the raw reference, and A_g is the absorbance measurement of the gelatinized reference, subtracted from 1 to give the fraction of gelatinization.

2.2.6 Statistical analysis

Statistics were conducted using the R statistical package free software, version 2.15.1 (2012-06-22) and the *lme4* package for fitting linear and generalized linear mixed-effects models (GLMM) (2012-06-23) (R Core Team 2013). Due to non-normal distribution, repeat measures, and the influence of fixed and random effects on the response, we performed a linear mixed model using the function [lmer] to analyze the relationship between species, position sampled, and the percent of starch gelatinization for our five minute roasting trial (Baayen et al., 2008; Bolker et al., 2009; Winter, 2013). The response was selected as the percentage of total starch that was stained per observation square (because of non-convergence errors, the continuous count of stained and unstained starch as a response could not be used). Species and position were interacting fixed effects while the individual specimens (that is, the "subjects" of our experiment) and the observation number were random intercepts. We also included by-individual and by-observation-number random slopes for the effect of diameter measurements. Since the presence of starch within the sampling area of the microscope slide was biased towards the center of the square area, we weighted the model by the total starch counted per observation. A step-wise comparison between the full and the null model using likelihood ratio testing with the function

[anova] informed us of the significance of the effect and interactions between species and position. Visual inspection of residual plots did not reveal deviations from homoscedasticity, and a histogram and Q-Q plot of residuals did not indicate any violation of normality (Supplementary Figure 2). To look for overly influential observations, we ran a loop to perform "leave-one-out" diagnostics, which reruns the model with each observation point excluded one by one and reports the impact of that observation on the model outcome. Visual inspection of the resulting values against their intercepts indicated that the model was a good fit.

2.3 Results

2.3.1 Visual assessment

After five minutes of cooking, all specimens exhibited charred exteriors and some flaking of the outer skin. We noticed that it was difficult to remove specimens from the fire using cooking tongs because the outer skin was too soft and broke apart easily. Once specimens were brought back to the lab for handling and sampling, all exhibited this same softening and ease of peeling. Much care had to be taken to disturb the outer layers as little as possible while cutting and sampling the starch. Upon cutting into the middle of the five and 10 minute specimens, the interior flesh was firm, juicy, and largely indistinguishable from the raw control cuts. Only in some specimens was a ring of faint discoloration noticeable, which seemed to mark the boundary between raw and gelatinized starch. The specimens cooked for 15 and 20 minutes all had charred exteriors, crumbling interior tissue, and a considerable reduction in moisture. Fiber content of the domestic specimens used is notably much less and starch content much greater than what is often found in wild plants (O'Dea, 1991). However, the main factors affecting starch gelatinization are water and temperature, and as long as the moisture content is greater than 60 percent, then there is very little interference from the presence or absence of non-starch components such as fiber or protein (Wang and Kim, 1998).

2.3.2 Histological results

Example histological views of native, partially gelatinized, and fully gelatinized starch with Congo Red staining are shown in Figure 3. The result of the final linear mixed model using only the five minute roasting data from the first roasting trial is summarized in Table 4 and the average and standard deviation of gelatinization fraction are reported in Table 5. The interaction between species and position sampled significantly influence the amount of gelatinization after five minute roasting (χ^2

(17) = 268.45, $p < 0.0001$). An effect of species alone is present; however the effect of position is still significant independent of species. Therefore, we can expect that the gelatinization pattern of any starchy USO or fruit after five minutes will be largely raw in the center with increasing gelatinization towards the edge. Each five minute observation point for each position, and their average, is plotted in Figure 4, along with the 20 minute gelatinization fraction. Trend lines all corroborate the increase in gelatinization predicted by the linear model as observations traversed from C to E of each specimen. However, the spread of data is very large, particularly for *C. pepo* and *D. carota*, which is also apparent from the standard deviation values reported in Table 5.

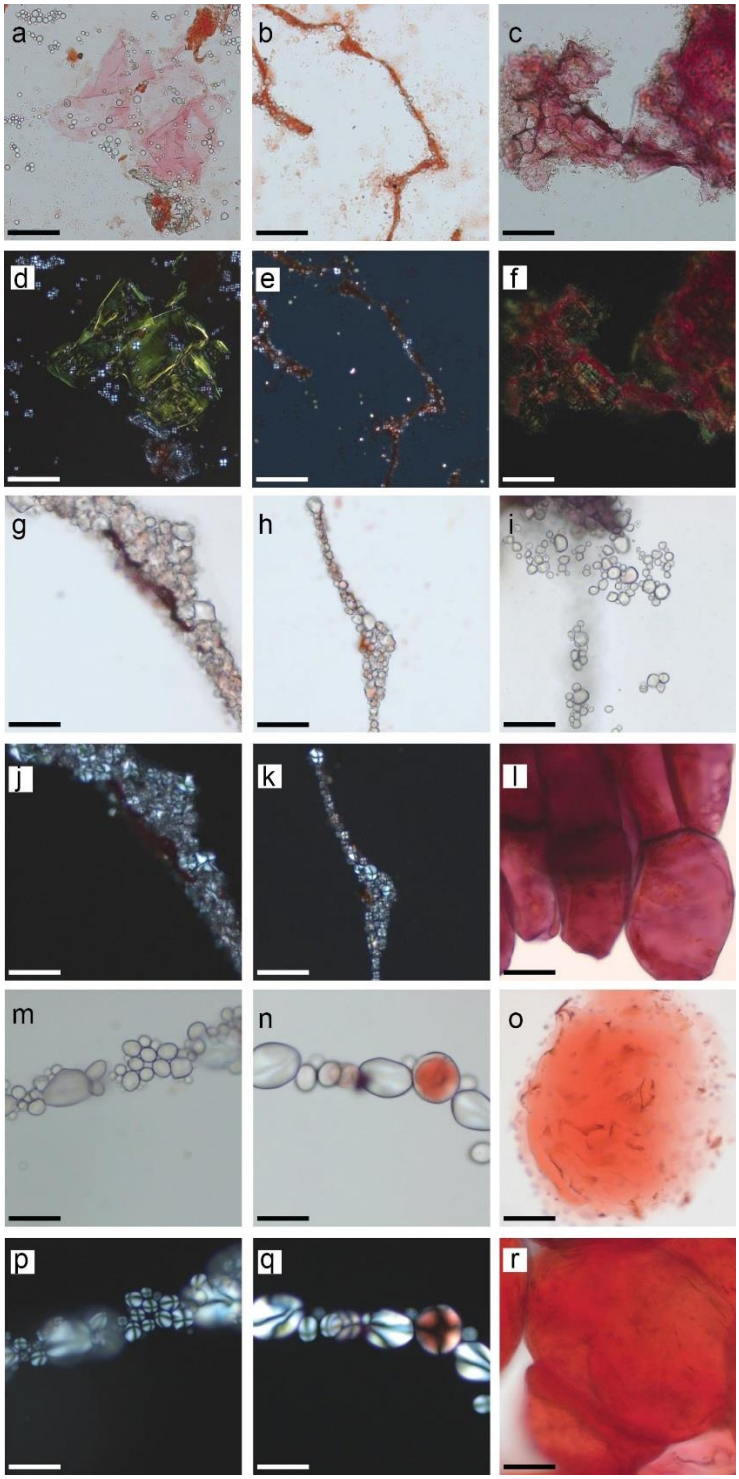

Fig. 3 Starch images of control (raw) and experimental (roasted) specimens show that the Congo Red stain reacts with partially and fully gelatinized starch, but not native starch. Images depict brightfield and polarized views of: *C. pepo* raw (**a**, **d**), partially gelatinized (**b**, **e**), and fully gelatinized (**c**, **f**); *I. batatas* raw and partially gelatinized (**g**-**k**), and fully gelatinized (**l**); and *S. tuberosum* raw (**m**, **p**), partially gelatinized (**n**, **q**), and fully gelatinized (**o**, **r**). Scale bars at 50µm.

Table 4. GLMM intercept estimates for degree of variable influence after five minutes of roasting[1]

Factor	Estimate	Std. Error	t value
(Intercept)	0.190	0.084	2.26
posE	0.555	0.068	8.22
posM	0.103	0.065	1.59
speciesD.carota	0.548	0.233	2.35
speciesD.rotundata	-0.197	0.115	-1.72
speciesI.batatas	-0.326	0.117	-2.78
speciesM.esculenta	-0.131	0.089	-1.47
speciesS. tuberosum	-0.199	0.107	-1.85
posE:speciesD.carota	-0.411	0.110	-3.75
posM:speciesD.carota	-0.171	0.105	-1.63
posE:speciesD.rotundata	-0.229	0.224	-1.02
posM:speciesD.rotundata	-0.098	0.119	-0.82
posE:speciesI.batatas	0.536	0.122	4.40
posM:speciesI.batatas	0.303	0.098	3.09
posE:speciesM.esculenta	0.119	0.089	1.34
posM:speciesM.esculenta	0.263	0.084	3.11
posE:speciesS.tuberosum	0.390	0.162	2.41
posM:speciesS.tuberosum	-0.088	0.090	-0.98

[1]Base levels represented here by (Intercept) are "posC" and "speciesC.pepo".

The average percentage of stained starches across all species remains low for both C and M positions during the five minute cooking and increases rapidly at the E position (Table 5, Fig. 4). In the 20 minute cooking treatment, all species except *I. batatas* are more than 50% gelatinized in the center, with gelatinization increasing precipitously to the edge position. The additional 10 and 15 minute time points that we obtained with a second roasting trial for *I. batatas* and *S. tuberosum* (Table 5) show that gelatinization increases slowly over these time periods. For both, gelatinization is on average below 40% in C positions after 10 and 15 minutes of roasting, and remains so at the M position for *I. batatas*. In *S. tuberosum* the percent of gelatinization jumps to between 40% and 60% after 10 and 15 minutes of roasting. Again, in both species, E positions were near or fully gelatinized.

Table 5. Mean ± standard deviation of gelatinized starch fraction for the CR experiment roasting time trials.

Species	C	M	E
C. pepo			
5 min	0.377 (± 0.39)	0.412 (± 0.35)	0.891 (± 0.24)
20 min	1.0 (± 0.43)	1.0 (± 0.39)	1.0 (± 0.20)
D. carota			
5 min	0.629 (± 0.48)	0.462 (± 0.52)	0.982 (± 0.06)
20 min	1.0 (± 0.42)	1.0 (± 0.50)	1.0 (± 0.05)
D. rotundata			
5 min	0 (± 0)	0 (± 0)	0.143 (± 0.38)
20 min	0.508 (± 0.33)	0.824 (± 0.47)	0.867 (± 0.50)
I. batatas			
5 min	0.005 (± 0.02)	0.105 (± 0.27)	0.970 (± 0.11)
10 min	0.091 (± 0.17)	0.349 (± 0.36)	1.0 (± 0)
15 min	0.146 (± 0.23)	0.209 (± 0.24)	0.971 (± 0.08)
20 min	0.318 (± 0.29)	0.400 (± 0.49)	1.0 (± 0)
M. esculenta			
5 min	0.093 (± 0.15)	0.324 (± 0.25)	0.528 (± 0.47)
20 min	1.0 (± 0.42)	1.0 (± 0.37)	0.981 (± 0.08)
S. tuberosum			
5 min	0.109 (± 0.29)	0.104 (± 0.24)	0.899 (± 0.30)
10 min	0.143 (± 0.20)	0.459 (± 0.36)	1.0 (± 0)
15 min	0.376 (± 0.39)	0.635 (± 0.48)	1.0 (± 0)
20 min	0.625 (± 0)	1.0 (± 0)	0.667 (± 0.58)

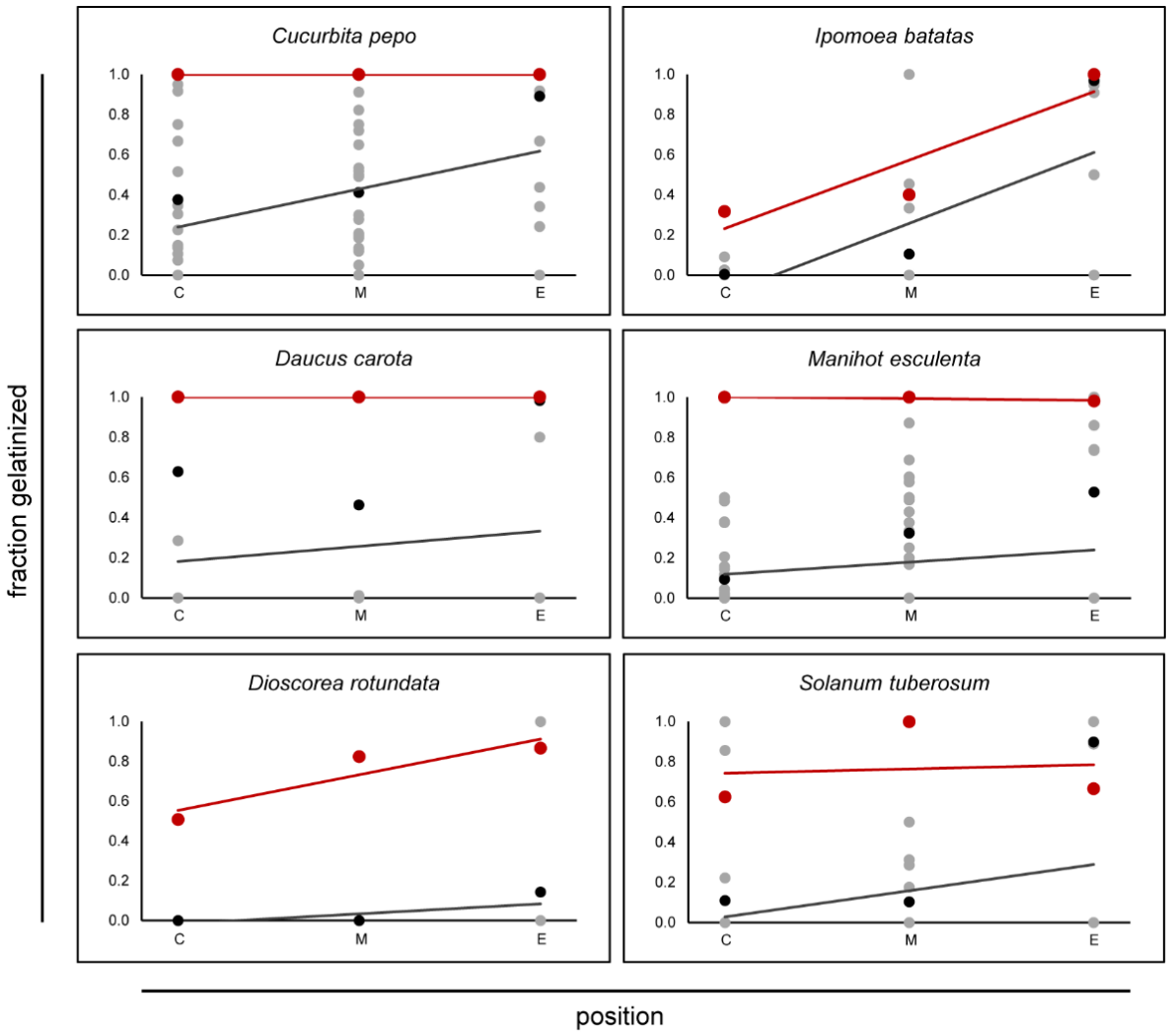

Fig. 4 Scatter plots of the observed gelatinization fraction in stained starch samples across C, M, and E positions for each species. Gray dots are individual observations for five minute roasting data, while black dots represent the mean of these observations. Red dots show the averaged fraction of gelatinization after 20 minutes of roasting. Gray and red trend lines indicate the linear response curve for both five and 20 minute roasting sessions respectively. Differences in number of data points are due to some slide observation coordinates containing no visible starch.

2.3.3 Gelatinization kinetics

The rather large standard deviation values and spread of data reported above indicate that histological staining methods are likely prone to a number of interpretive inconsistencies, such as unequal distribution of the stain across the slide, inability to distinguish gelatinized starches from other stained cellulose structures, and variation in starch susceptibility to Congo Red. Furthermore, staining and counting can easily miss starches buried in overlapping layers. Therefore, we elected to conduct a third trial using only *I. batatas* to validate our previous results in which gelatinization is inferred from a semi-quantitative and highly replicable photometric assay. The reduction in iodine binding affinity is indicative of the rapid diastase hydrolysis activity on damaged and gelatinized starch, and is our measure for gelatinization. The results of triplicate measures on triplicate specimens roasted at five, 10, 15, and 20 minutes are reported in Table 6 and Figure 5. Figure 5a shows the smoothed response curves predicting gelatinization across time-points based on the spread of observations, while Figure 5b displays the average and standard error of absorption measurements across time. Not surprisingly, the gelatinization kinetics found for *I. batatas* are in agreement with the general trend that gelatinization in the center of the specimen remains low, 26% on average, after only five minutes of roasting. Beyond that, gelatinization increases steadily to above 60% at 20 minutes. Measurements in the middle positions estimated a relatively higher level of gelatinization than did the histological staining methods, but average values held steady between 60% and 75% even after 15 minutes of roasting. We expect that perhaps the diastase activity is very efficient upon minor disturbances to the starch semi-crystalline structure, making the photometric assay much more sensitive to partial degradation of starch than CR staining.

Table 6. Percent and standard deviation of gelatinized starch from the kinetic experiment in the C and M position for all 12 specimens across all four time periods.

Time (mins)	Specimen	C	M
5			
	A	22.7 (± 1.7)	71.4 (± 4.8)
	B	23.7 (± 13.3)	77.1 (± 1.9)
	C	30.6 (± 2.2)	43.2 (± 4.4)
10			
	A	100.0 (± 0.2)	100.0 (± 0)
	B	44.4 (± 9.4)	70.4 (± 7.4)
	C	36.4 (± 3.7)	22.7 (± 14.2)
15			
	A	97.3 (± 4.8)	100.0 (± 0.8)
	B	28.9 (± 17.7)	71.1 (± 8.5)
	C	2.7 (± 1.0)	51.4 (± 2.5)
20			
	A	100.0 (± 0)	100.0 (± 0)
	B	50.9 (± 2.5)	78.9 (± 2.3)
	C	59.7 (± 5.3)	66.2 (± 2.2)

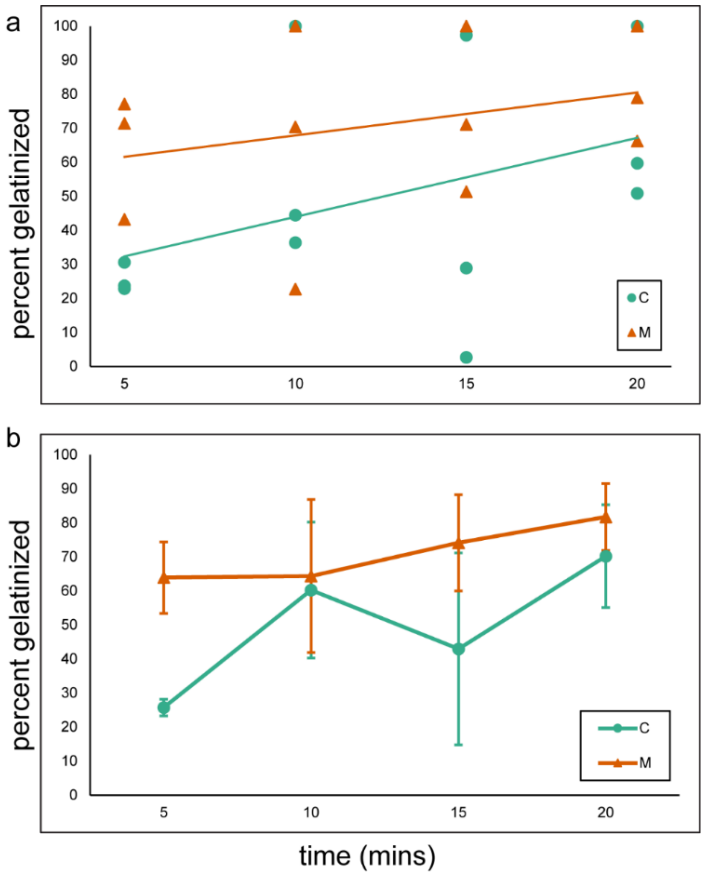

Fig. 5 Kinetics of gelatinization for *I. batatas* (experiment 3) across four time points showing the percent of gelatinization estimated by relative absorption values. (**a**) Individual gelatinization estimates for each of a, b, and c specimens at 5, 10, 15, and 20 minutes respectively. Smoothed trend lines allow an estimation of the gelatinization function by time. (**b**) Average overall gelatinization estimate and standard error per time point across all specimens by time. Green circles, C position; Orange triangles, M position.

2.4 Discussion and conclusions

Our experimental results indicate that brief five-minute fire roasting does not represent adequate cooking time to fully gelatinize native starch in five domestic USOs and one starchy fruit. Gelatinization of more than half of identified starch was on average only observed in the most exterior point sampled just below the peel, except for the center position of *D. carota*, which represents a unique case, likely due to the paucity of observable starch in this species. Gelatinization was rarely present in center (C) and middle (M) points and the red staining observed in these positions most often occurred on mildly damaged or swollen, but intact, starches. By comparison, the red stained starches in the edge (E) positions were largely gelatinized matrix. While significant differences in the gelatinization patterns did occur between species, the model as a whole indicated that the position sampled was the most significant factor in predicting gelatinization, indicating that gelatinization progresses quite slowly from exterior to the interior of the specimen. We acknowledge that this experiment should be repeated on non-cultivated starchy foods to further substantiate our claims. Recent work analyzing digestibility of raw and roasted wild tubers, however, also suggests that brief roasting has little effect on absorption of accessible glucose (Schnorr et al., 2015). Since the present experiment is a test of structural-molecular response to one particular method of thermal processing, we feel confident that our results are useful for helping to understand and predict the effect of brief roasting on any high moisture, intact, starchy organ or fruit.

Understanding the effects of different types of cooking methods on food properties is critical to our interpretations about the importance and necessity of cooking. Different toxic and nutritional properties of plant foods necessitate different processing techniques to navigate plant defense mechanisms and enable human consumption. Modern USO cultivars predominantly come from the families Dioscorea, Solanaceae, and Euphorbiaceae (*D. rotundata* [yam], *S. tuberosum* [potato], and *M. esculenta* [cassava] respectively) and often contain potent toxins such as alkaloids, saponins, glycosides, and raphides (Johns, 1996). Cooking the species of these families, then, is an important tool in the tradeoff between mitigating high toxicity and accessing concentrated starch. This is especially true for domestic varieties in which the lower allelochemical load is easily mitigated through heat treatment alone (Stahl, 1989; Johns, 1996). By comparison, wild plants often contain high amounts of oligosaccharides and phytochemicals (chemical compounds that come from plants such as carotenoids, carotenes, flavonoids, glucosinolates, and organic acids), likely because they have not been intensively selected for high starch content by human consumers (Johns, 1996; Fuller, 2007). Most plant toxins are stable at normal cooking temperatures (≥100 °C), making heating more useful for deactivating enzymes that liberate toxic compounds such as glucosinolates and cyanogenic glycosides (found in *M. esculenta*) or preventing protein activity by destroying the secondary molecular structure. Cooking, therefore, may be a less effective means for handling toxic plant compounds in wild plants. Instead, mechanical

processing or leaching are much more reliable strategies for disabling the majority of toxins found in wild USOs (Stahl, 1989; Johns, 1996).

If thermal processing is not intended to fully cook or gelatinize USO starch, then we must explore alternative desirable effects of fire treatment. Changes in mechanical properties as a result of brief roasting, proposed in Dominy et al. (2008), provide a salient alternative explanation for brief roasting. In particular, brief roasting can expedite removal of bark-like cortical tissue as well as reduce the fracture toughness of the interior parenchymatous tissue of certain wild tubers. In both cases, roasting facilitates improvements in mechanical processing, either from peeling or chewing, and may enable easier access and more thorough mastication (Dominy et al., 2008; Zink et al., 2014). Whether this confers a significant increase in calories alone is an interesting, yet still speculative, proposal. In fact, work by Wollstonecroft (2008) on the effect of thermal and mechanical processing on edibility of sea club-rush (*Bolboschoenus maritimus*) showed that when the plant cell walls and cell-cell adhesion are too strong, thermal treatment does not promote tissue softening despite gelatinizing interior starch. Instead, mechanical processing such as pounding or grinding are necessary to disrupt cell wall structure to soften the tissue and expose interior starch (Wollstonecroft et al., 2008). Therefore, fracturing, caused by tissue rupture (see Fig. 6), allows access to interior starch. Tissue fracture is instead a factor of cell wall structural integrity rather than starch gelatinization (Alvarez and Canet, 2001), and can be a greater factor to nutritional acquisition than gelatinization (Wollstonecroft et al., 2008; Tydeman et al., 2010). Since some plant tissue structures degrade from heat, then cooking can facilitate both access to cellular contents and an increase in their digestibility, but if cell-cell adhesion is weak, then tissue separation can occur before fracture (shown in Fig. 6), and severely limit nutritional acquisition (Tydeman et al., 2010).

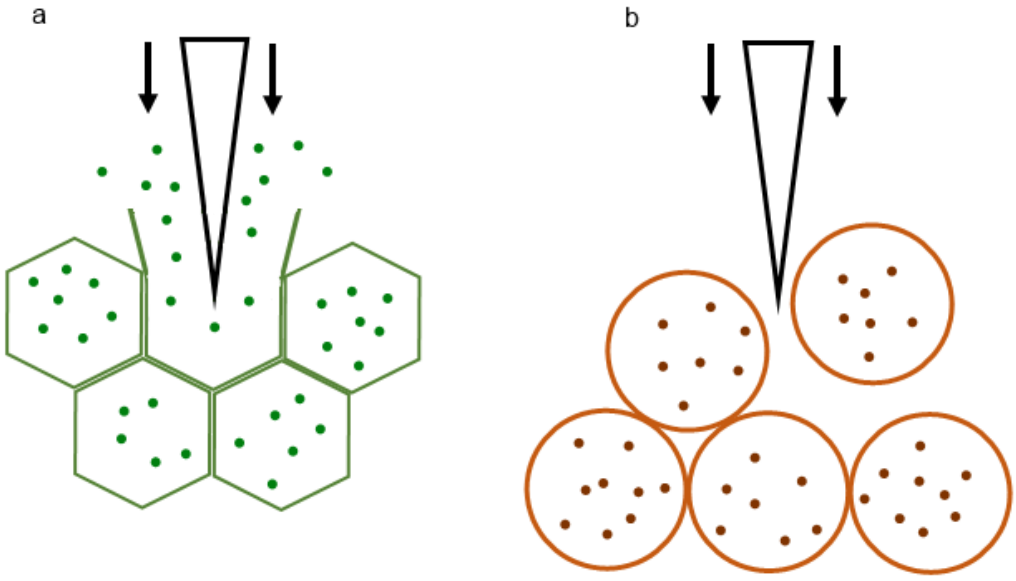

Fig. 6 Diagram representing cell rupture (**a**) resulting in tissue fracture where intercellular content is exposed, and (**b**) cell separation as a result of weak cell-cell adhesion where intercellular contents remain inaccessible, after Tydeman et al. 2010.

In the case of many of the tropical USOs, maximizing nutritional gain may be contingent upon brief roasting such that just enough internal turgor pressure exists to allow tissue fracture while the underlying cortical tissue softens just enough to separate the periderm and expedite consumption. Furthermore, the impact of cooking on other nutritional factors, such as water and micronutrients, is variable depending on the type of heat treatment and food type. Research suggests that cooking may actually be detrimental to vitamin retention and accessibility (Watson, 1976; Reddy et al., 1999; Mandalari et al., 2008; Wollstonecroft et al., 2008; Tydeman et al., 2010). Therefore, calorie content in plant foods may be ancillary to the selective acquisition of other vital nutrients such as carotenoids, ascorbic acid, and especially water (Silberbauer, 1981; Rothman et al., 2011; Jensen et al., 2012).

Brief fire roasting may enhance the culinary experience of foods in a number of ways that are not directly related to increasing caloric nutrition. Roasting may safeguard against pathogenic microorganisms, aid supplementation of particular minerals derived from wood ash (e.g. iron and calcium (Greenhouse, 1981)), or simply ease consumption and increase palatability, becoming a quality of life improvement. Since starchy plants are plentiful and reliable resources in a savanna-mosaic environment (Vincent, 1985), it would have been beneficial for human ancestors living in such regions to adopt strategies, either biological or technological, to enable plant food consumption. In light of our findings, the exploitation of starchy plant resources in and of itself, rather than cooking, may have been the answer to resource related selective pressures in environments occupied by early hominins (O'Connell et al., 1999). However, we recognize that cooking serves different functions in different environments, and it is necessary to model context-dependent variants of cooking technology to empirically test their value for human consumers in that particular environment.

The influence of cooking on starch food is well reported in the food science literature (Collison and Chilton, 1974; Valetudie et al., 1995; Hoover, 2001; Richardson et al., 2004; Stevenson et al., 2005; Nimsung et al., 2007; Sajeev et al., 2008; Singh et al., 2010; Nguyen Do Trong et al., 2011; Parada and Aguilera, 2011), however such research is inadequate for evolutionary-based hypothesis testing regarding the dietary ecology of human ancestors. Research models are needed that contextualize plant food cooking techniques with the archaeological evidence of early human and hominin technologies. We believe that a simple fire-roasting technique, which is regularly used by extant hunter-gatherers to cook USOs, is an appropriate analogy. Future experiments using wild plants are needed to improve our understanding of starch gelatinization properties under varied relevant thermal processing conditions. Such models can be powerful informants to how and why human ancestors experienced dietary diversification, especially with regards to plant foods, and exactly how cooking may have significantly influenced human evolution.

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CHAPTER 3

ASSESSING DIGESTIBILITY OF HADZA TUBERS USING A DYNAMIC *IN-VITRO* MODEL

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ABSTRACT 2

Bioaccessibility is a useful measure for assessing the biological value of a particular nutrient from food, especially foods such as tubers. The wild tubers exploited by Hadza foragers in Tanzania are of interest because they are nontoxic, consumed raw or briefly roasted, and entail substantial physical barriers to consumers. In this study we attempted to elucidate the biological value of Hadza tubers by measuring the absorption of glucose through *in-vitro* digestion. We quantified digestibility using data from 24 experimental trials on four species of Hadza tuber using a dynamic *in-vitro* model that replicates digestion in the stomach and small intestine. Analysis of glucose in the input meal and output dialysate revealed the accessible glucose fraction. We also conducted assays for protein, vitamin and mineral content on whole tubers and meal fractions. Bioaccessibility of glucose varies depending on tuber species. Holding effects of chewing constant, brief roasting had negligible effects, but high intraspecific variation precludes interpretive power. Overall, Hadza tubers are very resistant to digestion, with between one to two thirds of glucose absorbed on average. Glucose absorption negatively correlated with glucose concentration of the tubers. Roasting may provide other benefits such as ease of peeling and chewing to extract edible parenchymateous tissue. A powerful factor in glucose acquisition is tuber quality, placing emphasis on the skill of the forager. Other nutrient assays yielded unexpectedly high values for protein, iron, and iodine, making tubers potentially valuable resources beyond caloric content.

3.1 Introduction

Bioaccessibility, the portion of food that is available for absorption into the systemic circulation, is a newly appreciated metric in nutritional science and food ecology for assessing the contribution of food towards metabolism (Stahl et al., 2002). Also known as the biological value of food, bioaccessibility (also sometimes referred to as bioavailability) is normally expressed as a ratio or percentage of nutrition absorbed versus consumed and is descriptive beyond nutritional composition because it accounts for digestible and indigestible constituents. Applying this metric to nutritional ecology in anthropology is important because Plio-Pleistocene hominins survived on wild foods, which have not undergone artificial selection for nutritional content and digestibility (Johns, 1996; Purugganan and Fuller, 2009). Wild plants may be especially problematic because natural selection would favor plants that resist consumers, if they do not facilitate reproduction and dispersal, either through physical or chemical barriers (Johns, 1996).

One particular type of wild food, called plant underground storage organs (USOs), is thought to be of importance in hominin dietary ecology. While many types of USOs are a common component of the modern human diet (Couture et al., 1968; Woodburn, 1968; Hart and Hart, 1986; Bradbury and Holloway, 1988; Oke, 1990; Lindeberg et al., 1996; Cordain et al., 2000; Dounias, 2001), their edibility and nutrition is highly variable depending on many factors, such as the species, season, and age of the specimen (Bradbury and Holloway, 1988). As a result, USO processing and consumption is a regionally and culturally specific process. Research in human dietary ecology often questions whether the nutritional needs of human foragers living in East Africa could be met from the consumption of a diet consisting primarily of meat, USOs, and other plant foods (Ungar et al., 2006). USOs were likely a key resource for early hominins because of a number of appealing properties including: 1) wide distribution and high yield throughout a variety of environments (Vincent, 1985; Couture et al., 1986; Oke, 1990; Yasuoka, 2006) possibly enabling survival for early hominins ranging across and out of Africa; 2) a concentration of minerals and carbohydrates that are critical elements for brain growth (Finglas and Faulks, 1984; Bradbury and Holloway, 1988; Aiello and Wheeler, 1995; Ashworth and Antipatis, 2001; Laden and Wrangham, 2005; Wrangham et al., 2009; Deshmukh and Rathod, 2013); 3) recurrent exploitation by women in foraging societies for whom reproductive investment and constraints make hunting an unfavorable subsistence strategy (O'Dea, 1991; Hawkes, 1996; O'Connell et al., 2002; Marlowe, 2005; Berbesque et al., 2011); 4) and finally, USO foraging draws heavily on the experience and allomothering supplied by older post-reproductive female foragers, providing a compelling argument for the importance of grandmothers in human evolution (O'Connell et al., 1999; Crittenden and Marlowe, 2008). A reliance on USOs often implies the need for cooking to breakdown physical and chemical barriers in the plant so that a human consumer can access the nutrition commensurate to what was expended in acquisition (Stahl, 1989; Carmody et al., 2011). The Hadza hunter-gatherers of East

Africa offer a unique opportunity to test the bioaccessibility of nutrition in the wild USOs, specifically tubers from Fabaceae and Convolulaceae, that they consume year round (Marlowe and Berbesque, 2009). In this paper we explore the factors relating to digestibility and glucose absorption using a dynamic *in-vitro* model for four different species of wild tuber regularly consumed by the Hadza.

Image 3. A day's forage is shared back at camp amongst all camp members, including men and older children.

Traditional caloric measurements in nutritional science, based on quantitative assays, portray food in only one dimension. To complete the picture, we must understand the ability and limitations of humans to deal with complex macronutrients, micronutrients, and antinutrients through digestion, especially these latter compounds that occur mainly in plants and adversely affect nutritional absorption. While in theory we may recognize that the digestive tract is not a perfect nutrient harvester, however, in practice it is much harder to quantify the extent to which different food is broken down and absorbed, or passed on into the colon for fermentation (Stahl et al., 2002). Add to that the effect of different food processing techniques on nutrient accessibility, and the whole notion of exacting nutritional gain from food can seem a vexing process of navigating overwhelming complexity. The human body relies on mechanical, chemical, and enzymatic activity to break down many different complex molecules for absorption into the systemic circulation (Hadley and Levine, 2007). Therefore, to mimic these events *in-vitro*, one must account for varying pH, peristaltic movement, time dependent release of digestive enzymes, and most importantly, an absorption barrier or gradient that will inhibit

passage of larger molecules. From this perspective, it is easy to see that both physical and chemical aspects of different foods can affect the accessibility and efficiency of nutrient absorption, and that this may be further modulated by regional nuances both in culture and biology (Johns, 1996). To address such complexity, we use the TNO *in-vitro* gastro-intestinal model (TIM-1), which allows full simulation of human digestion with validated physiological accuracy to *in-vivo* activity (Minekus et al., 1995, Souliman et al., 2006). The TIM-1 has been validated for nutrients in several types of foods that are generally consumed, and validation has been performed for carbohydrate, protein, and fat digestion in a number of different whole foods (Minekus 1998; see Chapter 7). In addition, vitamin bioaccessibility has been validated for both water and fat-soluble vitamins. In all cases, the TIM-1 results closely correspond to clinical data (Minekus 1998). Therefore, it can be concluded that the system mimics the dynamic physiological process of digestion, and can be used with confidence for unknown meals, including wild foods.

Food processing is an incredibly important aspect of human dietary ecology (Stahl, 1989; Wollstonecroft et al., 2008). Historically, humans have overcome refractory or toxic compounds in food through simple or complex processing techniques that can cause physical or chemical changes to food properties. The archaeological precedence for these techniques is still not clearly understood, but certainly physical processing, such as cutting or pounding, has been in use since at least 2.6 million years ago, with good evidence for nearly one million years prior to that (Semaw et al., 2003; McPherron et al., 2010; Lemorini et al., 2014). Cooking, in particular, is a profoundly important technology for modern humans and it is proposed that the adoption of cooking by human ancestors has led to a universal human dependency on cooked foods (Wrangham and Conklin-Brittain, 2003; Carmody et al., 2011). However, evidence of fire in an archaeological context, whether implying a consistent presence or use in cooking, is still debated (Goren-Inbar et al., 2004; Roebroeks and Villa, 2011; Berna et al., 2012). Cooking breaks down complex molecular structures such as long-chain polysaccharides and proteins, making food softer, and exposing binding sites for digestive enzymes, which facilitates absorption in the gut (Englyst and Cummings, 1986; Holm et al., 1988; Bornet et al., 1989). In addition, thermal treatment of food, especially plants, can denature toxic compounds or enzymes responsible for releasing toxins, as well as kill pathogens, making food safe for human consumers (Stahl, 1984; Johns, 1996). For a human forager, cooking reduces pathogen load and can help buffer against food shortages from seasonal or climatic effects, niche competition, and marginal habitat occupation by enabling expansion of dietary breadth. Therefore, if the control of fire incurred limited costs in terms of time, energy, and resource management, then its use for cooking was clearly advantageous to hominin survival.

While many USOs are toxic when raw, most notably common cultivated yams such as *Dioscorea alata* and *D. rotundata*, cassava (*Manihot* spp.), and potato (*Solanum tuberosum*) (Bradbury and Holloway, 1988), the wild tubers exploited by the Hadza are all non-toxic and are consumed raw as well as cooked (Woodburn, 1968; Vincent, 1985). Instead of toxins, Hadza tubers maintain defense

against consumers through largely physical attributes. Edible parts are found deep underground, covered in a thick inedible bark-like skin, the peridermis and cortex, and are laced with inedible fibers to which the edible parenchymatous tissue (the pulp) adheres, necessitating considerable time and dedication to extract and consume (Schoeninger et al., 2001). The Hadza forage and consume these wild tubers year-round, which constitutes a significant portion of their diet, supplementing other foods such as game meat, fruits, legumes, and honeycomb (Marlowe and Berbesque, 2009). Roasting times reportedly range between three (Dominy et al., 2008) to thirty minutes, with the lower end of this range, described as "light roasting" (Tomita, 1966) and resulting in "lightly charred" tubers (Woodburn, 1966), cited as approximately five minutes (Woodburn, 1968; O'Connell et al., 1999; Mallol et al., 2007; Marlowe and Berbesque, 2009). Many researchers have questioned why the Hadza bother roasting the tubers for such brief durations (Marlowe and Berbesque, 2009), and how these roasting practices may affect nutritional gain from these tubers. The brief roasting technique employed by the Hadza to consume their tubers is a simple form of cooking, requiring only an open-flame fire, and no other extended processing common to plant food preparation such as grinding, leaching, or sprouting (Stahl, 1989). As such, on the basis of putative stone tool technologies and cranial architecture of early hominins, brief roasting may fall within the behavioral and cognitive capacity of early *Homo* (Neubauer and Hublin, 2012). Modern hunter-gatherers should not be mistaken as Paleolithic emulations, but rather referents to the behavioral and nutritional adaptations that may have been necessary for early hominin occupation of the East African savanna-mosaic environment. Therefore, it is of interest and relevance to anthropologists modeling hominin nutritional ecology to understand the extent to which the constituents of edible East African tubers become available through digestion to the consumer's metabolic physiology.

This study entails the first attempt to quantify the digestibility of wild East African tubers exploited by a modern population of hunter-gatherers. We analyzed the glucose absorption and protein and vitamin contents of four species of tubers consumed by the Hadza, in both raw and briefly roasted states, using the TIM-1 dynamic *in-vitro* model of the human gut (Minekus et al., 1995). Glucose absorption represents the bioaccessible fraction, and glucose bioaccessibility was assessed across and within tuber species for differences between cooked and raw specimens. Our findings indicate large intraspecific variation, which reduces statistical power, but in general that cooking has variable effects, depending on the species, and that tubers were highly resistant to digestion, resulting in low glucose accessibility.

3.2 Methods

3.2.1 Field collections

Tuber collections were conducted during the rainy season in January 2013 in two different camps in Yaeda Valley, near Lake Eyasi, northern Tanzania. Camps were selected on the basis of having at least 10 adults, both men and women, with women foraging daily for tubers. Upon arrival into the camp, members were verbally informed of the study and asked whether or not they would like to participate before researcher presence was established. All data were collected with research permission from the Tanzanian Commission for Science and Technology (COSTECH), permit number 2012-315-NA-2000-80. We collected tubers during foraging trips in the mornings and evenings over a period of four to five days at a time. We only obtained tuber species that the women were actively gathering for consumption. In the end, tubers present in sufficient quantity to complete the study included five main species that are regularly consumed by the Hadza, which is summarized in Table 1: *//ekwa* (*Vigna frutescens*), *shumuko* (*Vatovaea pseudolablab*), *mak'alitako* (*Eminia entennulifa*), *panjuko* (*Ipomoea transvaalensis*), and *penzepenze* (*Vigna* sp.). The women were asked to forage as normal and only tubers offered willingly to the research team were used for subsequent analysis. In order to avoid taking all the foraged food (which was offered), and to check whether offered tubers were actually worth consuming and not just sub-optimal tubers, we would randomly reject an offered tuber and observe whether the women kept or discarded it. In all cases, the tubers were kept and consumed.

Image 4. Collecting tubers with Hadza women meant carrying my share of the load of willingly offered forage.

Women were observed during roasting sessions in camp and measurements of tuber circumference and roasting time recorded for available species brought back to camp for reference (Table 2). Once back in camp, collected tubers were immediately cleaned of adherent dirt and soil with a dry towel and toothbrush, weighed using a hanging balance (KERN HBD-N), and then stored raw, whole, and intact in a dark, dry, breathable container at ambient temperature in the shade for no more than six days while in camp.

Image 5. A large haul of *I. transvaalensis* tubers and one long *V. frutescens* root from an afternoon forage.

This period of time in a warm, humid, but ventilated container is an important step in tuber preservation, constituting a curing phase that lasts from five to 20 days. This is the standard procedure recommended by the Food and Agriculture Organization of the United Nations (FAO) for tropical tuber crops that are shipped world-wide for human consumption (Diop, 1998; see Chapter 3, section 3.1.1), and is the ideal storage procedure for our purposes of simulating human consumption and digestion. The curing phase allows roots and tubers to heal surface wounds and strengthen the peridermal tissue when held at relatively high temperatures and humidity for some days after harvest. This also helps mitigate water loss, cellular respiration, and invasion by pathogens (Diop, 1998). During the time of our visit, temperatures recorded at the nearest weather station in Arusha, TZ ranged between 13°C and 31°C for the daily lows and highs, with an average high temperature of 28°C, and average high humidity between 70-90%. Optimal conditions for curing include temperatures between 15-40°C and relative humidity at 85-90% (Diop, 1998; see Chapter 3, Table 3.1). After a maximum of six days in our field camp, tubers were transported in the container to a cool ventilated storage shed for the remaining duration of our field work (a maximum time of one week) until we could transport all tubers directly on the airplane back to our labs in Leipzig, Germany. Once we arrived at the lab, all tubers were immediately refrigerated at 4°C, the recommended temperature for long-term storage, until further processing (a maximum of one week). Throughout this time, the condition of the tubers was closely monitored, and those that showed major damage such as cuts or bruises were not accepted. Cutting and attempting to air-dry samples in the field introduces numerous mechanistic vectors for loss of nutrition such as from

sun scorch, microbial contamination, and insect infestation (Diop 1998; see Chapter 2, Table 2.1). No characteristic signs of rot were detected by sight (mold, browning, bruising), smell (acrid or very sweet), or taste (sour or acidic) in all 68 tubers except for two specimens, which were realized to have sustained surface damage and subsequently discarded (see Figure 1).

Table 1. Taxonomic identifications of Hadza tuber species with brief physical quality descriptions.

Latin name	Hadza name	Physical traits
Vigna frutescens	*//ekwa*	Elongated tuber with woody bark-like peridermal and cortical tissue. Parenchyma adheres to thick longitudinal inedible fibers that are chewed and expelled.
Eminia entennulifa	*mak'alitako*	Large round tubers with thinner cortical peel than *V. frutescens* but consisting of cortical layers and a juicy parenchyma filled with small thin inedible fibers.
Ipomoea transvaalensis	*panjuko*	Smaller tubers similar in shape and size to *I. batatas* (sweet potato). Peel is thin and no inedible fibers restrict consumption. Most commonly consumed raw.
Vatovaea pseudolablab	*shumuko*	Large round juicy tuber with thick layered cortical tissue and very fine internal fibers.
Vigna sp.	*penzepenze*	Elongate and fibrous, similar to *V. frutescens* but slightly smaller and with distinct nodules. Cortical layer is thinner but difficult to remove without a knife or fire.

Table 2. Field measurements of circumference and cooking times for *V. frutescens* and *V. pseudolablab*.

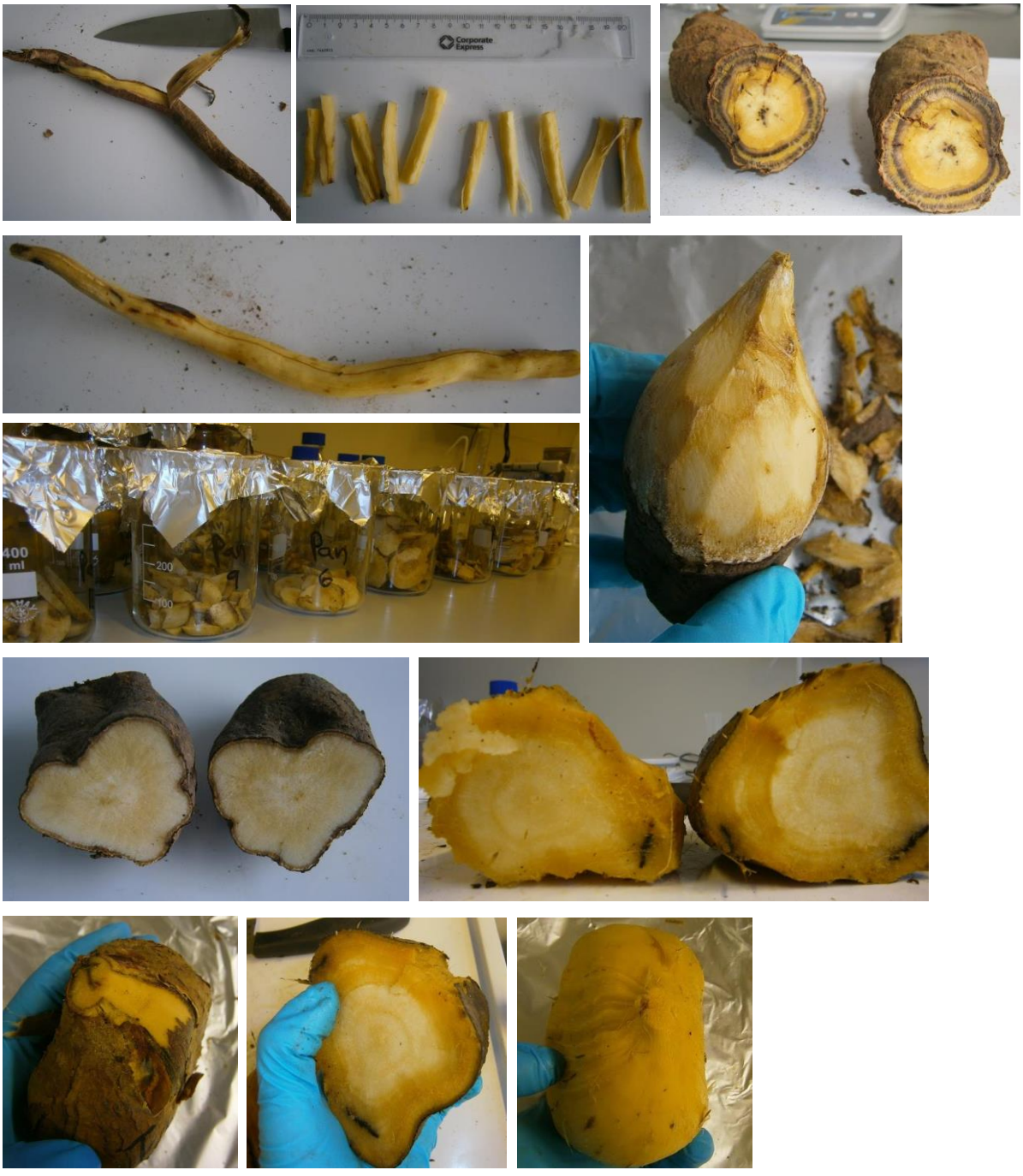
Specimen	Circumference (cm)[1]	Cook time (min)[2]	Notes[3]
V. frutescens 1	13.34	7	moved at 3 mins, only distal end in fire, first half out
V. frutescens 2	11.43	5	moved at 3 mins, only distal end in fire, first half out
V. frutescens 3	18.42	5	
V. frutescens 4	15.88	5	turned after 2 minutes and after 3 minutes
V. frutescens 5	13.97	5	
V. frutescens 6	10.80	13	turned at 4 and 5 mins, only ends in fire at 9 mins
V. frutescens 7	12.07	13	turned at 4 and 5 mins, only ends in fire at 9 mins
V. frutescens 8	7.62	9	turned at 4 and 5 minutes
V. frutescens 9	10.80	13	turned at 4 and 5 mins, only ends in fire at 9 mins
V. frutescens 10	10.16	11	turned at 4 and 5 mins, only ends in fire at 9 mins
V. frutescens 11	10.16	11	turned at 4 and 5 mins, only ends in fire at 9 mins
V. frutescens 12	12.07	13	turned at 4 and 5 mins, only ends in fire at 9 mins
V. pseudolablab 1	31.12	6	turned at minutes 3 and 4
V. pseudolablab 2	41.28	10	turned at minute 5
V. pseudolablab 3	26.04	5	

[1] Circumference was measured at the widest section of the tuber.

[2] Average cook time is 8.73 minutes, not accounting for adjustments that removed a portion of the tuber outside of the fire, and is therefore a conservative calculation.

[3] We observed that tubers are not static during roasting and are often turned or removed from the fire to reposition or reorient the tubers so that all portions are evenly roasted. This means that cooking times can be errantly overestimated if only one half of the tuber is roasted at a time, as is often the case for the longer *V. frutescens* tubers.

a.



b.

c.

Fig 1. Uncooked (**a**) and cooked (**b**) tuber specimens brought back from Hadza land show no characteristic sign of rot, except for two tubers (**c**), which were subsequently discarded.

3.2.2 Roasting and pre-processing

We conducted the roasting and pre-processing (chewing) phase in the plant foods research lab at the MPI-EVA in Leipzig, Germany. The tubers consumed by Hadza are largely composed of tough outer peridermal "peel" and internal inedible fiber, necessitating considerable pre-processing to extract the edible parenchyma. After peeling and chewing for approximately thirty seconds to three minutes, Hadza expel a mass of the inedible fibers, termed a "quid" (Schoeninger et al., 2001). The peel and quid are not subject to gastro-intestinal (GI) digestion, posing a unique challenge for the TIM-1 system, and were therefore removed through peeling and processing in a homogenizer, or "stomacher" (Seward stomacher 400C, Fisher Scientific), prior to our *in-vitro* digestion simulation. Normally, an immersion blender is used to completely liquefy TIM-1 input meals followed by incubation with α-amylase. Therefore, we chose the methods of Schoeninger et al. (2001) as it is the only study of Hadza tubers that fully appreciated and addressed this biologically relevant aspect of Hadza tuber consumption. We sought to replicate these methods for the chewing phase because they allowed us to control the α-amylase type and strength, and manually separate the edible parenchyma from indigestible fiber. The normal TIM-1 protocol was reinstated just before each trial run in which the samples were liquefied by immersion blending to allow passage through the TIM-1 chambers.

Tubers were weighed in the lab using the same hanging balance to determine moisture loss during storage and transit. The only two rotten tubers were discarded. When not in immediate use, specimens were stored at 4°C. Specimens were sorted by species into raw and roast groups, matched by weight, and the roast group was cooked on a high-flame open fire for five minutes (Table 3). This fire was produced to simulate a Hadza tuber roasting fire (bush fire) using small dry wood-sticks with a high initial flame and lasting about 10-15 minutes (Mallol et al., 2007). The temperature of the fire (500-700 °C) and duration of roasting time (5 min) are based on personal (see Table 2) and previous field observations (Woodburn, 1968; O'Connell et al., 1999; Mallol et al., 2007; Marlowe and Berbesque, 2009; for temperature data see Dominy et al., 2008; Table 3).

Table 3. Fire temperatures for simulated roasting trials to emulate Hadza tuber roasting fires.

Trial	Time (min)	Fire Temp °C (i)	Fire Temp °C (o)
1	5	550	600
2	5	550	600
3	5	500	600
4	5	500	590
5	5	500	590
6	5	600	700

Image 6. Setting up and carrying out tuber roasting emulations in Germany during a February snow.

Tubers were peeled by hand to ensure removal of the inedible peridermal and cortical tissues and the resultant peel weighed. The remaining parenchymal tissue was chopped coarsely and combined with water equal to the predetermined moisture loss. The chopped tuber and added water were transferred to a stomacher bag and combined with 50ml warm water (40°C) and 1000U α-amylase (Sigma A1031) per 100g tuber sample. This level of amylase activity was selected to approximate the estimated protein expression level for Hadza derived from a regression of the work of Perry et al. (2007) in their Figure 1C and Supplementary Table 1. Chewing was approximated using the stomacher at 250rpm for two 3-minute intervals. Homogenized tubers were placed on ice immediately for a minimum of 30 minutes to deactivate enzymes, and then fibers constituting the quid were removed manually, weighed, and frozen.

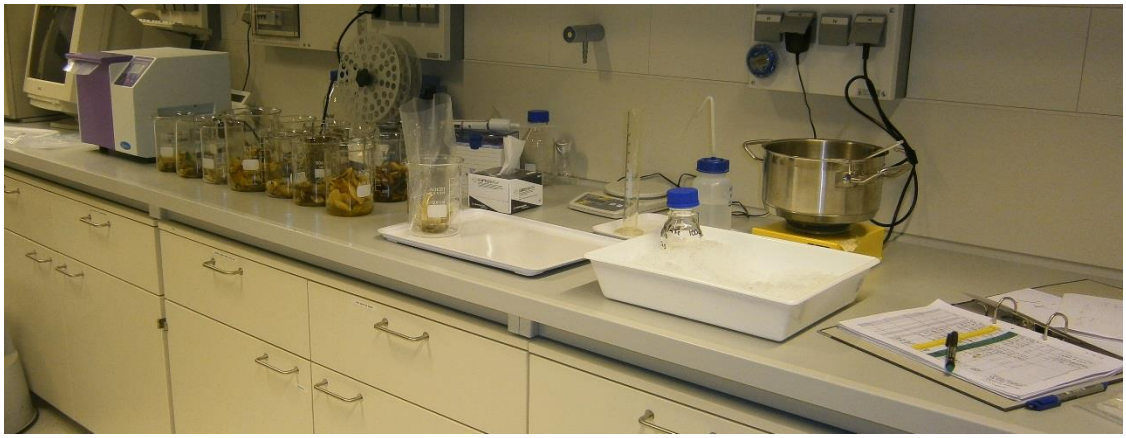


Image 7. Lab set up at MPI-EVA for the pre-processing steps to simulate chewing.

Final tuber samples deemed as the consumable portion were again weighed and then frozen at -20°C until further use. Because *I. transvaalensis* does not contain the same fiber mass and did not properly homogenize in the stomacher, these specimens were simply peeled and blended with an immersion blender and then incubated with α-amylase just prior to *in-vitro* digestion. All peeled and pre-processed specimens were brought on dry ice directly to TNO Department of Pharmacokinetics & Human Studies in Zeist, The Netherlands, to conduct the *in-vitro* digestion trials. All work was conducted by wet weight because this is necessary in the context of our work on the *in-vitro* system and in modeling human consumption of whole foods.

3.2.3 Dynamic *in-vitro* digestion

The TIM-1 system (**T**NO *In-vitro* gastro-intestinal **M**odel) is a unique, validated, computer-controlled simulation of the stomach and small intestine with great reproducibility and application in studies of human health (Minekus et al., 1995, Minekus, 1998; Souliman et al., 2006). A detailed description of the TIM-1 system and methodology has been previously reported (Blanquet et al., 2004; Minekus et al., 2005; Faessler et al., 2006). The model consists of four chambers representing the stomach, duodenum, jejunum, and ileum respectively (Figure 2). Meals comprising the amylase-treated tubers were inserted directly into the stomach compartment of the model and subsequently digested for six hours at 37°C. Peristaltic valve pumps, mixing, transit time, and fluid secretions were regulated automatically by preset values to mimic biological standards. As the introduced meal reached the jejunum and ileum compartments, contents were pumped across two semi-permeable membranes (hollow fiber limited to ca. 5kDa) that removed water, simple sugars and amino acids (products of digestion), through which the dialysate fluid was pumped at a rate of 10ml min^{-1}. This removal prevented build-up of metabolites that otherwise would inhibit further enzyme activity. Membrane absorption, dialysate and ileal delivery were collected every hour for sampling throughout the six hour run.

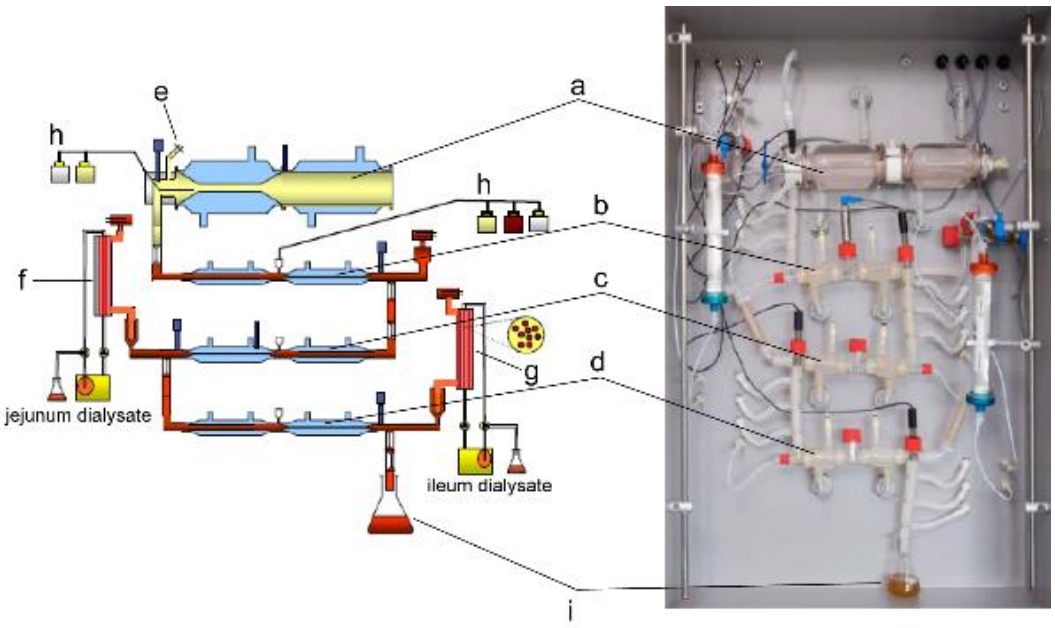


Fig 2. Schematic of the TIM-1 system illustrating the four main compartments: stomach (a), duodenum (b), jejunum (c), and ileum (d). Meal input begins at the stomach port (e), bioavailable nutrients are "absorbed" by passing the jejunum (f) and ileum (g) filters and collected in dialysate reservoirs to prevent buildup of metabolites. Acid and bicarbonate are used to control the pH, following preset values in each compartment while digestive fluids are secreted continually in the small-intestine during the experiment (h). Finally, the ileal efflux (i) is collected and analyzed, representing the ileo-caecal valve where residual meal contents unavailable for absorption would enter the colon. Image adapted from TNO TIM-1 reference manual, (Lelieveld et al., 2009).

3.2.4 Gastric and intestinal environment

The TIM-1 system was prepared prior to each experiment by rinsing and loading the compartments with starting fluids, following standard procedures (Faessler et al., 2006). The duodenal compartment was filled with 55ml of 50% bile solution (4g porcine bile in 250g water; Sigma B8631), 25% pancreatine solution (supernatant of 17.5g in 250g water, centrifuged 20 min at 9000 RPM at 4°C; Pancrex V, Paines and Byrne, Greenford, UK), 25% small intestine electrolyte solution (SIES; composed of NaCl 5g l^{-1}, KCl 0.6g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.3g l^{-1}, $NaHCO_3$) and 1g trypsin (Sigma T9201). Jejunal and ileal compartments were filled with 100ml of SIES. The pH was controlled in the stomach along pre-set values of 5.2, 3.2, 2.2, and 1.7 at 0, 30, 60, and 120-360 min, respectively, by secretions of 1M HCl or water. In the small intestine, the pH was held at 6.2, 6.5 and 7.4 in the duodenum, jejunum and ileum compartments, respectively, by secretions of 1M $NaHCO_3$. The model was allowed to heat to 37°C in all compartments prior to the experimental runs.

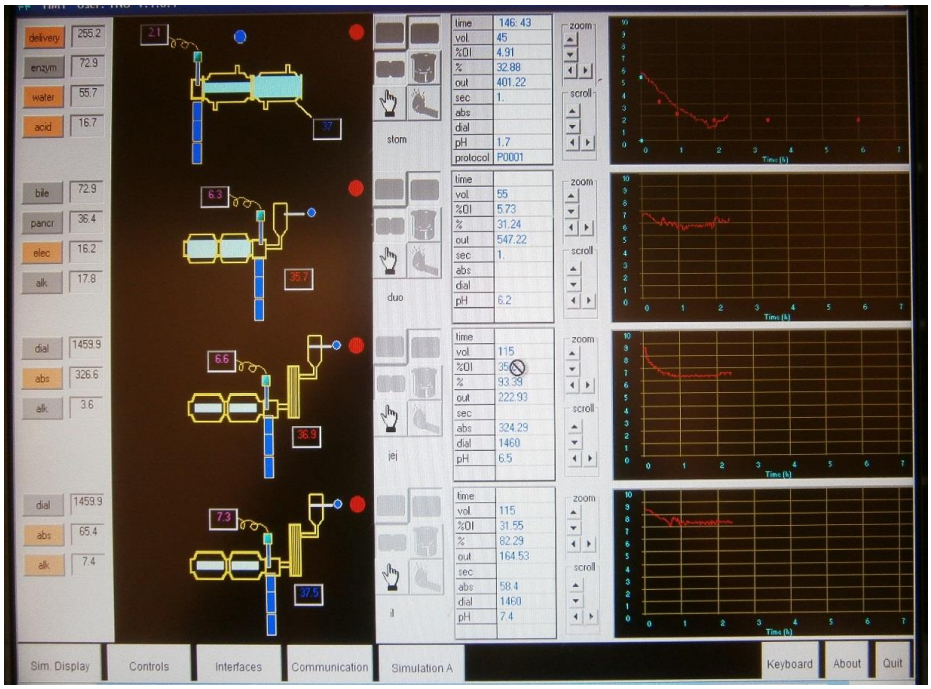


Image 8. The computer controlled TIM-1 interface showing preset values in action after start of a run.

3.2.5 Meal preparation

Meal preparation was handled according to standard procedure (Blanquet et al., 2004; Dominy et al., 2004). Initially, individual amylase-treated tuber specimens were run in duplicate for both cooked

and raw test states except for *I. transvaalensis*, which had to be combined due to small individual sizes. However, for some technical replicates we used multiple specimens because the material from one specimen was not enough to meet the minimum input amount for the TIM-1. A third trial was conducted using the remaining consolidated material, portioned by species and cooking state, to attempt to minimize effects from intra-species variation. All tuber samples and the specimen run schemes are detailed in Supplementary Table 1 (see Appendix A). Meals consisted of 200g of amylase-treated tuber, 30g gastric electrolyte solution (NaCl 3.0g, KCl 1.1g, $CaCl_2$ 0.14g dissolved in 46.8g water and adjusted again to 500ml with water), 20g water, 5g SIES, 1M HCl to adjust the pH to 5.2 and 45g rinse water for gastric introduction. For the nutritional reference values, one 2ml and two 1ml meal samples were saved and frozen immediately at -20°C prior to the experimental run.

Image 9. Loading the stomach compartment with the tuber meal and removing all excess air in the membrane.

3.2.6 Sampling and analysis

Digestion of tubers was simulated across six-hour experimental runs, mimicking a period in

which approximately 95% of the meal had transited the complete upper GI tract. At each hour, dialysis bottles were replaced and the absorption, dialysate and efflux weighed and 1ml samples saved to obtain digestion kinematics over time. Samples were stored at -20°C. At the end of each experiment, the residues remaining in the model were removed, weighed and 1ml samples stored at -20°C. The residues and ileal efflux are elements of the meal considered to be inaccessible for digestion and absorption in the small intestine while the dialysate represents the bioaccessible nutritional constituents (Faessler et al., 2006).

Dialysate samples were tested for glucose with glucoamylase (AMG) treatment using a commercial kit (Roche) on a Cobas Mira plus autoanalyzer (Roche, Almere, The Netherlands). With AMG treatment all small and easily degradable dextrins (maltose, maltotriose, and starch fragments with 1-4 and 1-6 bonds) are degraded by AMG. However, more complex molecules such as native starch can be missed by AMG. Therefore, the meal (the input volume consisting of pre-processed tuber plus the meal starting residues and amylase solution) and ileal efflux samples were first submitted to acid hydrolysis through treatment with 1M H_2SO_4 in order to degrade more resistant glucose polymer units that may be missed by standard AMG treatment. Since our aim was to model stomach and small intestine digestion, and because humans can only produce amylases specific for alpha linked glucose polymers, we did not assay for fructose as a product of inulin digestion, which is instead fermented by colonic microbiota (Roberfroid, 1999).

In addition to glucose, we were also interested in the protein content and digestibility of the tubers. We therefore measured amino acids by alpha-amino nitrogen (AAN) analysis using the modified Biuret assay (Noll et al., 1974) on dialysate and ileal efflux samples. A modified Biuret assay better accounts for native proteins using bovine serum albumin (BSA) as a standard and implemented on a Cobas Mira plus autoanalyzer. The total Kjeldahl nitrogen (TN) content of the meal samples was also measured so as to account for all sources of nitrogen (nitrate, nitrite, ammonia and organically bonded nitrogen). A standard calibration curve is generated by boiling known quantities of BSA for five minutes in 1M NaOH. After cooling, Cu^{2+} ions are added (copper sulphate solution), mixed and centrifuged. The absorbance of the supernatant is measured at A500nm and the crude protein concentration curve generated. The same procedure is applied to the test samples and the total protein concentration estimated with the calibration curve. Dividing the protein concentrations by 6.25 results in the calculated TN amount for the unknown samples and a correlation curve is generated with TN (Kjeldahl) and TN (Biuret) measured samples, allowing calculation of the actual TN of the test samples. All analyses were performed by Bio-aNAlytiX (Mook, The Netherlands).

3.2.7 Micronutrient analysis

To date there has been no attempt to quantify the micronutrient content of Hadza tubers. Since this is an important determinant of food quality, we made preliminary assessments of the mineral content of select "meal" doses from the consolidated (within species) samples and the mineral and vitamin content of whole tubers from one species, *penzepenze* (*Vigna* sp.) that were not submitted to *in-vitro* digestion. Ideally we would also analyze the dialysate to obtain measures of micronutrient digestibility, but practicality deemed that establishing baseline values for micronutrients was the first priority. During meal preparation, ~15ml aliquots of each meal (with starting residues) were withdrawn and frozen immediately at -20°C in opaque containers for further analysis. One aliquot was saved for each raw and cooked specimen of the consolidated material, totaling eight meal analyses in all. Because the meals were made up of specimens that had been pre-processed in the form of peeling, pulverizing, exposure to enzymes and one freeze-thaw cycle, we expected that some water-soluble vitamin concentrations could be altered, and so only minerals are reported with confidence for meals (Reddy et al., 1999). The *penzepenze* tubers were left whole and frozen immediately at -20° upon arrival from Tanzania to our laboratory in Germany. They were not used for digestion trials because of insufficient material. However, these specimens were ideal candidates for full micronutrient assays, including vitamins and minerals, because of very limited handling, and can provide some initial estimate of the micronutrient content of some Hadza tubers, namely those also belonging to *Vigna* (e.g. *V. esculenta*, *V. frutescens*, and *V. macrorhyncha*) (Marlowe, 2010), in the legume family, Fabaceae. All analyses were performed by TNO Triskelion, Zeist, Netherlands. Specific analytical methods are reported in full in Supporting Information.

3.2.8 Statistical analysis

Data were compiled and analyzed using Microsoft Excel and R (version 3.0.1) (RCoreTeam, 2013). Kendall rank correlation coefficients were determined using the [cor] function in the R 'Stats' package and visualized using the [pairs] function. Correlation heat maps were drawn using [levelplot] function in the 'Lattice' package (Sarkar, 2008). Significance testing was accomplished using the [cor.test] function with the confidence level threshold set at "0.95". All mean values are reported with ± standard deviation.

3.3 Results

Data obtained from the experimental *in-vitro* trials to measure absorption of glucose from Hadza tubers provide interesting and unexpected results. Where the term "glucose" is used, we recognize that this encompasses free and starch-derived glucose. However, our quantification does not differentiate between free glucose and starch-derived glucose (measured as glucose after acid hydrolysis and AMG) and so we refer to both simply as "glucose" to be in keeping with our direct measurements. Glucose bioaccessibility was assessed by the amount of glucose absorbed relative to the total amount measured in the meal volume and is therefore a percent value. When we compare percent bioaccessibility of glucose with the absolute gram amount of glucose absorbed, we obtain an indication of not only digestibility but also of potential caloric contribution from tuber glucose to the diet. Furthermore, one unique aspect of the TIM-1 dynamic *in-vitro* system is the realistic peristaltic movement and transportation of food material through each anatomical chamber of the GI model. With this feature we are able to witness the digestion process over time, termed the kinetic properties of digestion, which informs us about the rate of passage and how readily nutrients are liberated by digestive enzymes.

3.3.1 Tuber physical properties

In total, four species of tuber were acquired in sufficient quantity for analysis using *in-vitro* digestion: *V. frutescens*, n=11 tubers; *E. entennulifa*, n=10; *V. pseudolablab*, n=14; *I. transvaalensis*, n=31 (Supplementary Table 1). During pre-processing to simulate peeling and chewing, fresh weight, peel weight, fiber weight and resulting edible fraction were recorded to understand how these features may affect the nutritional quality of wild tubers (Fig. 3 and Supplementary Table 1). *Vigna frutescens* has the lowest average edible fraction (26% ± 8%) with the majority of its total fresh weight deriving from the inedible peel and quid fiber. The edible fractions of *E. entennulifa* and *V. pseudolablab* comprise on average half of the total tuber fresh weights (49% ± 11% and 52% ± 10% respectively) with peel and then fiber making up the remaining fraction. The edible fraction of *I. transvaalensis* is 73% ± 7% and dependent only on peel weight since it contains no inedible fiber. As a result, consumption of *V. frutescens* is to a greater extent inhibited by the presence of tough physical barriers to the edible fraction than the other commonly consumed wild tubers in the Hadza diet.

Image 10. The peel from one *V. frutescens* specimen.

3.3.2 Kinetic data

Absorption curves over the six hour duration of the TIM-1 experiments are shown in Figure 4 for each tuber species. Peak glucose absorption occurs during the second hour for all runs except raw *I. transvaalensis*, which instead peaks at the third hour, indicating a much more protracted rate of digestion (first column). The cumulative percent of glucose absorption (second column) is not predictive of the cumulative absolute amount of glucose absorption (third column), due to large differences in initial glucose content of the tubers (discussed further below). In addition, we observe wide variation in glucose absorption regardless of cooking treatment. No clear or consistent pattern is seen with regard to cooking. Finally, except for *V. frutescens*, glucose absorption values are not consistent within or across species, indicating large variability in glucose accessibility and absolute absorption between individual specimens.

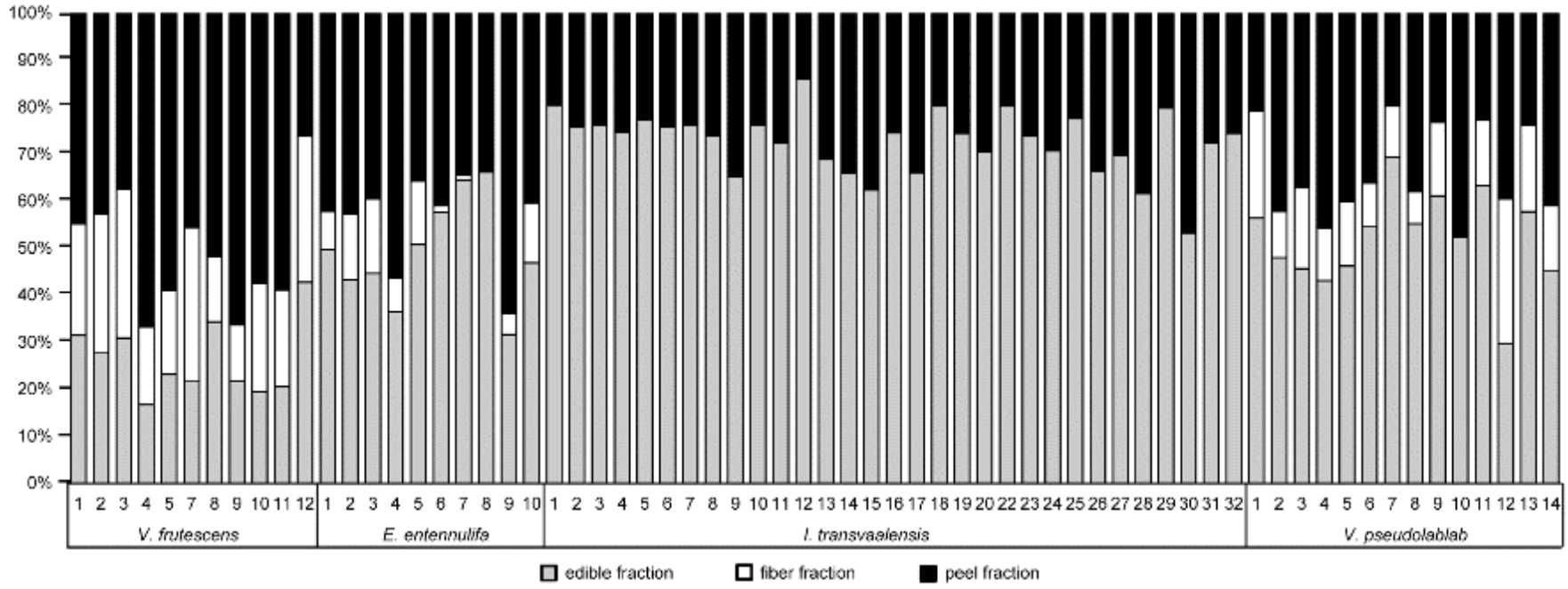


Fig 3. Summary of physical properties of Hadza tubers. Tubers were peeled and processed to remove inedible fiber, called the quid, and extract the edible portion. Each component was weighed to obtain the relative proportion of peel, fiber and edible pulp for different species of tuber. Note that *I. transvaalensis* does not contain inedible fiber.

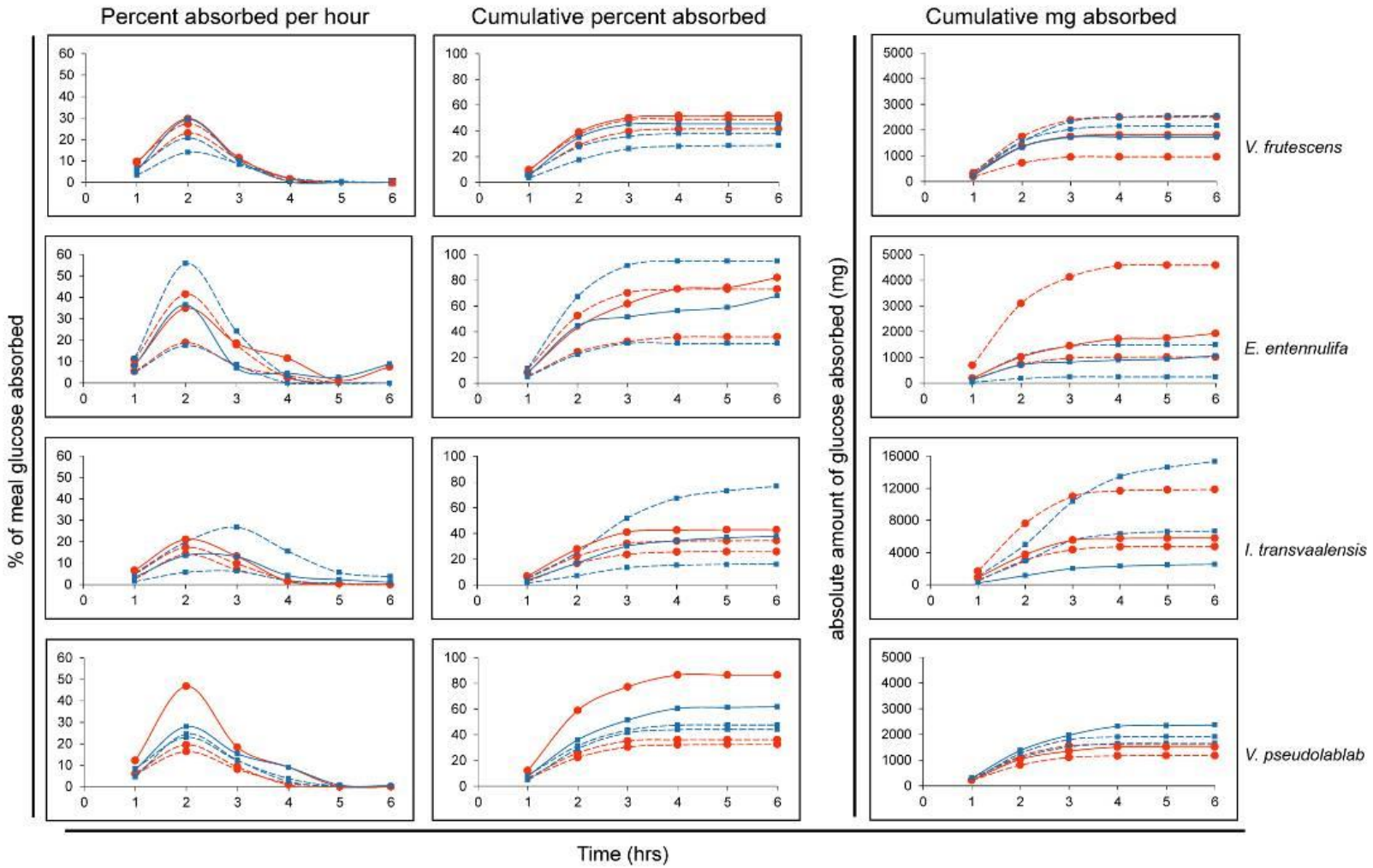


Fig 4. Kinetic data for TIM-1 runs, shown here by tuber species (right axis) with six runs per species; three raw runs (blue squares) and three cooked runs (red circles). Solid lines indicate runs from pooled samples. Columns depict the percent of glucose absorbed at each hour, the cumulative percent of glucose absorbed at each hour, and the cumulative absolute amount (measured in milligrams) of glucose absorbed at each hour, respectively.

This observation is consistent with previous work that demonstrates great variability in the nutritional concentration of Hadza tubers, which is normally attributed to their being non-cultivated wild plants (Vincent, 1985; Schoeninger et al., 2001). Our data now demonstrate that, like initial concentration, digestibility of glucose derived carbohydrate in Hadza tubers is also extremely variable.

3.3.3 Nutritional properties

A summary of the starting glucose and crude protein (TN measured in meals and multiplied by 6.25) concentrations as grams per kilogram of edible wet weight for each run specimen is provided in Figure 5. These values represent the input meal concentration of glucose and protein for each individual *in-vitro* digestion run. On average, *I. transvaalensis* has the highest glucose concentration (78.45g kg^{-1} ± 40.34), followed by *V. frutescens* (26.08g kg^{-1} ± 13.03), *V. pseudolablab* (13.97g kg^{-1} ± 3.96) and finally *E. entennulifa* (12.62g kg^{-1} ± 15.21) (hatched bars in Fig. 5a). Protein values were highest on average for *V. frutescens* (25.63g kg^{-1} ± 11.02) followed by *V. pseudolablab* (21.02g kg^{-1} ± 13.19), *I. transvaalensis* (20.25g kg^{-1} ± 8.55) and finally *E. entennulifa* (11.97g kg^{-1} ± 9.55) (hatched bars in Fig. 5b). We note that glucose values may not be representative of the total carbohydrate content of the tubers since other simple sugars, such as fructose or galactose, were not directly measured. In addition, these averages summarize a very large range of variation within species, as indicated by their standard deviation values, and so it is difficult to resolve an average value of calories from glucose-based carbohydrate for Hadza tubers without sampling many more specimens across both time (season) and space (geographical location).

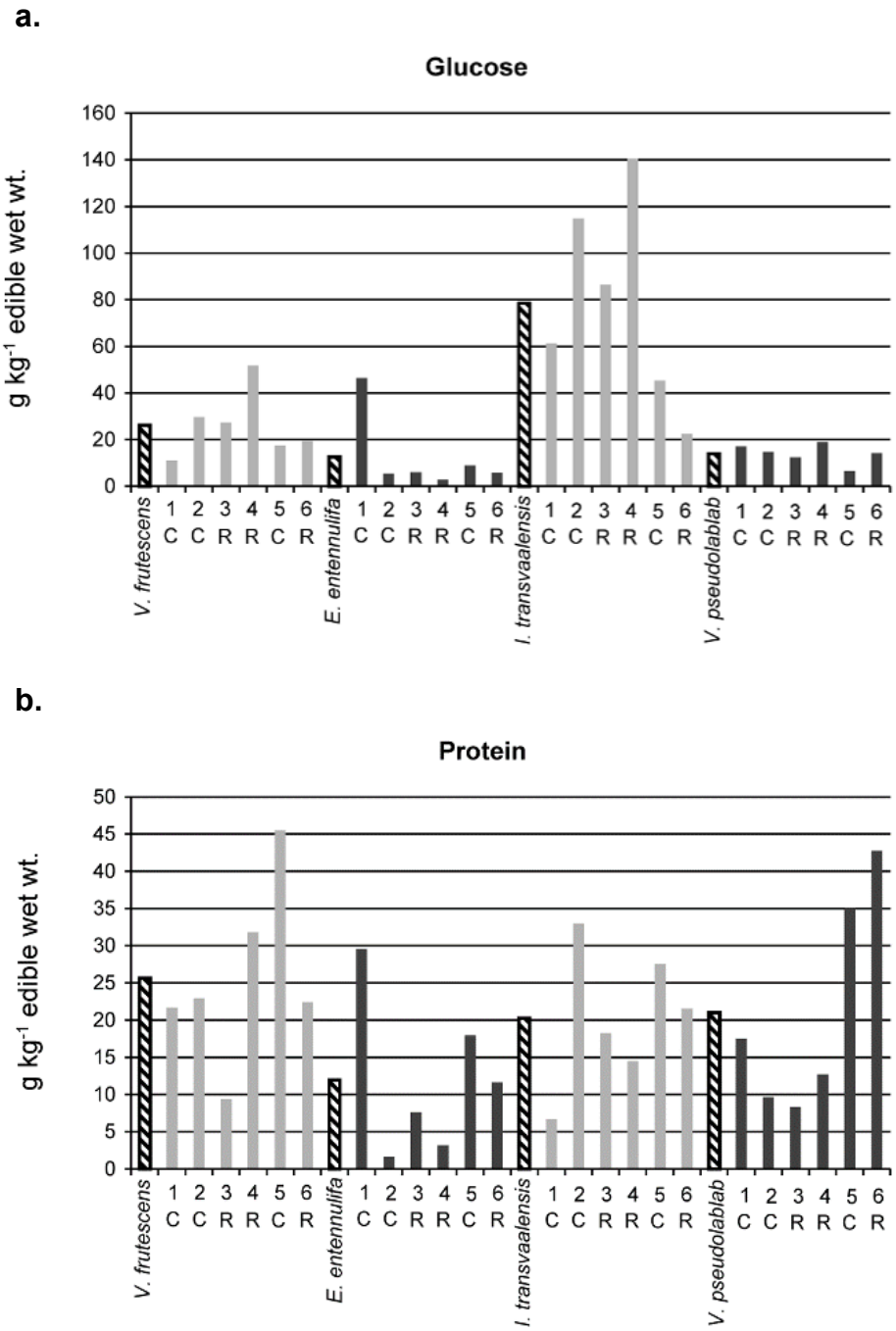


Fig 5. Glucose concentration (**a**) and protein (**b**) concentration in g kg-1 of edible wet weight as measured from starting meal samples for each run. Hatched black bars are averaged values for each tuber species. Concentrations have been corrected for dilution during the processing stage (simulated chewing) and are directly comparable. C cooked, R raw.

However, to fit our data within the context of previous work, we compared our glucose measurements to previous work on Hadza tuber nutrition (Schoeninger et al., 2001; Crittenden, 2009). Direct comparison is difficult because of different reporting schemes and analysis techniques. Despite this methodological challenge, we have attempted to make all work broadly comparable by converting values into grams per kilogram of whole wet weight, which we report in Supplementary Tables 2a-c. We found that Schoeninger et al. (2001) report the most similar values to our own data (Supplementary Tables 2a & b). Since we replicated the preprocessing techniques described by Schoeninger et al. (2001), this perhaps explains the relative match between these two studies, while other reports are consistently much higher in carbohydrate (starch plus simple sugars) values (Supplementary Table 2c) (Vincent, 1985; Crittenden, 2009). Protein values remain relatively consistent across studies.

3.3.4 Bioaccessibility

Nutritional bioaccessibility of wild tubers, presented here as the amount of glucose absorbed in the TIM-1 system relative to the input amount, was found to be quite variable in the small intestine (Figure 6). Tuber protein concentration was too low to reliably discriminate from the endogenous digestive enzyme matrix in the TIM-1 system and so absorption efficacy of nitrogen is not reported. Specimens with the highest absolute glucose absorption, *V. frutescens* and *I. transvaalensis*, had the lowest average percent glucose absorption (Table 4). This is not likely to be the result of any technical limitations of the TIM-1 system because of the continuous flow of high concentration digestive enzymes, mimicking physiological conditions (Minekus et al., 1995). Percent bioaccessibility of glucose averaged over the cooked and raw runs does indicate an effect of cooking for *V. frutescens* and *I. transvaalensis*. The absorption of glucose between raw and cooked *V. frutescens* differed by 10% (47.5% cooked versus 37.5% raw), whereas glucose absorption of raw *I. transvaalensis* is 9.1% greater than cooked (43.5% raw versus 34.4% cooked). On average, *E. entennulifa* and *V. pseudolablab* specimens did not show any appreciable difference in glucose absorption between raw and cooked runs. However, these results are limited owing to the low number of technical replicates and high degree of intraspecific variation. To resolve the issue of whether cooking affects bioaccessibility in a more statistically rigorous manner, we believe different methods should be used to allow for many more replicates and better isolation of variables.

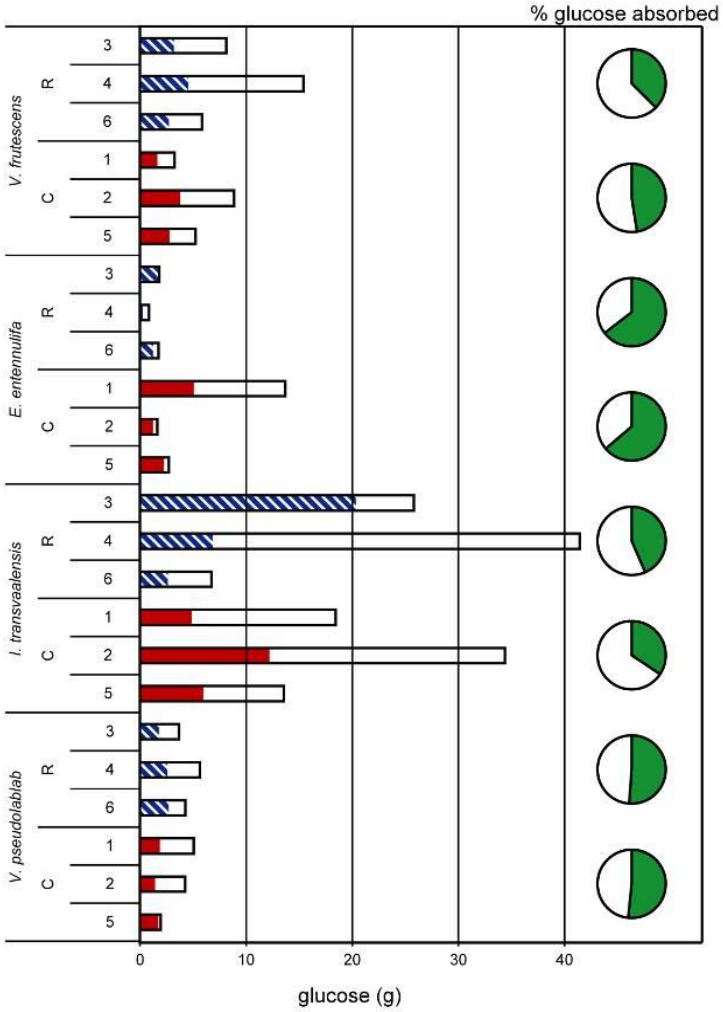


Fig 6. Summary of glucose absorption relative to input (g) and average bioaccessibility across all runs organized by specimen and state: cooked (C) or raw (R). Horizontal bars show the gram amount of input glucose (total hollow bar) and the gram amount absorbed (shaded area). These absolute values have been corrected for the dilution factor and are directly comparable to each other. Pie graphs show the percent of bioaccessible glucose (light shading) averaged over the three raw and three cooked runs for each tuber species.

Table 4 Summary of parameters to assess raw and cooked outcomes during TIM-1 digestion.[1]

Tuber	% bioaccessibility		loss coefficient[2]		hours until 95% max absorption[3]	
	Raw	**Cook**	**Raw**	**Cook**	**Raw**	**Cook**
V. frutescens	37.5 (±8)	47.5 (±5)	1.56	1.03	11 (4, 4, 3)	9 (3,3,3)
E. entennulifa	64.5 (±32)	63.7 (±24)	0.51	0.52	12 (3, 3, 6)	13 (4,3,6)
I. transvaalensis	43.5 (±31)	34.4 (±8)	0.98	1.61	15 (5, 5, 5)	11 (4, 4, 3)
V. pseudolablab	51.2 (±9)	51.6 (±30)	0.86	0.86	12 (4, 4, 4)	11 (3, 4, 4)

[1] According to three metrics- bioaccessibility, loss coefficient, and absorption timing- we predict that *V. frutescens* is best in cooked form, *I. transvaalensis* is best raw, and *E. entennulifa* and *V. pseudolablab* are not greatly affected by cooking. Values reported for bioaccessibility are mean ± standard deviation (in parentheses).

[2] Values greater than 1 indicate more glucose lost than absorbed (e.g. raw *V. frutescens* has 1.56 times glucose lost as is bioaccessible).

[3] Value is the sum total of hours for each run. In parentheses are the individual run hours for each of the three runs that were performed.

To explore the relationship between glucose absorption and glucose concentration of the starting meal, we plotted these two variables against each other for each species individually and tested for significance using the [cor.test] function in R. Outliers that produced an erroneous "two-point" correlation were removed for significance testing. We find that starting meal concentration significantly negatively correlates with the bioaccessibility of *V. frutescens* alone (cor.test, Spearman, $p = 0.033$), indicating that glucose concentration may be tied to other resistant or refractory carbohydrate. When we plotted this same relationship across all species, we see a clear significant negative relationship between glucose concentration of the meal (most likely deriving from starch and free D-glucose) and absorption of glucose (cor.test, Spearman, $p < 0.001$) (Figure 7). This means that absorption of glucose may be inhibited by the higher glucose content, such as found in starch, since these glucose polymers can resist digestion or overwhelm the enzyme activity in the small intestine. Therefore, a "glucose" rich tuber does not necessarily mean more glucose, proportionally, is directly available to the consumer. However, the undigested glucose is not entirely lost. Colonic microbiota preferentially use glucose based carbohydrate for fermentation into beneficial metabolic compounds, namely short chain fatty acids (SCFAs), which contribute to both host and microbial metabolism (Koropatkin et al., 2012). Because the TIM-1 model replicates only the upper digestive tract we are unable to assess the potential for the glucose in Hadza tubers to be digested by colonic bacteria.

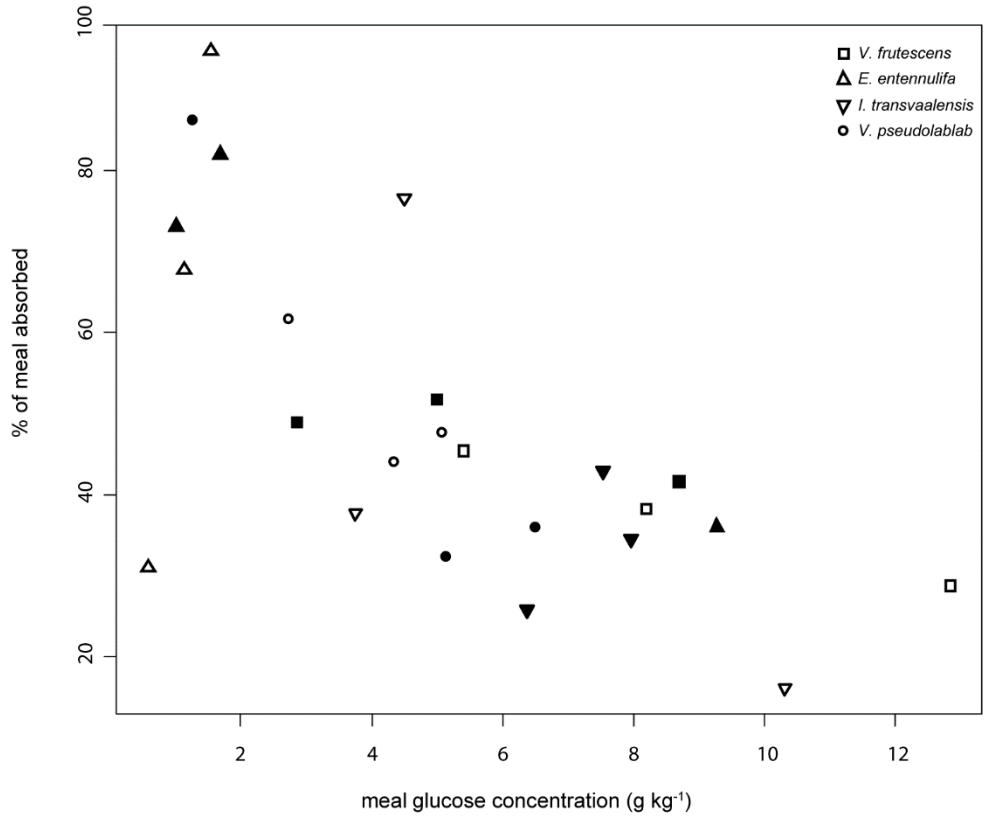


Fig 7. Plotted values of meal concentration and percent bioaccessibility of glucose for all specimens shows an overall negative relationship between these two variables, suggesting that more glucose dense specimens are more resistant to digestion. In contrast, lower glucose concentration is associated with higher glucose bioaccessibility, suggesting reduced concentration of refractory polysaccharides or higher concentration of free simple sugars, or perhaps a combination of both. Solid symbols are cooked specimens.

Finally, to fully assess the effect of brief roasting on the outcome of the digestion trials, we evaluated and summarized three different parameters taken from the results reported above. These parameters are 1) bioaccessibility, 2) loss coefficient, and 3) time until 95% of maximum absorption (Table 4). While bioaccessibility has been discussed previously and is relatively straight forward, loss coefficient is calculated from the difference between glucose accessibility of intake (percent of the meal glucose absorbed), and the glucose accessibility of recovery (percent of the dialysate, efflux, and residual glucose absorbed), divided back into the glucose accessibility of intake. The resulting value is a coefficient of the amount of glucose lost (for absorption) or inaccessible relative to the amount absorbed during digestion. This loss is attributed to refractory polysaccharides or clumping, making material inaccessible to the digestive enzymes in the model. Lastly, the time until 95% of maximum absorption was found from the kinetic data in which we have hourly absorption values over a six hour

period for all runs. Absorption typically peaked at two hours, however the rate of fall off noticeably varied, which gives an indication of resistance to digestion and length of time food is expected to remain in the gut. This is because a longer absorption curve over time means glucose is liberated and absorbed more slowly, potentially because of resistant starch and refractory fiber in the meal matrix. Therefore, a longer time until maximum absorption may indicate prolonged satiation, lower glycemic load, and use and motility of the entire small intestine, including the ileum, in digestion (a positive trait, implicated in helping maintain proper intestinal function). Based on these three parameters, a consumer interested in maximizing calories from glucose should roast their *V. frutescens*, since it provides more glucose and much less loss when cooked (Table 4). By the same token, the consumer should eat the *I. transvaalensis* raw, since it has greater glucose absorption and less loss when not cooked. We also observe the most protracted absorption period for raw *I. transvaalensis* (five hours for each of three runs) (Table 4).

3.3.5 Correlation of physical and nutritional properties

The physical properties of tubers described previously provide valuable contextual information to determine if these factors may relate to and be predictive of nutritional properties, including initial glucose concentration and final absorption. Kendall rank correlation coefficients were determined for correlations between physical properties: fresh weight, peel weight, fiber weight, and edible fraction; and nutritional properties: glucose concentration, protein concentration, glucose accessibility of the meal, and glucose accessibility of recovered meal after digestion (absorption plus efflux plus residual). Correlations were calculated only for those runs in which the meal dose comprised only one tuber specimen ($n = 8$, Supplementary Table 1). The calculation was also repeated with *I. transvaalensis* removed, since this was a considerable outlier, and interpretations are based only on significant or near significant tau (τ) values for both sets of calculations. Results are summarized in Tables 5 & 6 and Figures 8 & 9. Interestingly, fresh weight, fiber weight, peel weight, and edible weight all negatively correlate with the absolute value of both protein and glucose initial "meal" concentrations. Fresh weight in particular had the strongest negative associations, trending significant with regard to protein concentration. This suggests that larger and heavier tubers of these particular species may be dilute of caloric nutrients due to high water content.

Table 5. Kendall rank correlation coefficients (τ) determined by the [cor] function in R of tuber and nutritional properties for runs using single specimens (n = 8)[1].

nutritional properties	weight	fiber weight	peel weight	edible weight	edible fraction
glucose g/kg	-0.500	-0.400	-0.500	-0.286	0.286
percent of intake	-0.214	-0.400	-0.071	-0.286	0.143
percent of recovery	0.643**	0.546*	0.500	0.286	-0.429
glucose absorbed (abs)	-0.500	-0.400	-0.500	-0.286	0.286
protein g/kg	-0.571*	-0.255	-0.429	-0.500	0.071

*approaching significance ($p \sim 0.05$)

**significant ($p < 0.05$)

[1] Significance tested for individual correlations using the [cor.test] function in R with confidence level threshold set at 0.95.

```
z <- cor(y,x, use="complete.obs",method="kendall")
p <- cor.test(a,b, alternative="two.sided",method="kendall",conf.level=0.95)
```

Table 6. Kendall rank correlation coefficients (τ) determined by the [cor] function in R without the outlier species, *I transvaalensis*, using single specimens (n = 7)[1].

nutritional properties	weight	fiber weight	peel weight	edible weight	edible fraction
glucose g/kg	-0.333	-0.234	-0.333	-0.238	0.048
percent of intake	0.048	-0.238	0.238	-0.238	-0.143
percent of recovery	0.524	0.429	0.333	0.238	-0.238
glucose absorbed (abs)	-0.333	-0.238	-0.333	-0.238	0.048
protein g/kg	-0.619*	-0.143	-0.429	-0.524	-0.048

*approaching significance ($p \sim 0.05$)

[1] Significance tested for individual correlations using [cor.test] function in R with confidence level threshold set at 0.95.

```
z <- cor(b,a, use="complete.obs",method="kendall")
p <- cor.test(a,b, alternative="two.sided",method="kendall",conf.level=0.95)
```

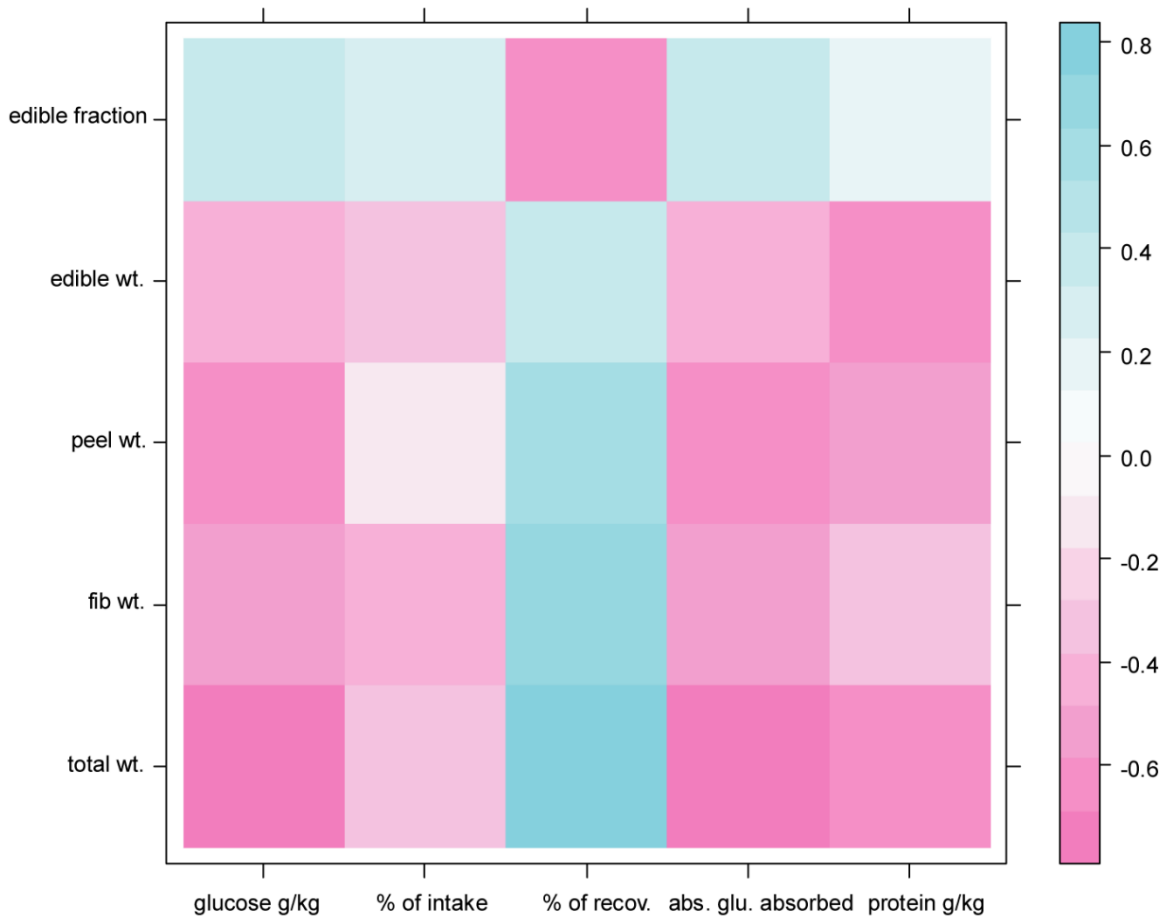


Fig 8. Kendall correlation heat map of single runs (n = 8), comparing physical properties (y-axis) to nutritional and digestion properties (x-axis). Glucose g/kg is the glucose concentration measured in the starting meal. Percent categories refer to the amount of glucose absorbed relative to the meal (intake), or recovery (recov.) glucose concentration. Teal shading indicates positive correlation while magenta shading indicates negative correlation. Abbreviations: abs. glu. absorbed (absolute glucose absorbed); % of recov. (% of recovery); fib. (fiber); wt. (weight).

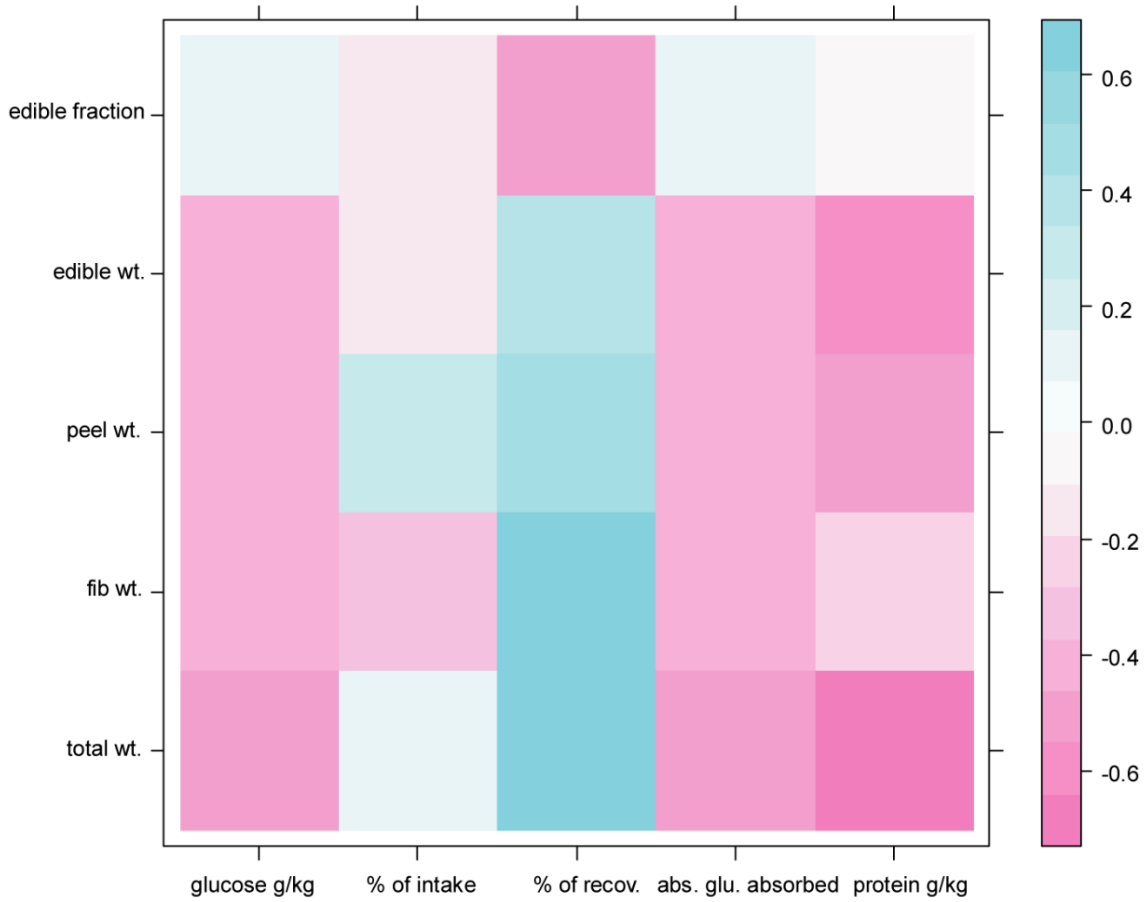


Fig 9. Kendall correlation heat map of singleton runs with *I. transvaalensis* outlier removed (n = 7), comparing physical properties (y-axis) to nutritional and digestion properties (x-axis). Glucose g/kg is the glucose concentration measured in the starting meal. Percent categories refer to the amount of glucose absorbed relative to the meal (intake), or recovery (recov.) glucose concentration. Teal shading indicates positive correlation while magenta shading indicates negative correlation. Abbreviations: abs. glu. absorbed (absolute glucose absorbed); % of recov. (% of recovery); fib. (fiber); wt. (weight).

3.3.6 Micronutrient analysis

Mineral concentration data for the consolidated meal aliquots and of the whole tuber specimens is reported in Table 7 and vitamin concentration data for the whole tuber specimens is reported in Table 8. For comparison we included published mineral and vitamin concentration data of cultivated and wild tuber species from the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference (http://ndb.nal.usda.gov/) and several other published sources (Finglas and Faulks, 1984; Bradbury and Holloway, 1988; Deshmukh and Rathod, 2013). We also included as reference the recommended dietary allowances (RDA) provided by the USDA based on the report from the Institute of Medicine's (IOM) Food and Nutrition Board (IOM, 1997, 1998, 2000, 2001, 2005, 2011). In the context of these comparisons, preliminary analyses suggest that Hadza tubers are rich in important minerals including calcium, potassium, magnesium, sulfur, iron, and manganese. When matched against RDA values, we see that Hadza tubers may be important contributors to daily dietary calcium, potassium, magnesium, iron, copper, manganese, iodine, chromium and molybdenum. The *penzepenze* "whole" tubers are especially rich in iron (22.0 mg $100g^{-1}$ fresh weight) and iodine (2.6 mg $100g^{-1}$), an interesting finding that we discuss later. Due to the starting residue content of calcium, potassium, and sodium salts that were part of the initial meal volume, however, the values for these elements were omitted for the Hadza tuber meal fractions in Table 7 (indicated as "NA"). Mineral and vitamin analysis for *penzepenze* are the average of five specimens reported per 100 grams of fresh weight. Vitamin values indicate that compared with domestic tubers, *penzepenze* is a relatively poor source of water soluble vitamins except, curiously, for B12, which is normally rare in plant resources and mainly acquired from animal derived foods. Vitamin B12 is critical for cellular metabolism, the nervous system, and brain function, but can only be synthesized by bacteria and archaea (Albert et al., 1980; Roth et al., 1996). The presence of B12 in plants stems from microbial biosynthesis and may be an indicator of microbial colonization of the plant tissue in this particular plant species. Nitrogen fixation is common in almost all of Fabaceae (legumes), supported by the presence of bacteria in root nodules (Bergersen, 1971). Earlier studies indicated that legume species grown with symbiotically fixed nitrogen are benefitted by the presence of cobalt in the soil, which is necessary for cobalamin (B12) production (Reisenauer, 1960; Evans and Kliewer, 1964). Vitamin B12 has been found to be concentrated in root nodules of legume plants (Reisenauer, 1960). Therefore, a mutualistic relationship may exist whereby bacteria colonize and facilitate nitrogen fixation for the plant, while the plant root system may help capture cobalt from the soil for microbial synthesis of essential B12 vitamins. Measurements of fat soluble vitamins in *penzepenze* are largely consistent with domestic varieties, which are a generally poor source of such nutrients, with the exception of vitamin A and carotenoids.

Table 7. Mineral analysis of Hadza tuber meal fractions and *penzepenze* with other wild and cultivated tubers[†]

Minerals	**Ca**	**P**	**Mg**	**Na**	**K**	**S**	**Fe**	**Cu**	**Zn**	**Mn**	**I**	**Cl**	**Se**	**Co**	**Cr**	**Mo**
RDA (mg/day)[1]	1000	700	420	1500	4700	ND	8	0.9	11	2.3	0.15	2300	0.055	ND	0.035	0.045
species	mg/100g fresh weight												µg/100g fresh weight			
Mak'alitako (*E. entennulifa*), raw	NA	6.6	63.3	NA	NA	5.2	1.5	0.2	0.2	0.1	ND	ND	0.6	2.9	49.7	1.5
Mak'alitako (*E. entennulifa*), roasted	NA	6.6	76.9	NA	NA	6.1	2.6	0.1	0.2	0.3	ND	ND	0.5	4.6	12.2	0.7
Shumuko (*V. pseudolablab*), raw	NA	23.4	107.8	NA	NA	9.3	2.3	0.1	0.2	0.1	ND	ND	0.9	1.8	7.7	2.5
Shumuko (*V. pseudolablab*), roasted	NA	13.0	90.2	NA	NA	3.4	2.1	0.1	0.2	0.1	ND	ND	0.4	1.7	8.9	1.9
//ekwa (*V. frutescens*), raw	NA	27.0	119.6	NA	NA	19.3	2.7	0.1	0.1	0.3	ND	ND	0.4	2.2	6.4	4.7
//ekwa (*V. frutescens*), roasted	NA	26.7	114.0	NA	NA	18.9	2.0	0.1	0.2	0.3	ND	ND	0.4	2.0	5.4	4.4
Panjuko (*I. transvaalensis*), raw	NA	45.8	55.8	NA	NA	26.4	1.4	0.1	0.4	1.1	ND	ND	1.1	1.6	7.5	30.0
Panjuko (*I. transvaalensis*), roasted	NA	21.9	31.1	NA	NA	14.1	1.1	0.1	0.2	0.5	ND	ND	0.5	0.9	5.9	16.9
Penzepenze (*Vigna* sp.), raw	210.0	37.0	82.0	8.9	590.0	15.0	22.0	0.1	0.4	0.8	2.6	110.0	0.5	13.0	300.0	41.0
Galya (*Brachystelma edulis*)[3]	93.0	28.7	37.3	1.9	83.3	ND	8.1	0.2	0.2	0.7	ND	ND	ND	ND	ND	ND
Kharpudi (*Ceropegia bulbosa var.*)[3]	96.2	34.1	32.6	2.7	88.1	ND	10.8	0.2	0.3	0.7	ND	ND	ND	ND	ND	ND
Haaman (*Ceropegia hirsuta*)[3]	103.6	36.4	36.9	2.5	106.5	ND	11.0	0.2	0.3	0.8	ND	ND	ND	ND	ND	ND
Sweet potato (*I. batatas*), raw[2]	29.0	51.0	26.0	52.0	260.0	13.0	0.5	0.2	0.6	0.1	ND	ND	ND	ND	ND	ND
Sweet potato (*I. batatas*), baked[2]	27.0	52.0	25.4	49.3	297.0	13.8	0.4	0.2	0.7	0.1	ND	ND	ND	ND	ND	ND
Taro (*C. esculenta*), raw[2]	32.0	70.0	115.0	1.8	448.0	8.5	0.4	0.2	3.8	0.4	ND	ND	ND	ND	ND	ND
Taro (*C. esculenta*), baked[2]	31.1	74.5	115.3	1.6	442.0	8.1	0.3	0.2	3.9	0.4	ND	ND	ND	ND	ND	ND
Giant taro (*A. macrorrhiza*), raw[2]	38.0	44.0	52.0	30.0	267.0	12.0	0.8	0.1	1.6	0.6	ND	ND	ND	ND	ND	ND
Giant taro (*A. macrorrhiza*),cooked[2]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Yam (*D. alata*), raw[2]	8.2	38.0	17.0	3.3	318.0	12.0	0.6	0.2	0.4	0.0	ND	ND	ND	ND	ND	ND
Yam (*D. alata*), baked[2]	7.7	35.5	15.9	2.5	295.0	11.9	0.5	0.2	0.4	0.0	ND	ND	ND	ND	ND	ND
Cassava (*M. esculenta*), raw[2]	20.0	46.0	30.0	7.2	302.0	6.4	0.2	0.1	0.5	0.1	ND	ND	ND	ND	ND	ND
Cassava (*M. esculenta*), baked[2]	19.1	42.4	12.2	6.3	281.0	6.2	0.2	0.1	0.6	0.1	ND	ND	ND	ND	ND	ND
Potato (*S. tuberosum*), raw[3]	5.6	35.0	14.1	10.7	320.0	ND	0.3	0.1	0.2	ND	ND	ND	ND	ND	ND	ND
Potato (*S. tuberosum*), roasted[3]	9.2	55.0	24.6	9.2	565.0	ND	0.6	0.1	0.4	ND	ND	ND	ND	ND	ND	ND

[†] ND = no data; NA = not applicable

[1] Dietary Reference Intakes: RDA and AI for Vitamins and Elements based on male 19-30 yrs., accessed from [online] fnic.nal.usda.gov/dietary-guidance/dietary-reference-intakes/dri-tables; [2] Bradbury and Holloway, 1988; [3] Finglas and Faulks, 1984; [4] Deshmukh and Rathod, 2013

Table 8. Vitamin analysis of Hadza tuber, *penzepenze*, and other cultivars[†]

Vitamins	**B1**	**B2**	**B3**	**pantothenic acid**	**B6**	**C**	**E**	**biotin**	**folate**	**B12**	**K1**	**A**	**D**	**carotenoids**
RDA (mg/day)[1]	1.2	1.3	16	5	1.3	90	15	0.03	0.4	0.0024	0.12	0.9*	0.015	NA
species	mg/100g fresh weight							μg/100g fresh weight						
Penzepenze (*Vigna sp.*), raw	0.007	0.03	0.25	1.2	0.048	1.2	0.2	2.8	9.5	0.97	< 0.4	< 0.3	< 50	9.6
Sweet potato (*I. batatas*), raw	0.086[2]	0.031[2]	0.92[2]	ND	0.209[3]	24[2]	0.26[3]	ND	11[3]	0	1.8[3]	11*[2]	0	NA
Taro (*C. esculenta*), raw	0.032[2]	0.025[2]	0.95[2]	ND	0.283[3]	15[2]	2.38[3]	ND	22[3]	0[3]	1.0[3]	7*[2]	0[3]	NA
Giant taro (*A. macrorrhiza*), raw	0.021[2]	0.018[2]	0.94[2]	ND	ND	17[2]	ND	ND	ND	ND	ND	0*[2]	ND	NA
Yam (*D. alata*), raw	0.047[2]	0.03[2]	0.82[2]	ND	0.293[3]	28[2]	0.35[3]	ND	23[3]	0[3]	2.3[3]	18*[2]	0[3]	NA
Cassava (*M. esculenta*), raw	0.05[2]	0.04[2]	0.67[2]	ND	0.088[3]	15[2]	0.19[3]	ND	27[3]	0[3]	1.9[3]	4.9*[3]	0[3]	NA
Potato (*S. tuberosum*), raw	0.2[4]	0.02[4]	0.4[4]	ND	0.24[3]	16[4]	ND	ND	25[4]	0[3]	ND	0*[3]	0[3]	NA

[†] ND = no data; NA = not applicable

* includes both vitamin A and carotenoids

[1] Dietary Reference Intakes: RDA and AI for Vitamins and Elements based on male 19-30 yrs., accessed from [online] fnic.nal.usda.gov/dietary-guidance/dietary-reference-intakes/dri-tables; [2] Bradbury and Holloway, 1988; [3] USDA nutrition tool, accessed from [online] http://ndb.nal.usda.gov/; [4] Finglas and Faulks, 1984

3.4 Discussion and conclusions

The results of our digestion experiments on raw and briefly roasted Hadza tubers demonstrate that bioaccessibility plays a significant role in the nutritional value of foods. For the four Hadza tubers we tested, we found that glucose absorbed relative to total glucose is considerably limited in the *in-vitro* GI environment. This is in part due to motility of the fibrous parenchyma, which tended to clump and limit exposure to gastric and pancreatic enzymes, and to the presence of indigestible fiber or refractory carbohydrates. Previous nutritional studies that analyzed fiber content of Hadza tubers found a relatively high percentage of fiber, between approximately 10% and 30%, in the form of soluble (pectin) and insoluble (cellulose, hemicellulose, and lignin) fiber (Vincent, 1985; Schoeninger et al., 2001; Crittenden, 2009). Fiber as well as other nutrients, even those that are digestible, can form complexes with non-structural carbohydrates, proteins or fats to resist the activities of digestive enzymes and diminish caloric returns. For this reason, discovering the bioaccessible components of Hadza tubers, and even more, their site of activity in the upper or lower gut, is essential to our understanding and interpretation of the overall nutritional contribution of these foods to human foragers. However, this study does not take into account the cost of chewing, and there is good evidence that the oral comminution phase of digestion is in fact a significant bottleneck to obtaining edible matter from Hadza tubers. Indeed, chewing efficiency is a known aspect of tuber digestibility that measurably increases as a result of cooking (Dominy et al., 2008).

In this experiment, we found that absorption was limited to anywhere between 35% and 65% of total glucose availability in the edible fraction of four Hadza tubers. Factoring this fraction in to the original tuber glucose concentration produces very low estimates of actual glucose contribution per kilogram of whole tuber. However, Hadza tubers are a plentiful food resource (Vincent, 1985; Marlowe and Berbesque, 2009), available year round, and routinely exploited by Hadza women, who have been observed to procure an average of 6.1kg of tuber per foraging foray (McDowell, 1981; Vincent, 1985). Women frequently make more than one foray per day, once in the morning and then again in the early evening. Observations of much greater tuber acquisition (20+ kilos per woman) are not unheard of (Vincent, 1985). While some forage is consumed away from camp, the result of snacking, the majority is brought back to camp and shared with other members that did not participate in foraging; usually young children, men, and older adults. Even with snacking and sharing, we may suppose that an adult Hadza woman acquires approximately two to four kilograms of *V. frutescens* tuber per day (Vincent, 1985), which based on our measurements of glucose accessibility alone, could satisfy up to roughly one fifth to one quarter of daily calories based on a total energy expenditure of 1877 ± 364 kcal per day (Pontzer et al., 2012). This estimate does not include calories that may be obtained from protein, fat, or non-glucose based carbohydrate content of *V. frutescens*. In addition, it does not take into account the

energy obtained from fermentation by the gut microbiota in the large intestine, which leads to the production of SCFAs. These are also potential energy substrates for the host (Cummings et al., 1987; Louis et al., 2007; Flint et al., 2008). We therefore expect the true caloric values of these tubers to be even greater than our estimation based on upper GI digestion alone.

Interestingly, there was greater variation seen within species during the initial individual runs than when specimens of each species were pooled. However, the high intraspecific variation and inconsistent results with homogenized runs indicates that we have either not captured a "representative" sample, or, that a real representative sample is impossible, as we believe to be true. For wild foods in particular, there are a number of uncontrolled variables such as yearly rainfall patterns, plant location, plant age, soil composition, and plant health, to name a few. Although we cannot emphasize statistically conclusive results about the effect of cooking, we believe the consistent pattern of high intraspecific variation observed in all studies of Hadza tubers brings to light some very interesting implications about human forager reliance on plant food resources. This suggests that the acquisition of glucose hinges not on roasting, but on how adept the forager is at selecting the individual plant that bears the highest quality tuber. Foraging requires keen botanical knowledge of the best plants to target, and geographic awareness of locations that enable plant growth. In addition, a foraging subsistence likely induces physiological adaptations that selectively increase metabolic efficiency in a resource limiting or unpredictable environment. Such traits include a reduction of metabolic rate (Pontzer et al., 2012) or specialization of gut microbiota to harvest and produce energy for the host (Cummings et al., 1987; Louis et al., 2007). We note that considerable loss occurred during digestion because of clumping and high fiber content preventing exposure to digestive enzymes and absorption, and much of the tuber pulp may pass the ileocaecal valve into the large intestine where fermentation with gut microbiota can take place. We stress the importance of activating the whole gut in digestion and that resistant carbohydrate material, such as tubers, can help sustain the health of the colonic ecosystem (Hijova and Chmelarova, 2007; Flint et al., 2008; Kau et al., 2011; Koropatkin et al., 2012). Indeed, the colon can provide additional metabolic products to the host in the form of SCFAs when fed carbohydrates, and it is likely that Hadza possess a unique suite of microbiota specifically adapted to deal with a vast array of refractory and resistant fiber (Schnorr et al., 2014).

Different species of tuber harbor unique nutritional qualities that are not always related to caloric value. Water is a critical human requirement, and certainly so during the dry season in the arid East African climate. Highly edible wild tubers, both in terms of edible fraction and accessible glucose, may actually be more important for their moisture rather than caloric contribution. Our results and previous work demonstrate that *V. pseudolablab* and *E. entennulifa*, two of the regularly consumed Hadza tubers, contain low glucose concentrations and are approximately 70% and 90% moisture (Schoeninger et al., 2001; Crittenden, 2009). Taxonomically and physically similar tuber species were likely in existence in savanna-mosaic landscapes for a substantial portion of human evolution, and may have facilitated occupation of arid climates and expansion away from permanent water features such

as oceans, lakes and rivers (Schoeninger and Bunn, 2009). We found that fresh weight correlated negatively with glucose and protein concentration. If indeed larger tubers do retain more water, then this dilution effect can be problematic for nutritional reporting depending on whether values are expressed on a dry or wet weight basis. Expressing values on a dry weight basis, while remaining the common convention in the literature, was not only impossible for our study, but may also alter the perceived value of a resource. When the nutritional profile of a food is reported relative to the total dry weight, calories alone become the "currency" rather than the entire edible wet weight (i.e. nutrients are reported on a condensed weight scale). While reporting dry weight remains valuable, particularly for comparative studies, it is also important to consider the entire wet weight material for studies modeling human food consumption because the water content inflates the amount of mass consumed relative to nutrition, meaning a consumer may get physically full from the amount of material eaten, even when fewer calories are consumed. Using fresh weight as the basis of measure is important in the context of modeling forager food intake because tubers are consumed fresh, and so consumption may be physically limited on a fresh-weight basis due to volume of the tuber and capacity of the gut.

Non-caloric nutrients, called micronutrients, are necessary for metabolic and immune function, rendering these vitamins and minerals critical for survival. Micronutrient deficiency, or malnutrition, can lead to a number of diseases due to metabolic dysregulation or increased susceptibility to infection; the recognition and understanding of which has only been thoroughly articulated in the last century (Keusch, 2003). Therefore, by knowing human nutritional requirements, we can extrapolate this information to infer whether a particular environment is sustainable for a human forager, or to what extent limitations are overcome through behavioral adaptations. Since there has been very little work to date on the plant micronutrient landscape in human forager/forager-horticulturalist environments, our preliminary assessment of the vitamin and mineral content of four tuber meal fractions and one whole tuber offers an initial assessment for how Hadza tubers contribute to nutritional homeostasis in an East African savanna-mosaic environment. The mineral content is quite favorable for certain elements essential to metabolism and functioning of the immune and nervous system, such as magnesium, iron, potassium, calcium, and iodine. Iodine concentration in *penzepenze* is especially interesting since inland environments are often considered iodine deficient, to the extent that many modern foods are fortified with iodine. In fact, some of the most important tropical tuber crop cultivars, such as sweet potato (*I. batatas*) and cassava (*M. esculenta*), are notably goitrogenic, meaning they prevent uptake of iodine, causing suppression and enlargement of the thyroid gland (Johns, 1996; Venturi and Begin, 2010). Iodine is essential for proper thyroid function and acts directly on brain growth by helping regulate thyroid hormone production and signaling development in a dose-dependent manner (Venturi and Begin, 2010). Most dietary iodine comes from aquatic or marine resources, an observation favoring the idea (and mounting evidence) that aquatic environments were a central presence in human evolution (Braun et al., 2010; Colonese et al., 2011; Archer et al., 2014). While we do not have any information on the iodine restriction potential of *penzepenze*, our preliminary

analysis of the iodine concentration is interesting and merits further investigation. Given the concurrent enrichment of iron in all Hadza tubers, especially *penzepenze*, which is twice that reported for wild tubers in India (Deshmukh and Rathod, 2013) and nearly three-fold greater than the RDA for iron, we posit that volcanic and Rift Valley geological activity over time may have enriched soils with mineral deposits that are in turn harvested by the plants of that region. Vitamin analysis of *penzepenze* (the only Hadza tuber for which vitamin results are reported with confidence) again shows an unexpectedly high concentration of vitamin B12, which is not common in plant food resources. Animals require B12 for metabolism and proper functioning of the central nervous system, and must obtain it from the diet or from microbial biosynthesis. The curious presence of this water-soluble vitamin in *penzepenze* could be an indication of rich microbial activity in the soil, setting up a mutualistic relation between the soil microbiota and the plants for exchange of nutrients, particularly for Fabaceae plants (Ramirez-Puebla et al., 2013). In this way, B12 enrichment may occur for Hadza tubers, placing increasing importance on tuber nutritional contribution in the form of micronutrients, rather than macronutrients.

Control of fire and cooking is considered a watershed moment in human evolutionary history because of its potential to greatly increase the nutritional value of foraged foods and dramatically extend the breadth of edible resources (Stahl, 1984; Laden and Wrangham, 2005). Cooking is widely regarded to improve the nutritional value of foods through a number of physical and chemical changes that enable more efficient digestion (Stahl, 1984; Johns, 1996). Therefore, we would expect that light roasting, as practiced by Hadza on wild tubers, would enable more efficient acquisition of calories. Our results indicate that cooking had a range of effects on glucose absorption depending on both specimen and species, however, these results lack statistical power given the low number of replicates. Most interesting is that cooking, on average, improved glucose absorption for *V. frutescens*, which is the species most favored by Hadza and almost always roasted (Crittenden, 2009; Crittenden, in press). Cooking had no effect on glucose absorption for *E. entennulifa* and *V. pseudolablab*, and curiously negatively impacted absorption for *I. transvaalensis*, which is typically consumed raw. Therefore, we now have empirical data that aligns with observed behavior of different tuber roasting practices among the Hadza. Since *E. entennulifa* and *V. pseudolablab* are large high moisture tubers with low glucose concentration by fresh weight, brief roasting likely does not gelatinize interior starches. Furthermore, low concentrations of native starch are probably efficiently hydrolyzed by salivary and pancreatic amylase. The negative impact of brief roasting on glucose absorption of *I. transvaalensis* tubers may stem from the high degree of clumping that occurred for cooked rather than raw specimens, reducing their exposure to digestive enzymes in the model.

While roasting had variable effective outcomes on tuber digestibility, one general effect of brief roasting is that it not only softens the parenchymatous tissue, but in addition, it dramatically softens the cortex of some species of Hadza tubers, particularly *penzepenze*, hastening tuber peeling, cutting, and fiber extraction (Dominy et al., 2008). Therefore, softening for ease of peeling and

chewing is a likely key motivator for the brief roasting behavior (Woodburn, 1966; Tomita, 1968). In our experience with peeling and simulated chewing of raw and roasted Hadza tubers, brief roasting greatly increased manual ease of access to the internal and edible tuber pulp. Therefore, we hypothesize that brief roasting has universal appeal for the changes induced to mechanical and physical properties and limited improvement in the direct biological value of Hadza tubers. However, this study did not take into account the energetic cost of chewing. There is good evidence that the oral comminution phase of digestion is in fact a significant bottleneck to the overall bioaccessibility of Hadza tubers. Indeed, the net caloric gain for a human consumer would certainly be higher for roasted tubers due to a measurable increase in chewing efficiency (Dominy et al., 2008; Zink et al., 2014). Compared to modern domestic cultivars such as yam, potato, cassava, sweet potato, and taro, all of which except sweet potato must be fully cooked for human consumption, Hadza tubers have relatively low glucose and carbohydrate concentrations and can be consumed raw as well as lightly roasted, much like the high moisture, low starch Jicama (*Pachyrhizus erosus*) (Finglas and Faulks, 1984; Vincent, 1985; Bradbury and Holloway, 1988; Schoeninger et al., 2001; Stevenson et al., 2007), a finding supported by multiple independent measurements. The relatively low density of starches and high concentration of simple sugars and moisture may make cooking of little relevance for effective digestion, but critical to physical access and mastication, especially for young children (Schoeninger et al., 2001; Crittenden, 2009). However, Hadza tubers may be uniquely deplete in carbohydrates, even among other wild roots and tubers that are targeted by other foraging populations. The composition of wild yams (*Dioscorea* spp.) from central Africa analyzed by Hladik et al. (1984) included between 54.2 and 79.8 percent of dry weight of starch and soluble sugars combined (a min - max of 146 - 324 g kg^{-1} wet weight respectively). Analysis of edible plant foods in South Africa, however, yielded many comparable carbohydrate values to our current assessment of the edible portions of Hadza tubers (Youngblood, 2003). In addition, of the five edible species selected for laboratory analysis in Youngblood (2003), none exhibited cyanogenetic glycoside poisons that are typical of many tropical bulbous plants (such as cassava), and several species are known to be targeted by children and consumed raw or slightly roasted. The disparity in these findings clearly illustrates that wild plant food nutrition is highly variable by region, and that nutritional data from a single region (even if comprehensive), should not be used to generalize interpretations about plant food contribution to the human diet.

The result of 24 *in-vitro* digestion trials with four species of tuber regularly consumed by Hadza foragers indicates that anywhere from one third to more than half of the available glucose, namely from simple sugars and starch, is not absorbed. These results, coupled with the overall finding that Hadza

tubers have low edible fractions, suggest that while wild tubers are an undeniably important resource, their caloric contribution from glucose is significantly hampered by resistant carbohydrate and fiber. Removal of physical barriers such as the peel and inedible fiber is greatly expedited through the use of roasting, whereas the absorption of glucose in tubers was not appreciably affected by roasting. We conclude that food processing, and cooking in particular, can be useful for reasons that may not necessarily involve nutrition or bioaccessibility directly. Instead, in the case of the wild tubers roasted and consumed by Hadza, ease of peeling, softening, separation of fibers, bulking and gut transit time seem to play a greater role in affecting glucose absorption through digestion. Future work is needed to fully establish the specific role of cooking, vitamin and mineral concentrations, and the digestibility of such micronutrients and proteins. Preliminary analyses indicate that these nutritional components especially may contribute to the overall resource value of Hadza tubers.

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CHAPTER 4

GUT MICROBIOME OF THE HADZA HUNTER-GATHERERS

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ABSTRACT 3

Human gut microbiota directly influences health and provides an extra means of adaptive potential to different lifestyles. To explore variation in gut microbiota and to understand how these bacteria may have co-evolved with humans, here we present the first investigation of phylogenetic diversity and metabolite production of the gut microbiota from a community of human hunter-gatherers, the Hadza of Tanzania. We show that the Hadza have higher levels of microbial richness and biodiversity than Italian urban controls. Further comparisons with two rural farming African groups illustrate other features unique to Hadza that can be linked to a foraging lifestyle. These include absence of *Bifidobacterium* and differences in microbial composition between the sexes that probably reflect sexual division of labor. Furthermore, enrichment in *Prevotella*, *Treponema*, and unclassified *Bacteroidetes*, as well as a peculiar arrangement of *Clostridiales* taxa, may enhance the Hadza's ability to digest and extract valuable nutrition from fibrous plant foods.

4.1 Introduction

The human gut microbiota (GM) is vital for host nutrition, metabolism, pathogen resistance, and immune function (Nicholson et al., 2012), and varies with diet, lifestyle, and environment (De Filippo et al., 2010; Kau et al., 2011; Wu et al., 2011). Together, the host and microbiome have been termed a "supra-organism" whose combined activities represent both a shared target for natural selection and a driver of adaptive responses (Turnbaugh et al., 2007). By studying GM variation across human populations, we are able to explore the limits of our genetic and metabolic potential, and the extent to which GM-host co-evolution is responsible for our physiological flexibility and environmental adaptation (Hehemann et al., 2010; Candela et al., 2012; Rinke et al., 2013).

Comparative studies between unindustrialized rural communities from Africa and South America and industrialized Western communities from Europe and North America have revealed specific GM adaptations to their respective lifestyles. These adaptations include higher biodiversity and enrichment of Bacteroidetes and Actinobacteria in rural communities, and an overall reduction in microbial diversity and stability in Western populations (De Filippo et al., 2010; Yatsunenko et al., 2012). Unindustrialized, small-scale, rural societies are targets for understanding trends in human-GM interactions because they rely less on antibiotics and sterile cleaners, and often consume a greater breadth of unrefined seasonally available foods (Ehlers and Kaufmann, 2010). Yet despite recent focus on rural societies, there remains a significant gap in our knowledge of the microbe-host relationship among hunter-gatherer populations. This is especially problematic because humans have relied on hunting and gathering for 95% of our evolutionary history.

Here, to explore for the first time how a foraging subsistence strategy influences GM profiles, we analyze fecal microbiota from 27 Hadza hunter-gatherers from two separate camp sites (Figure 1).

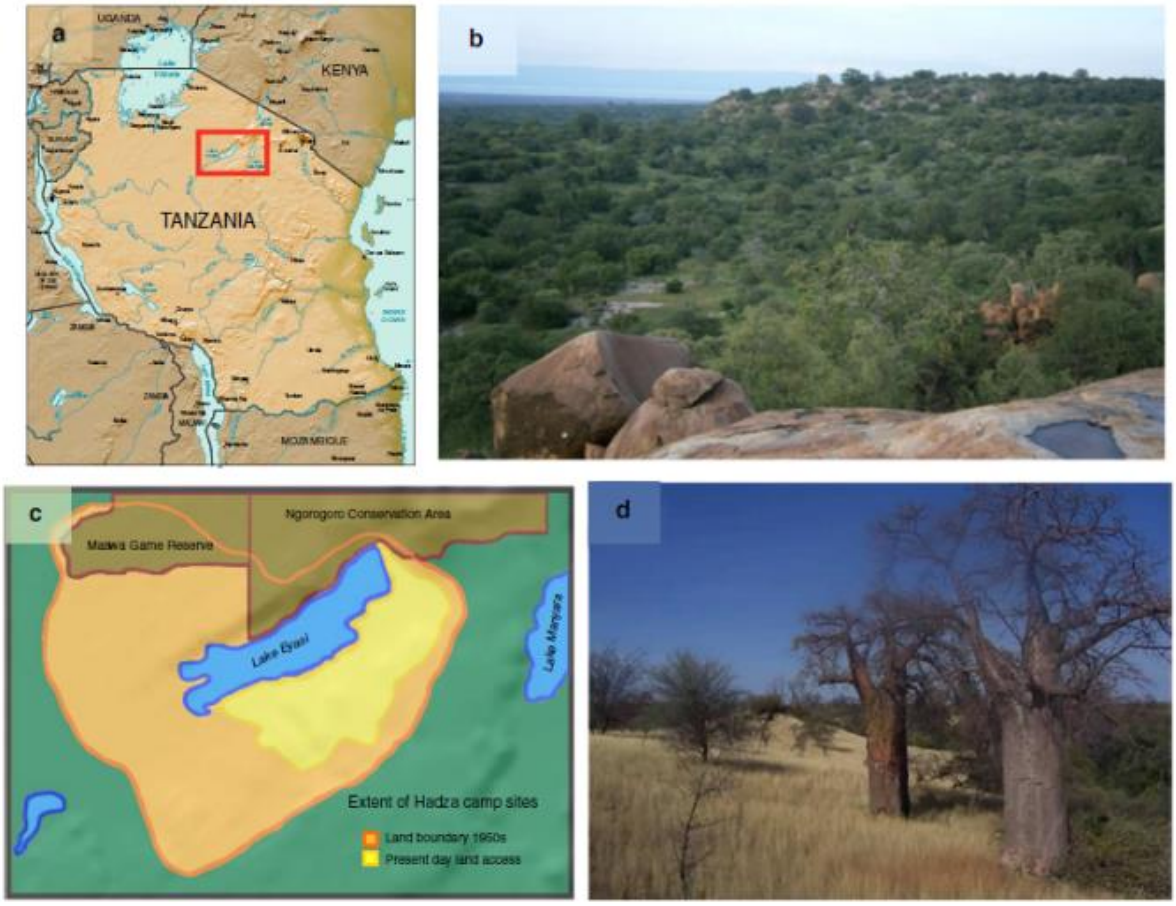

Fig 1. Location and scenery of Hadza land in Tanzania, Africa.

In deep bush camps, hunting and gathering still make up the majority (>90%) of subsistence. (**a**) Location of Hadza land in northern Tanzania; (**b**) top of a rock ridge near Sengele camp overlooking a lush landscape in between two phases of the rainy season; (**c**) extent of the land surrounding Lake Eyasi where Hadza make their campsites, orange line and shading denotes land area in 1950s, and yellow line and shading shows the reduced area Hadza occupy today; (**d**) view of baobab trees within Hadza land during the early dry season. Photos **b** and **d** by S. L. Schnorr and A. N. Crittenden.

The Hadza who chose to participate in this study came from the Dedauko and Sengele camps, situated in the Rift Valley ecosystem around the shores of Lake Eyasi in northwestern Tanzania. These participants are part of the approximately 200-300 traditionally living Hadza, who are one of the last remaining hunting and gathering communities in the world. The Hadza live in small mobile camps with fluid membership, usually comprising a core group of approximately 30 people and target native wild foods, both hunted and foraged, for the bulk of their subsistence (Blurton Jones et al., 1992). While the Hadza are a modern human population, they live in a key geographic region for studies of human evolution and target resources similar to those exploited by our hominin ancestors. The Hadza lifestyle therefore is thought to most closely resemble that of Paleolithic humans.

Phylogenetic diversity, taxonomic relative abundance, and the short chain fatty acid profile of the Hadza microbiome are compared to those of 16 urban living Italian adults from Bologna, Italy. We then compare these data with previously published data on two different rural African groups from

Burkina Faso and Malawi (De Filippo et al., 2010; Yatsunenko et al., 2012) to identify GM features unique to the Hadza lifestyle. This study presents the first characterization of any forager GM through work with the Hadza hunter-gatherers, and will allow us to understand how the human microbiota aligns with a foraging lifestyle, one in which all human ancestors participated prior to the Neolithic transition.

4.2 Methods

4.2.1 Subject enrollment

The 27 Hadza volunteers who participated in this study came from the Dedauko and Sengele camps and are part of the approximately 200-300 traditionally living Hadza. Fecal samples were collected over a period of two weeks in January 2013 from consenting healthy participants. All participants were first told of the study, its objectives, and their role as volunteers. Since Hadza are non-literate, verbal consent was obtained by those who agreed to participate, and this was documented by a separate witness. In the case of young Hadza, we obtained verbal assent from the youths and verbal consent from the parents, and again documented by a separate witness. Samples were matched with subject interviews to record age, sex, and health status; but because of ambiguity with regards to age of some of the participants, this information was excluded from further analysis. All work was approved by the University of Leipzig Ethik-Kommission review board on May 29, 2012, reference number 164-12-21052012. Permission for this work was granted from the Tanzanian Commission for Science and Technology (COSTECH), permit No. 2012-315-NA-2000-80.

Sixteen Italian adults (age: 20-40 years) were recruited for this study in the greater Bologna metropolitan area. All subjects were healthy and had not received antibiotics, probiotics or prebiotics for at least 3 months prior to sampling (Table 1). Written informed consent was obtained from the subjects enrolled. Samples were collected between March and April 2013. Twenty-four hour dietary recalls were provided by each enrolled subject for three days. We used the standard method in nutritional science of sampling two week days and one weekend day in an attempt to fully account for dietary habit and fluctuation. Records were entered and analyzed using the Food Processor SQL version 10.13.0 and compiled for summary reporting of the main caloric contributions by food group and macronutrient.

4.2.2 Sample collection and storage

Hadza samples were handled and stored following previously described methods (Nsubuga et al., 2004). Briefly, samples were submerged in 30 mL of 97% ethanol for 24-36 hours, after which the ethanol was carefully poured out and the remaining solid material was transferred to 50 mL tubes containing silica beads (Sigma 10087). All Hadza samples were transported by express to Bologna, Italy where further analysis was performed. Italian samples were collected, dried using the ethanol and silica two-step procedure, and stored at -80 °C in Bologna until further use.

Image 11. Bush lab for fecal sampling and storage in Hadza land.

Table 1. Age, sex and provenience of study enrolled participants.

HADZA			
SAMPLE ID	AGE	SEX	CAMP
H1	43	F	Dedauko
H2	37	M	Dedauko
H3	22	M	Dedauko
H4	24	M	Dedauko
H5	70	F	Dedauko
H6	30	M	Dedauko
H7	38	F	Dedauko
H8	20	F	Dedauko
H9	47	F	Dedauko
H10	40	F	Dedauko
H11	29	F	Dedauko
H12	8	F	Dedauko
H13	34	M	Dedauko
H14	30	M	Dedauko
H15	16	M	Dedauko
H16	21	M	Dedauko
H17	23	M	Dedauko
H18	17	M	Dedauko
H19	15	M	Dedauko
H20	13	M	Dedauko
H21	43	M	Sengele
H22	38*	M	Sengele
H23	24*	M	Sengele
H24	65*	M	Sengele
H25	40*	M	Sengele
H26	48*	M	Sengele
H27	29	F	Sengele

ITALIANS			
SAMPLE ID	AGE	SEX	PROVENIENCE
IT1	38	M	Bologna
IT2	34	F	Bologna
IT3	29	F	Bologna
IT4	27	M	Bologna
IT5	30	M	Bologna
IT6	32	F	Bologna
IT7	25	F	Bologna
IT8	34	M	Bologna
IT9	39	F	Bologna
IT10	40	F	Bologna
IT11	40	F	Bologna
IT12	30	F	Bologna
IT13	30	F	Bologna
IT14	21	F	Bologna
IT15	29	M	Bologna
IT16	32	F	Bologna

*Asterisks indicate individuals for whom age was less certain.

4.2.3 Comparison of dry and frozen fecal samples

Hadza stool samples could not remain frozen during their removal from Tanzania due to unreliable sourcing of dry ice shipping materials, so we first performed a comparison of DNA extraction and amplification and SCFA quantitation on split samples of Germany-living westerners. Stool samples were split into two segments, one fraction was stored at -80 °C and the second was dried using the two-step ethanol/silica procedure as described above. Total DNA extraction yield, pyrosequencing of the 16S rDNA V4 gene region and SCFA relative abundance quantification were performed (as described below) in parallel from frozen and dried sample aliquots. According to our data, we obtained comparable DNA yield, GM profiles, and SCFA relative abundance profiles from frozen and dry aliquots of the same stool (Table 2). GM profiles were shown to cluster by subject independent of the storage method (Figure 2). Taken together, these data support the reliability of the drying method for use in stool storage.

Table 2. Comparison of dried and frozen storage protocols on test fecal samples shows total DNA yield and absorbance values (**a**) and SCFA relative abundance (**b**)

a.

Sample ID	DNA ng ul^{-1}	A260	A280	260/280	260/230
101 frozen	423.86	8.477	4.494	1.89	1.95
101 dry	610.86	12.217	6.299	1.94	2.06
103 frozen	468.69	9.374	5.213	1.8	1.39
103 dry	260.74	5.215	2.879	1.81	1.54

b.

Sample	Acetic acid (%)	Propionic acid (%)	Butyric acid (%)	Valeric acid (%)
101 frozen	77.23	14.56	6.82	1.39
101 dry	80.45	11.17	7.44	0.94
103 frozen	67.89	20.76	9.53	1.83
103 dry	72.60	18.58	7.87	0.94

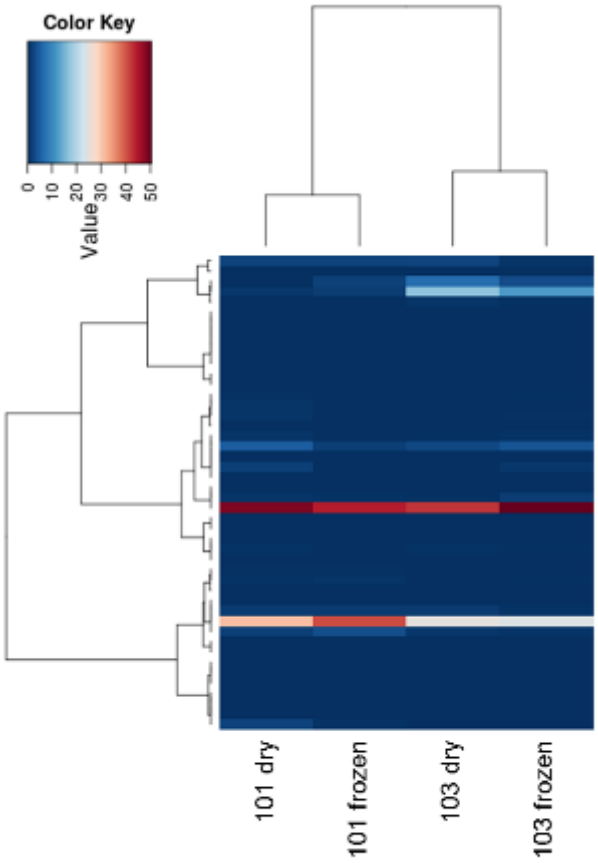

Fig 2. Comparison of dried and frozen storage protocols on test fecal samples. In test experiments, stools from two Western individuals (101 and 103) were collected and split into two samples each. One fraction was stored immediately at -80 ºC (frozen) and the second fraction was dried following a two-step ethanol submersion and silica storage procedure (dried). Hierarchical ward-linkage clustering based on the Spearman correlation coefficients of bacterial family proportion is shown. Subjects are clustered in the top of the panel. Families are clustered by the vertical tree.

4.2.4 DNA extraction from fecal samples

Total DNA from fecal material was extracted using QIAamp DNA Stool Mini Kit (QIAGEN) with a modified protocol. Briefly, 250 milligrams of feces were suspended in 1 mL of lysis buffer (500 mM NaCl, 50 mM Tris-HCl pH 8, 50 mM EDTA, 4% SDS). Four 3 mm glass beads and 0.5 g of 0.1 mm zirconia beads (BioSpec Products) were added, and the samples were treated in FastPrep (MP Biomedicals) at 5.5 movements per second for 3 min. Samples were heated at 95 °C for 15 min, then centrifuged for 5 min at full speed to pellet stool particles. Supernatants were collected and 260 µL of 10 M ammonium acetate was added, followed by incubation in ice for 5 min and centrifugation at full speed for 10 min. One volume of isopropanol was added to each supernatant and incubated in ice for 30 min. The precipitated nucleic acids were collected by centrifugation for 15 min at full speed and washed with ethanol 70%. Pellets were resuspended in 100 µL of TE buffer and treated with 2 µL of DNase-free RNase (10 mg mL^{-1}) at 37 °C for 15 min. Protein removal by Proteinase K treatment and DNA purification with QIAamp Mini Spin columns were performed following the kit protocol. Final DNA concentration was determined by using NanoDrop ND-1000 (NanoDrop® Technologies).

4.2.5 16S rDNA gene amplification

For the amplification of the V4 region of the 16S rDNA gene the primer set 520F (5'-AYTGGGYDTAAAGNG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') (with Y=C/T, D=A/G/T, N=any base, V=A/C/G) was utilized. These primers were designed to include at their 5' end one of the two adaptor sequences used in the 454-sequencing library preparation protocol (adaptor A and B), linked to a unique MID tag barcode of 10 bases allowing the identification of the different samples. PCR mixtures contained 0.5 µM of each forward and reverse primer, 100 ng of template DNA, 2.5 U of GoTaq Flexi Polymerase (Promega), 200 µM of dNTPs and 2 mM of $MgCl_2$ in a final volume of 50 µL. Thermal cycling consisted of an initial denaturation step at 95 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 40 °C for 30 s, and extension at 72 °C for 60 s, with a final extension step at 72 °C for 5 min (Claesson et al., 2009). PCR amplifications were carried out in a Biometra Thermal Cycler T Gradient (Biometra).

4.2.6 qPCR for Bifidobacterium quantification

qPCR was carried out in a LightCycler instrument (Roche). Quantification of the 16S rRNA gene of *Bifidobacterium* was performed with previously described genus-specific primers bif-164 and bif-662 (Candela et al., 2012). For quantification, standard curves were generated by using 10-fold serial

dilution of genomic DNA from *B. animalis* subsp. *lactis* BI07. Amplification was carried out in a 20 µl final volume containing 100 ng of fecal DNA, 0.5 µM of each primer and 4 µl of LightCycler-FastStart DNA Master SYBR Green I (Roche). Amplifications were done under the following conditions: (i) starting pre-incubation at 95 ºC for 10 min; (ii) amplification including 35 cycles of 4 steps each at the temperature transition rate of 20 ºC s^{-1}: denaturation at 95 ºC for 15 s, annealing at 63 ºC for 20 s, extension at 72 ºC for 30 s, and fluorescence acquisition at 90 ºC for 5 s; (iii) melting curve analysis.

4.2.7 Pyrosequencing of fecal slurries

The PCR products derived from amplification of the specific 16S rDNA V4 hypervariable region were individually purified with MinElute PCR Purification Kit (QIAGEN) and then quantified using the Quant-iT PicoGreen dsDNA kit (Invitrogen). After the individual quantification step, amplicons were pooled in equal amounts (thus, creating three 9-plex for Hadza samples and two 8-plex pools for Italian samples) and again purified by 454-Roche Double Ampure size selection protocol with Agencourt AMPure XP DNA purification beads (Beckman Coulter Genomics GmbH), in order to remove primer dimers, according to the manufacturer's instructions (454 LifeSciences, Roche). Amplicon pools were fixed to microbeads to be clonally amplified by performing an emulsion PCR following the GS-FLX protocol Titanium emPCR LIB-A (454 LifeSciences, Roche). Following this amplification step, the beads were enriched in order to keep only those carrying identical PCR products on their surface, and then loaded onto a picotiter plate for pyrosequencing reactions, according to the GS-FLX Titanium sequencing protocol. All pools were sequenced in one eighth of a plate each.

4.2.8 Bioinformatic analysis of 16S rDNA and statistical methods

Sequencing reads were analyzed using the QIIME pipeline (Caporaso et al., 2010) as described previously (Claesson et al., 2012). Briefly, V4 sequences were filtered according to the following criteria: (i) read length not shorter than 150 bp and not longer than 350 bp; (ii) no ambiguous bases (Ns); (iii) a minimum average quality score over a 50-bp rolling window of 25; (iv) exact match to primer sequences and maximum 1 error in barcode tags. For bacterial taxonomy assignment we utilized RDP-classifier (version 2.2) with 50% as confidence value threshold. Trimmed reads were clustered into OTUs at 97% identity level and further filtered for chimeric sequences using ChimeraSlayer (http://microbiomeutil.sourceforge.net/#A_CS). Alpha-diversity and rarefaction plots were computed using four different metrics: Shannon, PD whole tree, chao1 and observed species. Weighted and unweighted UniFrac distances and Euclidean distance of genus level relative abundance were used to perform Principal Coordinates Analysis (PCoA). PCoA, and heatmap and bar plots were built using the packages *Made4* (Culhane et al., 2005) and *Vegan* (http://cran.r-project.org/package=vegan).

The R packages *Stats* and *Vegan* were used to perform statistical analysis. In particular, to compare gut microbiota structure among different populations for α and β diversity we used a Wilcoxon signed rank test. Data separation in the PCoA was tested using a permutation test with pseudo F-ratios (function [adonis] in the *Vegan* package). Cluster separation in hierarchical clustering analyses was assessed for significance using Fisher's exact test. Significant differences in phylum or genus level abundance between Hadza and Italians, and between Hadza males and females, were assessed by Mann-Whitney U tests, and corrected for multiple comparisons using the Benjamini-Hochberg method when appropriate. False discovery rate (FDR) < 0.05 was considered as statistically significant.

Kendall correlation test between SCFA levels and the relative abundance of genera was achieved using function [cor.test] of the package *Stats* of R. Sequences from De Filippo et al. (2010) and Yatsunenko et al. (2012) were obtained from Metagenomics Rapid Annotation using Subsystem Technology (MGRAST), project I.D. 201, and European Nucleotide Archive (ENA), project number ERP000133, repositories, respectively, and processed and assigned following the QIIME pipeline. Bacterial co-abundance groups (CAGs) were determined as described previously (Claesson et al., 2012). Briefly the associations among the genera were evaluated using Kendall correlation test, visualized using hierarchical Ward clustering with a Spearman correlation distance metrics and used to define co-abundant genera groups. The significant associations were controlled for multiple testing using the qvalue method (FDR<0.05) (Dabney et al., 2013). Permutational MANOVA (Anderson, 2001) was used to determine if the CAGs were significantly different from each other. The Wiggum plot network analysis was created as previously described (Claesson et al., 2012) using cytoscape software (http://www.cytoscape.org/). Circle size represents genus abundance and connections between nodes represent positive and significant Kendall correlations between genera (FDR < 0.05).

4.2.9 GC-MS determination of SCFAs in fecal samples

Aliquots of dried fecal samples (about 250 mg) were briefly homogenized after the addition of 1 mL of 10% perchloric acid in water and centrifuged at 15,000 x g for 5 min at 4 °C. 500 µL of supernatant were diluted 1:10 in water, 10 µL of D8-butyric acid (internal standard, IS) were added to the sample at the final concentration of 20 µg mL^{-1}. The calibration curves were prepared adding the IS to scalar amounts of the acids in diluted samples or water (for external standardization). All the standards (purity > 99%), acetic, propionic, butyric, valeric acids and IS were provided by Sigma and were used to prepare calibration solutions for quantitation (linear response) and identification. HS-SPME was performed by using a 75 µm Carboxen™/polydimethylsiloxane fiber (Supelco). The optimized final extraction conditions were: temperature 70 °C, 10 min of equilibration time, 30 min of extraction time. The analytes were desorbed into the GC injector port at 250 °C for 10 min, including fiber cleaning. GC-MS analysis was carried out on a TRACE GC 2000 Series (ThermoQuest CE Instruments) gas

chromatograph, interfaced with GCQ Plus (ThermoQuest) mass detector with ion trap analyzer, operating in EI mode (70 eV). The capillary GC column was a Phenomenex ZB-WAX (30 m x 0.25 mm ID, 0.15 μm film thickness), consisting of 100% Polyethylene Glycol. Helium (He) was the carrier gas at a flow rate of 1.0 mL min^{-1}. An oven temperature program was adopted: initial 40 °C (hold time: 5 min), then ramped by 10 °C min^{-1} to 220 °C (hold time: 5 min). The temperature of transfer line and ionization source was maintained at 250 and 200 °C, respectively.

The GC was operated in splitless mode; the injector base temperature was set at 250 °C. The mass spectra were recorded in full scan mode (34-200 amu) to collect the total ion current (TIC) chromatograms. Quantitation was carried out by using the extracted ion chromatograms (XIC) by selecting fragment ions of the studied analytes (43 and 60 amu for acetic acid, 55 and 73 amu for propionic acid, 60 and 73 amu for butyric and valeric acids and 63 and 77 amu for IS). The SCFAs concentration in fecal samples was expressed in micromoles per gram (μmol g^{-1}) of feces. Limit of detection (LOD) ranged from 4 to 68 nmol g^{-1}.

4.3 Results

4.3.1 Dietary information for sampled cohorts

The Hadza diet consists of wild foods that fall into five main categories: meat, honey, baobab, berries, and tubers (Table 3) (Murray et al., 2001; Schoeninger et al., 2001; Marlowe and Berbesque, 2009). They practice no cultivation or domestication of plants and animals and receive minimal amounts of agricultural products (<5% of calories) from external sources (Marlowe, 2010). By comparison, the diet of the Italian cohort derives almost entirely from commercial agricultural products and adheres largely to the Mediterranean diet: abundant plant foods, fresh fruit, pasta, bread, and olive oil; low to moderate amounts of dairy, poultry, fish, and red meat (Table 4). Additionally, the majority of carbohydrates (based on gram amount) came from easily digestible starch (54%) and sugar (36%) while very little was derived from fiber- soluble or insoluble (10%) (Figure 3).

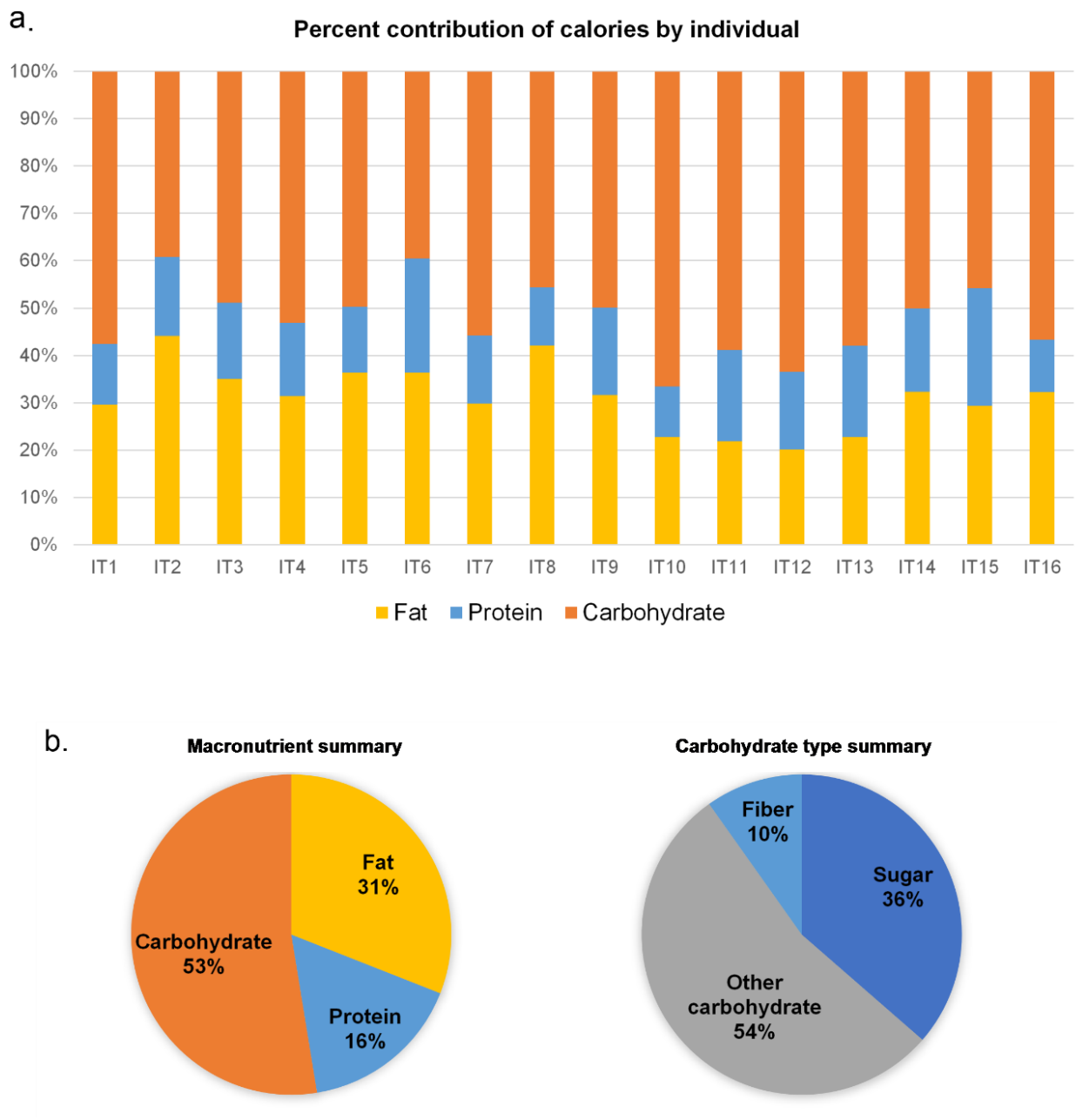

Fig 3. Macronutrient summaries of Italian cohort diet. (**a**) The percent macronutrient intake is reported by subject based upon three days of dietary recalls collected during two week days and one weekend day. Recorded diets represent the typical daily diet of each subject. (**b**) Pie graphs show the summary of the average macronutrient intake by percent of kilocalories consumed for the entire subject cohort as well as a breakdown of carbohydrate type.

Table 3. Summary of Hadza foods and nutrition.

					g 100g^{-1} dry wt.					kcal 100g^{-1} dry wt.	
Food type	**Hadza name**	**English name**	**Species name**	**Moisture (%)**	**Edible portion**	**Carbohydrate**	**Protein**	**Fat**	**Fiber**	**Energy**	**References**
Baobab	N//obabe (pulp)**	Baobab	*Adansonia digitata*	4.7	na	46.6	2.5	0.7	45.1	203.0	*1*
	N//obabe (seed)**	Baobab	*Adansonia digitata*	4.8	na	11.2	36.3	29.3	14.1	454.0	*1*
Berry	Kisinubi	na	*Cordia sinensis*	73.0	na	68.8	12.6	1.8	11.6	342.0	*1*
	Undushibi**	na	*Cordia sinensis*	71.0	na	61.4	15.2	na	13.6	324.0	*1*
	Masakapi	na	*Cordia crenata*	69.0	na	62.5	12.7	1.9	17.8	318.0	*1*
	Hlukayebe	na	*Grewia villosa*	24.0	na	72.7	7.1	na	13.4	337.0	*1*
	Kongolubi	na	*Grewia bicolor*	26.0	na	66.1	12.0	2.0	13.2	330.0	*1*
	Pawe	na	*Sclerocarya birrea*	83.0	na	49.9	3.6	na	37.7	232.0	*1*
Honey	Ba'alako**	Honey (stinging)	*Apis mellifera*	15.1	na	89.1	3.3	7.2	na	434.0	*1*
	N//ateko**	Honey (stingless)	*Meliponinae sp.*	23.5	na	92.8	3.2	3.2	na	412.5	*1*
Meat	Gewedako**	Dik-dik	*Madoqua kirkii*	na	na	na	na	na	na	na	*2*
	Tsokwonako**	Giraffe	*Giraffa camelopardalis*	na	na	na	na	na	na	na	*2*
	Chacha**	Galago (lesser)	*Galago senegalensis*	na	na	na	na	na	na	na	*2*
	Ndonoko**	Galago (greater)	*Otolemur crassicaudatus*	na	na	na	na	na	na	na	*2*
	Tsunako**	Bee larvae	*Apis mellifera*	na	na	na	na	na	na	na	*2*
Tuber	//Ekwa hasa**	na	*Vigna frutescens*	68.8	39.4	60.8	7.2	0.8	28.2	282.3	*3*
	//Ekwa gadabi	na	*Vigna fructescens*	70.0	na	60.6	8.0	1.9	22.7	84.5	*4*
	Shumuko**	na	*Vatoraea pseudolablab*	90.1	na	50.8	3.3	0.6	35.2	225.0	*4*
	Do'aiko	na	*Vigna macrorhyncha*	85.0	na	58.6	10.4	0.1	22.4	292.0	*4*
	Matukwaiko	na	*Coccinea surantiaca*	86.5	na	69.5	12.4	0.0	13.3	337.0	*4*
	Mak'alitako**	na	*Eminia entennulifa*	71.7	56.9	26.4	4.1	1.1	17.1	110.1	*3, 4*
	Panjuko**	na	*Ipomoea transvaalensis*	70.6	91.8	54.8	4.2	1.7	2.6	233.0	*4*

[1] Murray et al., (2001); [2] Marlowe, (2010); [3] Schoeninger et al., (2001); [4] Vincent, (1985)

Table 4. Percent contribution of food categories and corresponding nutrients to average daily Italian cohort diet[1].

Food Category	Most common food items	Energy (kcal)	Fat (g)	Protein (g)	All carbohydrate (g)	Fiber (g)	Sugar (g)	Other carbohydrate (g)
Bread, cereal, pasta, grain		37.2%	25.3%	33.6%	48.3%	45.2%	16.0%	74.0%
	flatbread, brioche, brown bread							
	pasta: tortellini, lasagna, noodles							
	cereals, biscuits, crackers, pizza crust							
Other starch		3.7%	1.2%	2.3%	5.7%	6.3%	0.7%	8.5%
	beans, legumes							
	rice, potatoes, couscous							
Meats, egg, meat broths		10.7%	16.0%	31.2%	0.7%	0.4%	1.0%	0.5%
	pork: cured, steak, roast, salami							
	chicken: baked, roasted							
	beef: steak, roast, sausage,							
	fish: tuna, sole, cod, bass, flounder							
Dairy		13.0%	22.3%	19.9%	5.1%	0.5%	13.0%	0.7%
	milk, cream, yogurt							
	cheese: parmesan, mozzarella, sheep							
Fruit, fruit juice		8.8%	0.8%	2.4%	17.2%	19.9%	29.4%	5.6%
	banana, apple, pear, persimmon, citrus							
	orange juice, pear juice							
Vegetables, sauces, herbs		4.6%	3.1%	5.4%	6.1%	23.1%	4.9%	4.6%
	lettuce, carrot, onion							
	tomato sauce, mixed vegetables							
	boiled cabbage, squash							
Desserts, sugary treats		12.6%	14.6%	4.0%	14.6%	2.8%	33.3%	4.8%
	sugar, honey, cake, jam, soda							
	chocolate, pastries, ice cream							
Oil, butter, fat		4.4%	13.6%	0.0%	0.0%	0.0%	0.0%	0.0%
	olive oil, butter							
Alcohol		3.5%	0.0%	0.3%	1.4%	0.0%	0.3%	0.8%
	beer, wine							

Nuts		1.1%	3.1%	0.8%	0.2%	1.2%	0.1%	0.2%
	walnut, peanut							
Other		0.4%	0.1%	0.1%	0.6%	0.5%	1.3%	0.2%
	espresso, coffee, barley coffee, tea							
	vinegar, mustard, salt							
Average daily amount		**1852.3**	**66.3**	**75.0**	**236.5**	**20.0**	**84.5**	**120.6**

[1]Percent contribution of each food group for energy (kcal), fat (g), protein (g), and carbohydrate (g). Carbohydrate is further broken down into types of carbohydrate with percent contribution by gram of the following: fiber, sugar, and other carbohydrate (oligosaccharides). An average daily intake for each category is reported in the last row.

4.3.2 Characterization of Hadza microbiota

Fecal samples from 27 Hadza, aged 8-70 years, mean age 32 years, and 16 Italians aged 20-40 years, mean age also 32 years (Table 1), were collected and pyrosequenced in the V4 gene region of bacterial 16S rDNA, resulting in 309,952 high-quality reads and an average of 7208 ± 2650 reads per subject. Reads were clustered into 11,967 operational taxonomic units (OTUs) at 97% identity. We used several different metrics to calculate α-diversity, including phylogenetic diversity (Faith, 1992), OTU species count, the Chao1 index for microbial richness, and the Shannon index for biodiversity (Figure 4). Rarefaction curves for phylogenetic diversity plateaued after 4000 reads per sample, approximating a saturation phase. All measures indicate a much higher GM diversity within the Hadza than in Italian samples (P<0.001, Mann-Whitney U test).

The Hadza and Italian samples show many notable differences in microbiota relative abundance, as a percent of reads assigned, at both phylum and genus levels (Figure 5, Table 5). In particular, the Hadza GM is largely dominated by Firmicutes (72% ± 1.9%) and Bacteroidetes (17% ± 1.1%). Other represented phyla are Proteobacteria (6% ± 1.2%) and Spirochaetes (3% ± 0.9%), with 2% of phylum level OTUs remaining unclassified. The most represented families in the Hadza GM are *Ruminococcaceae* (34%), *Lachnospiraceae* (10%), *Prevotellaceae* (6%) Clostridiales Incertae Sedis XIV (3%), *Succinivibrionaceae* (3%), *Spirochetaceae* (2%) and *Eubacteriaceae* (2%). Interestingly, a large number of taxa, the majority belonging to Bacteroidetes, Clostridiales, Bacteroidales, and *Lachnospiraceae*, are unassigned at the level of family and genus, together representing 22% of the total community.

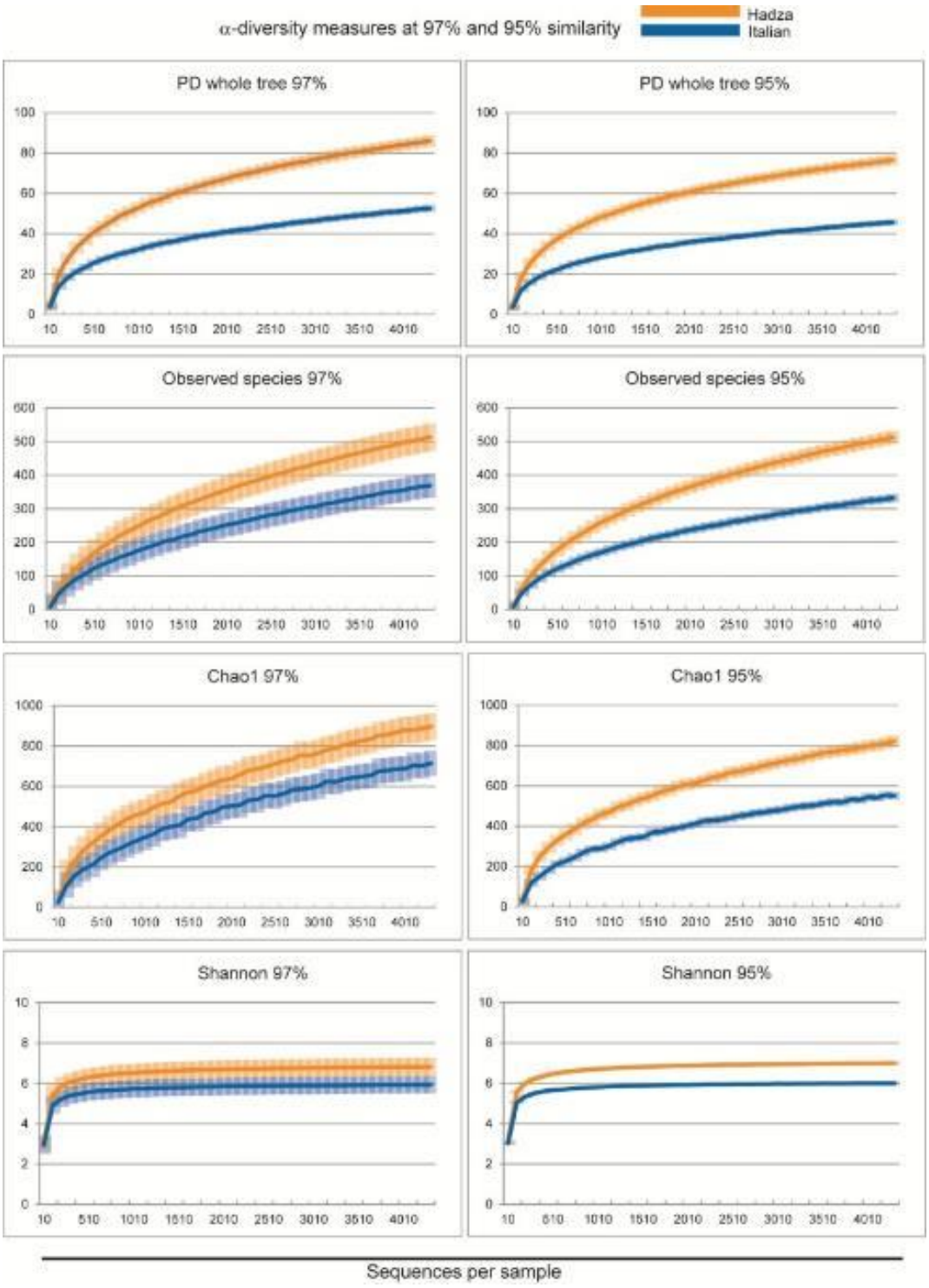

Fig 4. Rarefaction curves of different α-diversity metrics. The OTU table at 0.05 and 0.03 similarity thresholds was rarefied up to 4500 reads per sample and analyzed using various diversity metrics for both Hadza and Italian subjects. Similarity thresholds of 95% (family level) and 97% (genus level) were considered. Metrics used were Faith's Phylogenetic Diversity (PD whole tree), observed OTUs, the Chao1 measure of microbial richness, and the Shannon index of biodiversity. All four diversity metrics showed greater diversity in the Hadza microbiota compared to the Italian microbiota. Plateaus occurred by about 4000 reads per sample, approximating a saturation phase and indicating that the breadth of diversity has been well captured in the sequencing.

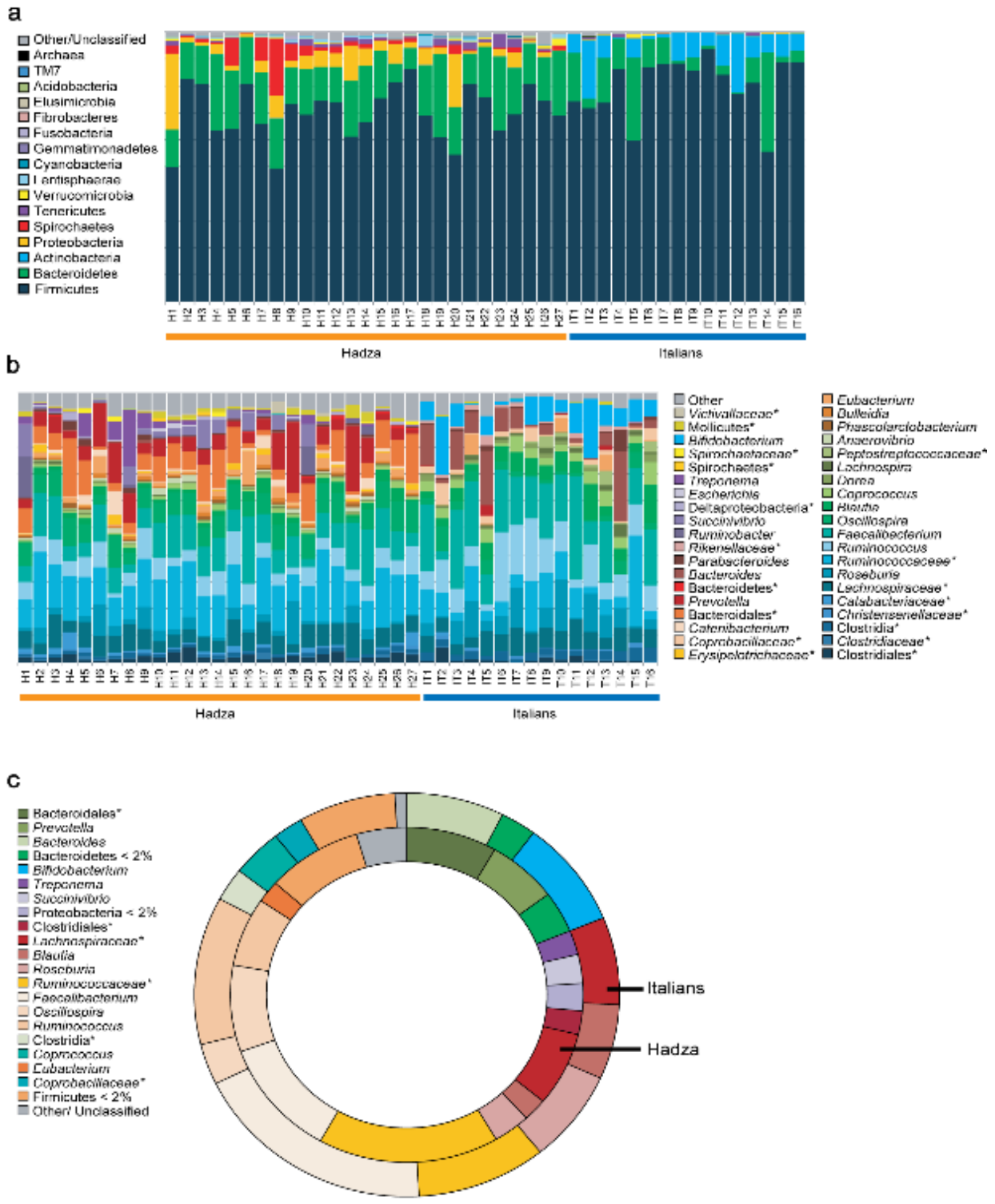

Fig 5. Bacterial relative abundance of Hadza and Italian subjects. 16S rDNA gene survey of the fecal microbiota of 27 Hadza (H1-H27) and 16 Italian (IT1-IT16) adults. Relative abundance of (a) phylum and (b) genus classified fecal microbiota is reported. Histograms are based on the proportion of OTUs per subject. Colors were assigned for all phyla detected, and for genera with a relative abundance ≥1% in at least 10% of subjects. (c) Donut charts summarizing genera relative abundance for Italians (outer donut) and Hadza (inner donut). Genera were filtered for those with ≥2% of total abundance in at least 10% of subjects. *denotes unclassified OTU reported at higher taxonomic level.

Table 5. Summary of taxa differences between Hadza and Italian subjects[1]

a.

over-represented in Hadza

Phylum	**Italians ± SEM**		**Hadza ± SEM**		**FDR**
Bacteroidetes	10.1	2.7	17.4	1.1	0.002
Bacteria unclassified	0.2	0.05	1.8	0.2	< 0.001
Proteobacteria	0.3	0.04	5.9	1.2	< 0.001
Spirochaetes	0	0	2.8	0.9	< 0.001
Genus					
Anaerophaga	0	0	0.6	0.1	< 0.001
Bacteroidales unclassified	0.07	0.02	0.8	0.1	< 0.001
Porphyromonadaceae unclassified	0.03	0.02	0.9	0.3	< 0.001
Prevotella	0.4	0.3	6.2	1.1	< 0.001
Bacteroidetes unclassified	0.2	0.07	6.6	0.8	< 0.001
Sphingobacteriales unclassified	0	0	0.6	0.1	< 0.001
Eubacterium	1.4	0.6	2.2	0.5	0.013
Robinsoniella	0.02	0	0.9	0.2	< 0.001
Clostridiales unclassified	7.4	0.8	11.1	0.6	< 0.001
Butyricicoccus	0.4	0.05	2.6	0.5	< 0.001
Hydrogenoanaerobacterium	0.07	0.03	1	0.1	< 0.001
Oscillibacter	1	0.3	3.8	0.4	< 0.001
Ruminococcaceae unclassified	3.6	0.6	8.6	0.7	< 0.001
Sporobacter	0.8	0.2	3.7	0.4	< 0.001
Veillonellaceae unclassified	0.02	0.01	0.8	0.2	< 0.001
Bulleidia	0	0	0.7	0.07	< 0.001
Catenibacterium	0.7	0.4	1.4	0.4	0.001
Firmicutes unclassified	0.3	0.1	0.8	0.1	< 0.001
Ruminobacter	0	0	1.2	0.7	0.045
Succinivibrio	0.02	0.02	2.8	0.7	< 0.001
Proteobacteria unclassified	0.02	0.01	0.5	0.2	< 0.001
Treponema	0	0	2.5	0.7	< 0.001

b.

over-represented in Italians

Phylum	Italians ± SEM		Hadza ± SEM		FDR
Actinobacteria	8.3	1.5	0.1	0.02	< 0.001
Firmicutes	80.9	2.7	71.8	1.9	0.006
Genus					
Bifidobacterium	8.1	1.5	0.02	0.01	< 0.001
Bacteroides	7.1	2	0.2	0.04	< 0.001
Alistipes	0.9	0.2	0	0	< 0.001
Blautia	9.5	1.3	3.5	0.4	< 0.001
Anaerosporobacter	0.6	0.1	0.2	0.04	0.003
Coprococcus	3.6	0.7	1.4	0.2	0.005
Dorea	1.4	0.2	0.4	0.06	< 0.001
Lachnospiraceae unclassified	6.4	0.7	3.9	0.2	0.001
Roseburia	7.7	1.3	3.9	0.8	0.014
Faecalibacterium	18.5	2.4	11.8	1	0.022
Ruminococcus	8.6	1.8	2.1	0.3	0.005
Erysipelotrichaceae unclassified	1.5	0.3	0.7	0.2	0.026

[1]Bacterial phyla and genera significantly over-represented in the gut ecosystem of Hadza (**a**) and Italians (**b**). For each microbial group, the mean relative abundance (%) ± SEM and *P*-value of the differences between the two populations (Mann-Whitney U test) are reported.

To explore variation within the Hadza GM, we used weighted and unweighted UniFrac distances to assess differences based on camp location and sex. We found no significant difference in phylogenetic diversity or relative abundance between camps (Supplementary Figure 1). However, unlike the Italian cohort, the Hadza GM does show significant separation by sex based on weighted UniFrac distance ($P<0.05$, permutation test with pseudo F-ratio). Analogous results were obtained when Euclidean and Bray-Curtis distance of genera relative abundance were considered ($P<0.05$) (Figure 6).

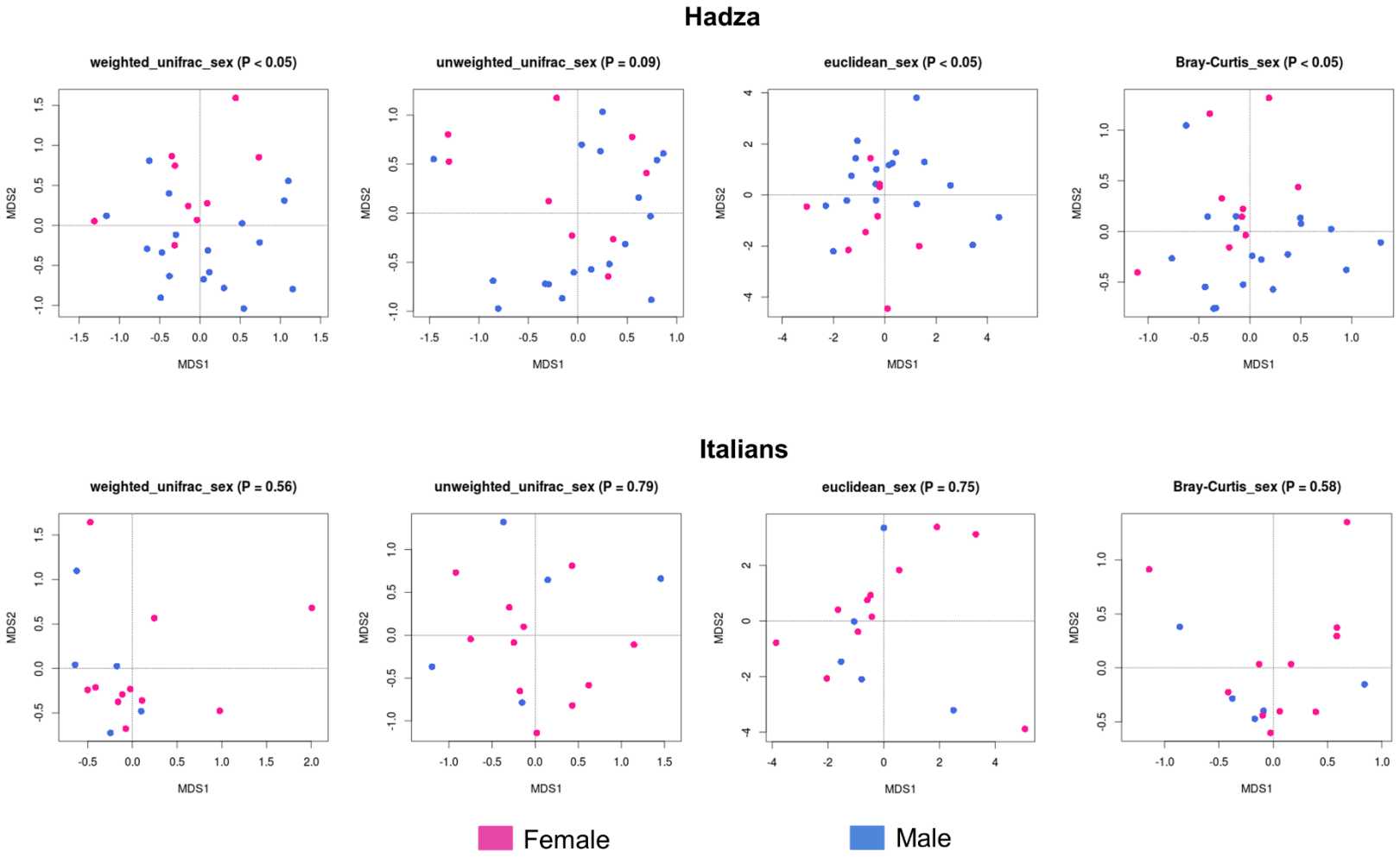

Fig 6. Sex difference in GM structure among Hadza and Italians. PCoAs based on unweighted and weighted UniFrac distances as well as Euclidean and Bray-Curtis distances show patterns of separation by sex within each subject cohort and their respective P-values. Significance was calculated by permutation test with pseudo F-ratio. Pink, females; blue, males.

To determine a structural basis for the observed separation, we compared genera relative abundance between Hadza men and women using a Mann-Whitney U test, and found a significantly increased abundance of *Treponema* ($P<0.05$) in women and increased *Eubacterium* ($P<0.05$) and *Blautia* ($P<0.001$) in men. These differences may result from the pronounced sexual division of labor and sex differences in diet composition among the Hadza (Marlowe, 2001). Women selectively forage for tubers and plant foods, and spend a great deal of time in camp with children, family members, and close friends. Men are highly mobile foragers and range far from the central camp site in order to obtain game meat and honey (Pontzer et al., 2012). Though all foods are brought back to camp and shared, men and women consume slightly more of their targeted foods from snacking throughout the day (Berbesque et al., 2011). The increased *Treponema* among women may be an adaptation to the higher amount of plant fiber in their diet, especially from tubers. *Treponema* is considered an opportunistic pathogen in industrialized populations due to *T. pallidum*, the bacterium responsible for syphilis and yaws (Antal et al., 2002). However, this genus also includes proficient cellulose and xylan hydrolyzers (Warnecke et al., 2007), and it is possible *Treponema* acts as a mutualistic component of the Hadza GM to help with fiber degradation. The sex-based divisions in the Hadza lifestyle likely play a role in altering composition abundance of the GM through different patterns of environmental and community exposure, such as those previously viewed across age, geography or diet (De Filippo et al., 2010; Yatsunenko et al., 2012). Further clarification of this division would require the inclusion of more Hadza women in the sample pool.

4.3.3 Detailed comparison to Italian controls

The Hadza and Italian GM profiles are quite distinct. Community structure visualized using PCoA of weighted and unweighted UniFrac distances reveal a sharp segregation along PCo1, indicating a strong core division in GM phylogeny between Hadza and Italian individuals ($P<0.001$, permutation test with pseudo F-ratio) (Figure 7). Mean values of unweighted UniFrac distances also reveal lower within-group variability of taxonomic diversity among Hadza than Italians ($P<0.001$). This similarity in breadth of phylogenetic diversity among Hadza is likely a result of close proximity community living with food sharing. Camp movement is usually resource driven (food and water) and the size and duration of camps vary greatly by season. In the dry season, many groups congregate around water holes, which also make hunting more productive. During the wet season groups are small and much more scattered with often five or fewer adults.

Though Firmicutes and Bacteroidetes are the dominant phyla in both Hadza and Italian GM, Hadza are characterized by a relatively higher abundance of Bacteroidetes and a lower abundance of Firmicutes (Table 5). The two GM ecosystems are remarkably different with respect to subdominant phyla (<10% relative abundance). Hadza are largely enriched in Proteobacteria and Spirochaetes, which are extremely rare in the Italian GM, while Actinobacteria, an important subdominant component of

the Italian GM, are almost completely absent from the Hadza microbiome. At the genus level, the Hadza GM is comparatively enriched in *Prevotella*, *Eubacterium*, *Oscillibacter*, *Butyricicoccus*, *Sporobacter*, *Succinivibrio* and *Treponema* and correspondingly depleted in *Bifidobacterium*, *Bacteroides*, *Blautia*, *Dorea*, unclassified *Lachnospiraceae*, *Roseburia*, *Faecalibacterium*, *Ruminococcus* and unclassified *Erysipelotrichaceae*. Moreover, there are many unclassified genera belonging to Bacteroidetes, Clostridiales and *Ruminococcaceae* in the Hadza GM, emphasizing our still limited ability to identify community dependent bacteria. The absence of *Bifidobacterium* in the Hadza GM was confirmed by qPCR (Supplementary Table 1). Taken together, data from our GM comparative analysis indicate a characteristic configuration for the Hadza gut microbial ecosystem that is profoundly depleted in *Bifidobacterium*, enriched in Bacteroidetes and *Prevotella*, and comprising an unusual arrangement of Clostridiales. This arrangement is defined by a general reduction of well-known butyrate producers, members of the *Clostridium* clusters IV and XIVa (den Besten et al., 2013), and a corresponding increase in unclassified Clostridiales and *Ruminococcaceae*. Interestingly, the Hadza GM is also characterized by a relevant enrichment in what are generally considered opportunistic microorganisms, such as members of Proteobacteria, *Succinivibrio*, and *Treponema*.

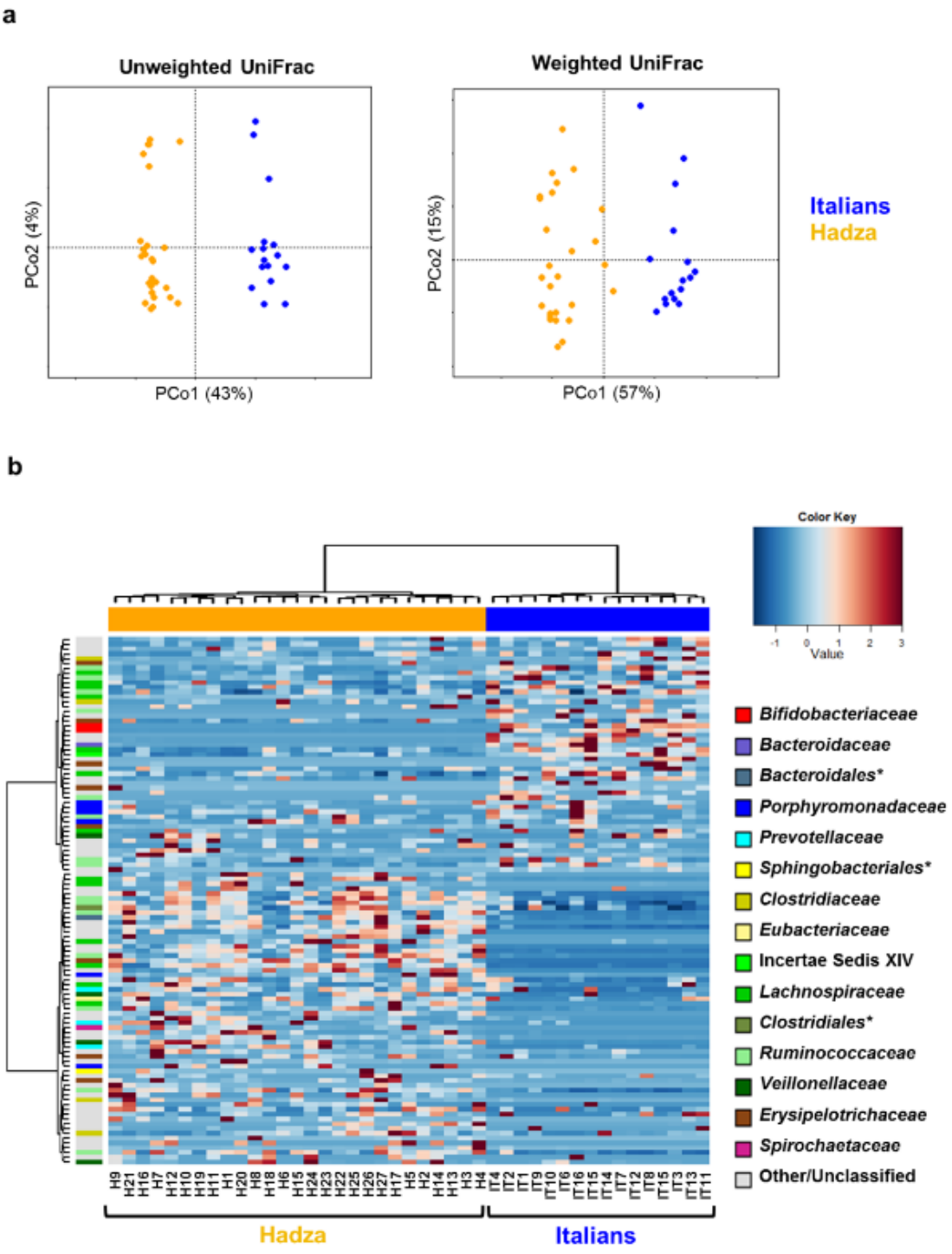

Fig 7. GM Phylogenetic difference between Hadza and Italian subjects. (**a**) Unweighted and weighted UniFrac distance PCoA of the fecal microbiota from 27 Hadza (orange dots) and 16 Italians (blue dots). (**b**) Hierarchical Ward-linkage clustering based on the Spearman correlation coefficients of genus proportion. Genera were filtered for subject prevalence of at least 30% within a population. Subjects are clustered on top of the panel and color-coded as in a. 110 genera clustered by the vertical tree are color-coded by family assignment. *denotes unclassified OTU reported at higher taxonomic level.

4.3.4 Comparison to African agricultural societies

The Hadza GM shares some features with other African populations, namely enrichment in *Prevotella, Succinivibrio* and *Treponema* (De Filippo et al., 2010; Yatsunenko et al., 2012; Ou et al., 2013). Therefore, in order to explore community-level relationships within the GM that may be unique to a foraging lifestyle, we sought associations among genera by including two previously published rural African groups with an agriculture-based subsistence and their respective Western controls: 11 Mossi children from the Boulpon village, Burkina Faso (BF) aged 5-6 years and 12 Italian children aged 3-6 years (De Filippo et al., 2010); 22 young adult members from four rural Malawian communities, Chamba, Makwhira, Mayaka and Mbiza aged 20-44 years and 17 US adults aged 24-40 years (Yatsunenko et al., 2012). Clustering analysis shows a significant (P<0.001, Fisher's test) separation among Hadza, Malawians, BF and Western controls (Figure 8a). PCoA based on Bray Curtis distances of genera relative abundance confirms this separation (P<0.001, permutation test with pseudo F-ratio) (Figure 8b). Interestingly, PC1, which represents 30% of the total variability, shows a clear separation between the Western controls and the African populations, while PC2, which explains a lower fraction of the total variability (19%), indicates a separation among Hadza, Malawians and BF. Separation along PC2 is also visualized among Western populations, but to a much lesser degree and with large interspersion between the U.S. and Italian children. Our data demonstrate biologically meaningful variation between the Western and non-Western GM profiles, showing that African populations with different lifestyle possess an overall more similar GM to each other than to Western populations. Although these results indicate a certain degree of GM variation among different African groups, we cannot exclude that a study effect may outweigh separation owing to actual differences in GM composition within these communities. While we do see that the US controls intersperse with the Italian children (green and light blue color coding respectively), the Italian adults from this study remain distinct from the other Western control samples, indicating that there may be some methodological bias that could also affect the observed GM differences among the African populations. Therefore, we urge caution in interpreting the strength of GM variation based on the separation seen among Hadza, BF, and Malawian populations in this single cross study comparison. Further caution is needed since subjects from all six populations are not age-matched.

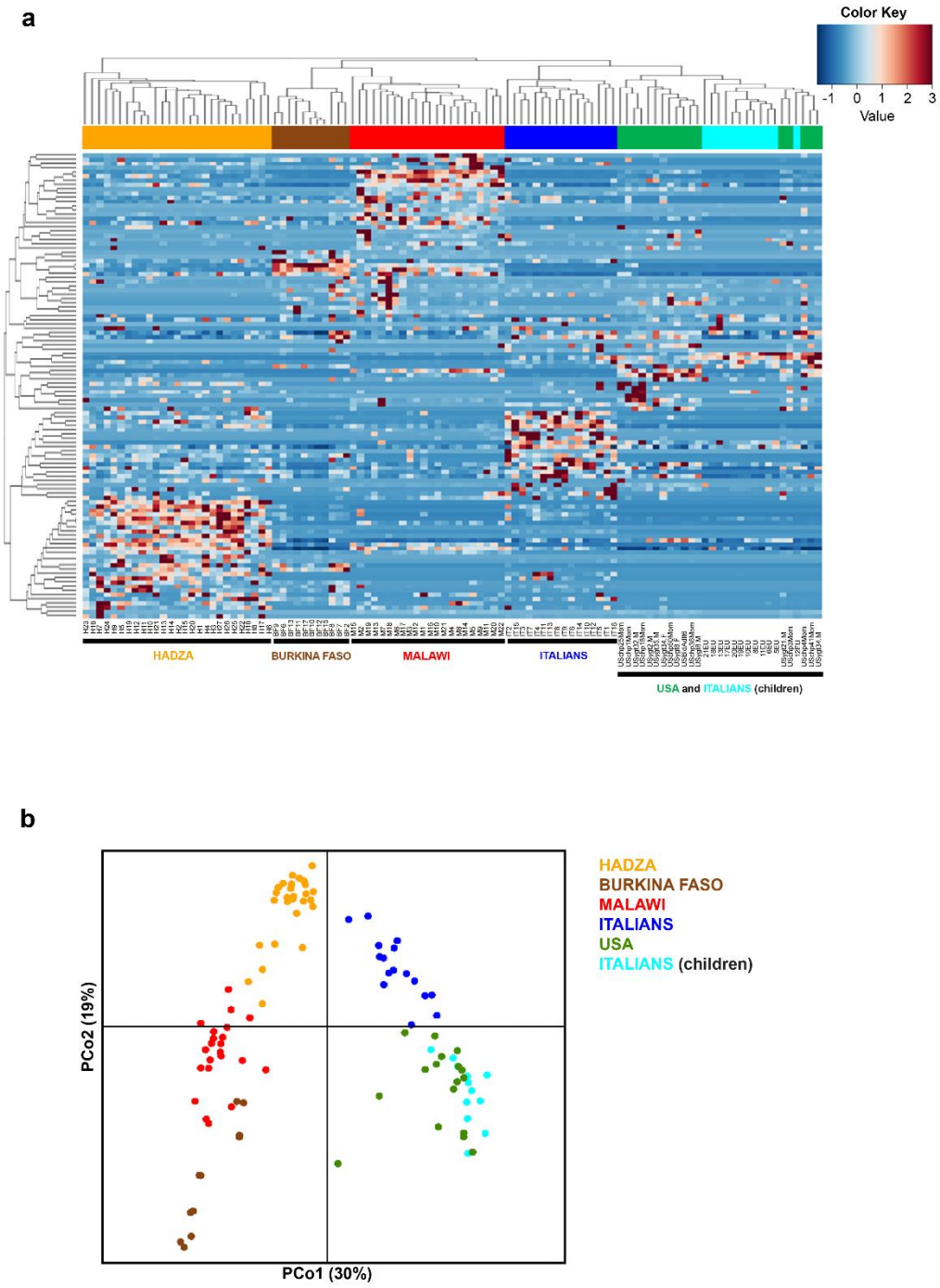

Fig 8. Comparison of GM relative abundance among populations reflects subsistence patterns. (**a**) Hierarchical clustering based on Eisen's formula of correlation similarity metric of bacterial genus proportion and average linkage clustering. Genera were filtered for subject prevalence of at least 30% of samples. Subjects are clustered in the top of panel and color-coded orange (Hadza), brown (Burkina Faso), red (Malawi), blue (Italian adult controls from this study), green (U.S. adults from Yatsunenko et al., 2012), and cyan (Italian children from De Filippo et al. 2010). 107 genera are visualized and clustered by the vertical tree. (**b**) PCoA based on Bray Curtis distances of the relative abundance of GM genera of each population.

To identify patterns of microbial community variation among Hadza, Malawian, BF and Western controls- Italian adults, Italian children, and US adults- we determined co-abundance associations between genera and then clustered them, resulting in six co-abundance groups (CAGs) (Supplementary Figure 2) (Claesson et al., 2012). In the context of this comparison, six CAGs define the microbial variation between populations (P<0.001, permutational MANOVA). CAGs have been named according to the dominant genera in each group as follows: *Dialister*, *Faecalibacterium*, *Prevotella*, *Blautia*, *Clostridiales*_unclassified and *Ruminococcaceae*_unclassified. The Wiggum (Claesson et al., 2012) plot depicts the GM compositional relationship for each of the six populations and shows a correspondingly unique pattern of abundance of the six CAGs (Figure 9). Interestingly, African populations are characterized by the *Prevotella* CAG, while Western controls show a distinctive overall dominance of the *Faecalibacterium* CAG. With respect to Malawian and BF, Hadza show a peculiar combined enrichment of *Clostridiales*_unclassified, *Ruminococcaceae*_unclassified and *Blautia* CAGs. Given the dietary and lifestyle distinctions of each population, the CAG distribution in Hadza, Malawians, BF, and Western controls could represent predictable GM community specificity to three different modes of subsistence: foraging, rural farming, and industrial agriculture respectively. The unique CAG distribution of Hadza with respect to the other groups corresponds to the higher abundance of *Treponema* and unclassified *Bacteroidetes* and *Ruminococcaceae* co-residents in the Hadza microbiome. All Hadza we sampled share this configuration, therefore we must posit the possibility that these bacteria and their co-residents confer a structural and functional asset responding to the specific needs of the Hadza lifestyle. However, more forager and subsistence agriculture communities should be sampled to learn what aspects of subsistence drive microbe community assimilation and whether variability is a result of environment, host selection, or both.

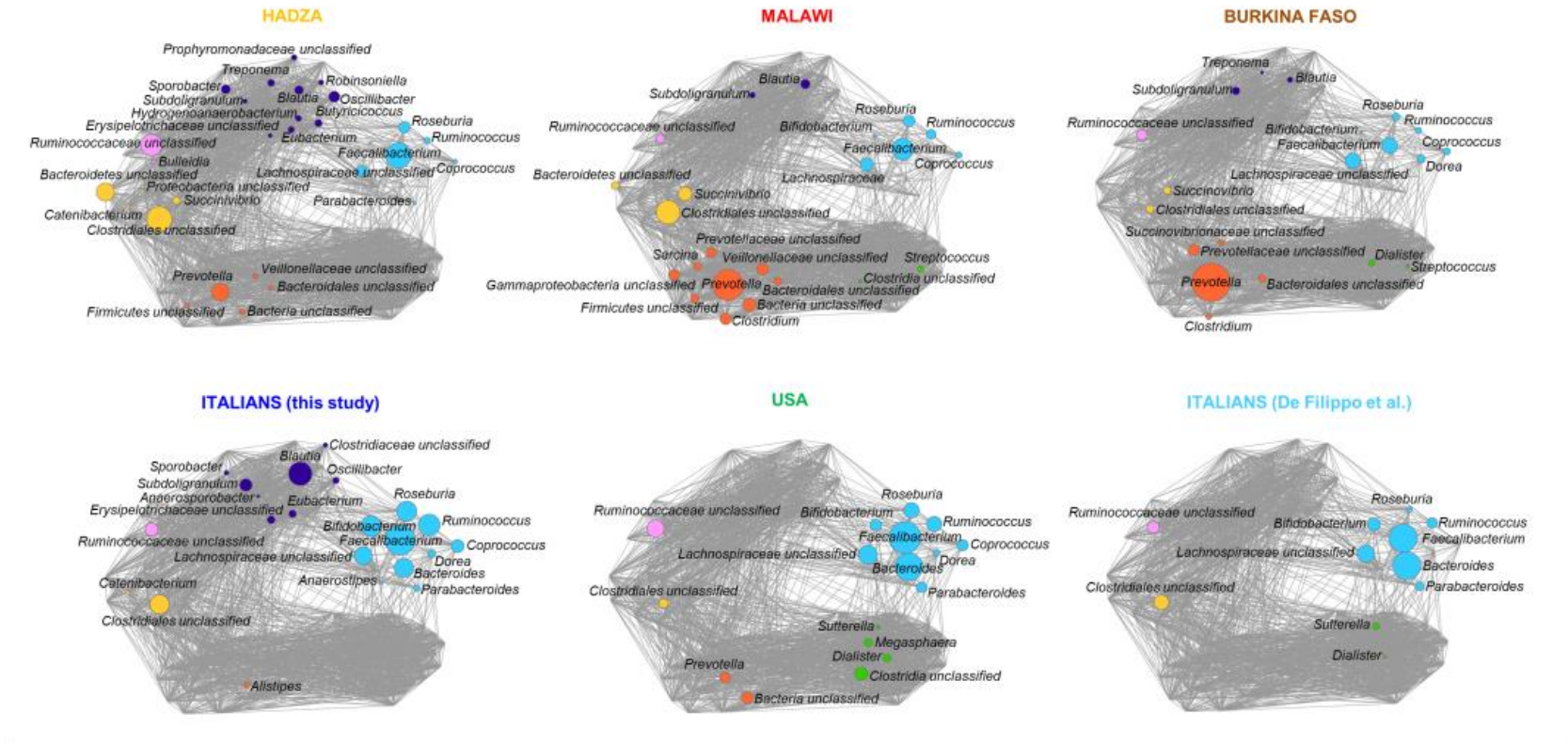

Fig 9. Distinct bacterial co-abundance groups define each population. Wiggum plots indicate pattern of variation of the six identified co-abundance groups (CAGs) in Hadza, Malawi, Burkina Faso, and Western controls. CAGs are named with the name of the most abundant genera and are color-coded as follows: Faecalibacterium (cyan), Dialister (green), Prevotella (orange), Clostridiales_unclassified (yellow), Ruminococcaceae_unclassified (pink), and Blautia (violet). Each node represents a bacterial genus and its dimension is proportional to the mean relative abundance within the population. Connections between nodes represent positive and significant Kendall correlation between genera (FDR < 0.05).

4.3.5 Short chain fatty acid profile of Hadza and Italians

End-products of bacterial fermentation are important for microbiota-host co-metabolism and evolution. Short chain fatty acids (SCFAs) are the dominant metabolites resulting from bacterial fermentation of plant derived substrates such as glycans and polysaccharides that pass undigested through the small intestine and into the colon. The SCFAs acetate, butyrate, and propionate are pivotal in several host physiological aspects such as nutrient acquisition, immune function, cell signaling, proliferation control, and pathogen protection (Tremaroli and Bäckhed, 2012).

Detected SCFA values for each sample are reported in Supplementary Table 2. PCA analysis of the SCFA relative abundance profiles show a segregation between Hadza and Italians (P=0.02, permutation test with pseudo F-ratio) (Figure 10). The Italian samples are characterized by a significantly (P<0.01, Mann-Whitney U test) greater relative abundance of butyrate, while Hadza samples are enriched in propionate (P<0.01). These differences may reflect dietary variation in both amount and type of fiber and carbohydrates consumed by Hadza and Italians, and the consequent relative depletion in butyrate producers belonging to the *Clostridium* cluster IV and XIVa in Hadza.

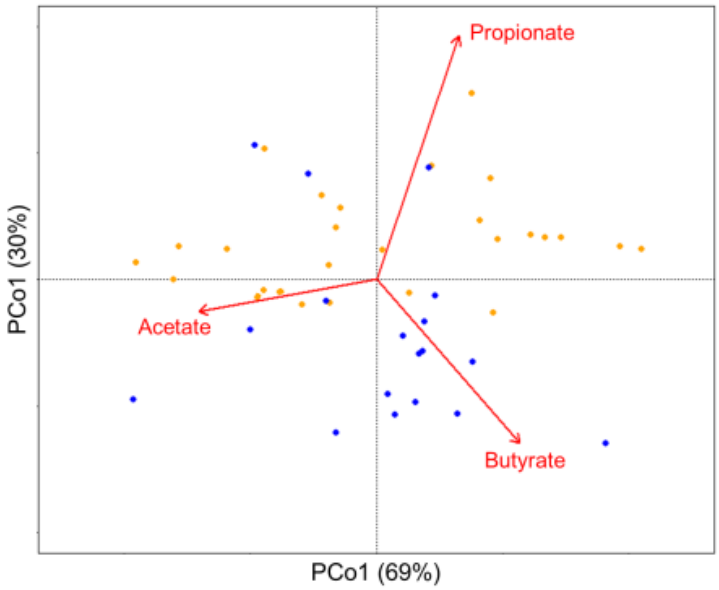

Fig 10. Comparison of metabolite production between Hadza and Italian samples. PCoA based on Euclidean distances of the profiles of short chain fatty acid relative abundance in Hadza (orange) and Italians (blue). Vectors show propionate, butyrate and acetate abundance.

However, because of the high degree of metabolic cross-feeding between members of the human gut microbial ecosystem (Flint et al., 2008), direct associative relations between bacteria presence/absence and SCFA production are not so simple. In order to investigate gut microbial networks on the basis of the observed differences in patterns of SCFA production in Hadza and Italians, we evaluated the GM

genera that correlate significantly with each SCFA (Supplementary Table 3). Among genera with greater than 5% relative abundance in at least one of the two populations, *Bifidobacterium*, *Bacteroides*, *Blautia*, *Faecalibacterium* and *Ruminococcus* are positively ($P<0.05$, Kendall tau rank correlation coefficient) correlated with butyrate, showing Kendall correlation values of 0.30, 0.31, 0.32, 0.52 and 0.30, respectively. In contrast, *Bifidobacterium*, *Blautia* and *Lachnospiraceae* show a significant ($P<0.05$, Kendall tau rank correlation) negative correlation with propionate of -0.36, -0.27 and -0.24, respectively, while *Prevotella* demonstrates a positive correlation of 0.41. The absence of *Bifidobacterium* and the lower relative abundance of *Blautia*, *Ruminococcus* and *Faecalibacterium* concurrent with greater relative abundance of *Prevotella* seen in the Hadza GM match a presence/absence scenario with SCFA concentrations that are enriched in propionate and reduced in butyrate with respect to Italians.

Though SCFAs are metabolic end-products for bacteria, they are important direct energy resources for the host. Butyrate is produced from dietary fiber, and when present in sufficient quantity, it becomes the major fuel source for colonic epithelial cells, reducing the need for energy allocation to these cells from the host (den Besten et al., 2013). Propionate is transferred to the liver where it serves as a precursor for hepatic gluconeogenesis (Nicholson et al., 2012; Tremaroli and Bäckhed, 2012; den Besten et al., 2013). The extra energy derived from these GM-produced SCFAs may provide nutritional support for the Hadza whose diet contains high amounts of fiber but is seasonally lean in lipids.

4.4 Discussion and conclusions

The Hadza represent a rare example of human subsistence through hunting and gathering that persists in the same East African region where early hominins lived. The Hadza maintain a direct interface with the natural environment, deriving their food, water, and shelter from a rich biosphere blanketed in the complexity of microbial communities and interactions. In our characterization of the Hadza GM, we report several findings that we feel support the conception of the microbiome as a diverse and responsive ecosystem adapting continuously as a commensal component of the host supra-organism. Keeping this framework in mind, we interpret the GM structure as an adaptation to the Hadza foraging lifestyle.

Image 12. Hadza woman stops to drink from a naturally occurring spring on the way to dig tubers.

The Hadza GM has characteristic features that are consistent with a heavily plant-based diet. Besides the presence of several well-known fiber-degrading Firmicutes that are also shared with Italians – e.g. members of *Lachnospiraceae*, *Ruminococcaceae*, *Veillonellaceae*, Clostridiales Incertae Sedis XIV and *Clostridiaceae*, the Hadza GM is enriched in *Prevotella, Treponema,* and unclassified members of Bacteroidetes, Clostridiales and *Ruminococcaceae*. These xylan-degrading *Prevotella* (Flint et al., 2008) and *Treponema* (Warnecke et al., 2007) and the abundance of still unclassified Bacteroidetes and Clostridiales, groups known for their fibrinolytic capabilities, may provide the Hadza GM with specific glycan-degrading abilities for Hadza to deal with a vast array of refractory and resistant organic materials introduced through diet.

Consistent with GM arrangements reported for other African groups (De Filippo et al., 2010; Yatsunenko et al., 2012; Ou et al., 2013), the Hadza GM shows a higher relative abundance of *Prevotella*, but with a correspondent reduction of *Bacteroides* in the gut ecosystem compared to the Italian control cohort. Thus, similar to what has been proposed for rural Africans consuming grain-based high-fiber diets (De Filippo et al., 2010), it is tempting to speculate that this microbe community within the Bacteroidetes phylum could harbor the necessary GM functions to deal with their especially unique, but also highly fibrous, plant food dietary constituents (Hehemann et al., 2010).

During our visit in January, 2013, between two rainy periods, there were a variety of foods available and acquired, the majority of which were derived from plants. These included at least four species of tuber, small and large game, honey from stinging and stingless bees, leafy green foliage, baobab fruit, and one species of berry. Tubers are an incredibly important food in the Hadza diet because they are consistently available and exploited year round, despite being the lowest ranked food

resource (Marlowe and Berbesque, 2009). Hadza tubers are uncultivated wild species belonging to the plant families Fabaceae (legumes), Convolvulaceae (morning glories and herbaceous vines), and Cucurbitaceae (squashes, melons, and gourds). Only the underground root is harvested and consumed either raw or briefly roasted. It is noteworthy that most of the tubers consumed by Hadza contain high moisture and tough indigestible fibers that must be expelled as a quid during chewing (Figure 11). The digestible fraction is thus incredibly variable but composed largely of water, simple sugars, starch and soluble fiber.

Fig 11. Insoluble fiber found in most Hadza tubers. Pictured above are two slices of one mak'alitako tuber (*Emminia entennulifa*). The left slice is pre-chewed and the right slice is post-chewing to show the resiliency of the insoluble fiber. These fibers are not swallowed by the Hadza.

Several publications have outlined the basics of Hadza diet (Vincent, 1985; Murray et al., 2001; Schoeninger et al., 2001; Marlowe, 2010; Crittenden, 2011; Peterson et al., 2012) and have converged on the following general characteristics. The majority of the annual Hadza diet (approximately 70% of kilocalories) comes from plant foods (Marlowe, 2010). Birds, small, medium, and large sized game meat comprise approximately 30% of the annual diet (Marlowe, 2010). See Marlowe (2010) and Peterson et al. (2012) for an exhaustive list of all species targeted. Small variation exists between published sources depending on whether kilogram wet weight or kilocalories per gram were used to calculate percent contribution to diet. Resource availability – both plant and animal – is highly correlated with rainfall patterns; therefore diet varies year to year as well as season to season. A general dietary pattern does emerge, however, and indicates that more meat is consumed during the dry season when people and game animals converge to target the same watering holes (Woodburn, 1968). Foods like baobab, tubers, and honey are targeted year round. Based on these data, the resulting picture is a diet rich in simple sugars, starch and protein while lean in fat. It would be of great interest to learn whether the shift from a largely plant-based diet to one that includes more meat, such as during the dry season, might show a concurrent change in GM structure amongst Hadza.

We find evidence of a sex-related divergence in Hadza GM structure, which is not documented in other human groups. This divergence corresponds to the Hadza sexual division of labor and sex

differences in diet composition. In the same environment with access to the same dietary resources, Hadza men and women are differentially adapted to their particular pattern of food consumption. The potential for Hadza women's GM to respond with significant structural differences to the increased consumption of plant foods represents a profound break with traditional thinking on the limited digestive capacity of the human gut and the constraint it imposes on nutritional provisioning for reproduction and brain growth (Aiello and Wheeler, 1995; Wrangham and Conklin-Brittain, 2003). Women's foraging must adequately provision for pregnancy and lactation, which is a strong adaptive pressure for the GM to derive the most energy from consistently available plant foods. In this regard, the GM aligns with the host nutrition acquisition strategy, thus potentially buffering women from resource "gaps" that may lead to nutritional deficiencies.

The reported presence of *Treponema* in now five geographically separate extant rural human communities from this and previous studies (Hadza, Burkinabe, Malawians, South Africans, and Venezuelan Amerindians) (Tito et al., 2012; Ou et al., 2013) supports an alternative functional role for this bacterial group whose expression in industrialized communities is normally attributed to pathogenic disease. De Filippo *et al.* hypothesize that the presence of *Treponema* in BF children enhance the host's ability to extract nutrients from the fibrous foods that comprise their traditional diet (De Filippo et al., 2010). While the Hadza do not eat agricultural or grain-based diets, they do rely heavily on fibrous tubers throughout the year, with women often consuming tubers for a greater percentage of daily calories than do men (Berbesque et al., 2011). These sources of fiber rich plant foods could similarly encourage a mutualistic *Treponema* population whose fibrinolytic specializations would be advantageous to Hadza nutritional acquisition, particularly in women.

Medical examinations conducted on Hadza found evidence of Treponematosis from serum samples at low rates (13 out of 215 sampled) with the highest prevalence in men of settled Hadza camps between 1966 and 1967 (Bennett et al., 1973). However, there was low but consistent prevalence for women in both settled and foraging Hadza groups with little clinical evidence of yaws, suggesting immunoregulation of *Treponema* pathogens.

Demographic reports of age structure, population density, growth and fertility indicate that the Hadza appear to be a healthy and stable savanna foraging population despite rapid encroachment of pastoralist groups in the same region (Blurton Jones et al., 1992). For a foraging population with little to no access to health care or medical facilities, the Hadza have relatively low rates of infectious disease, metabolic disease, and nutritional deficiencies in comparison to other settled groups in the northern Tanzania and southeastern Uganda region (Bennett et al., 1973; Work et al., 1973; Blurton Jones et al., 1992). However, these earlier assessments were more than 40 years ago, over two Hadza-generations, and many changes have since occurred to the land occupied by the Hadza. Re-evaluation of health and population metrics deserves renewed focus, especially now that research on the Hadza has garnered much attention.

The absence of Actinobacteria, particularly *Bifidobacterium*, in the Hadza GM is unexpected.

Bifidobacteria are associated with breastfeeding in infants and achieve large proportions of the GM in the first few months after birth (Turroni et al., 2009). Typically, in adults, bifidobacteria commonly make up 1-10% of the GM population. Complete absence of bifidobacteria, as observed in the Hadza, has never to our knowledge been reported for any other human group. We hypothesize that the lack of bifidobacteria in adult Hadza is a consequence of the post-weaning GM composition in the absence of agro-pastoral-derived foods. Support for this hypothesis comes from the observation that other populations in which meat and/or dairy consumption is low to absent, such as vegans and Koreans, also have very low representation of Actinobacteria and *Bifidobacterium* (Nam et al., 2011; Zimmer et al., 2012). The continued consumption of dairy into adulthood could be one reason most Western populations maintain a relatively large bifidobacterial presence. Aside from bifidobacterial species of human origin, the majority of *Bifidobacterium* have been isolated from livestock animals such as swine, cattle, and rabbit (Ventura et al., 2007; Turroni et al., 2009). The Hadza neither domesticate nor have direct contact with livestock animals. Thus, as they lack exposure to livestock bifidobacteria, this raises the question of whether the necessary conditions for interspecies transfer and colonization of bifidobacteria do not occur for the Hadza (Moeller et al., 2013). The Hadza retain a strong independent identity both in their native language and oral history, which says nothing about a previous pastoral or agricultural existence (Marlowe, 2010). Early Y chromosome and mitochondrial DNA analysis shows some of the highest genetic divergence between Hadza and members of the Khoisan/San language group, the Ju/'hoansi (!Kung), evidence suggestive of a very ancient lineage (Knight et al., 2003). Given their penchant for social timidity during early attempts at first contact and resistance to assimilation in the second half of the twentieth century, it is very likely the Hadza persist with a very ancient traditional lifestyle into present times (Marlowe, 2010).

Future work must focus on the GM of breast-fed Hadza infants to determine the role of bifidobacteria in the kinetics of assembly and development of the Hadza GM, and to learn whether this bacterial group is completely absent in all Hadza, including infants, or if it is definitively lost from the gut ecosystem post-weaning. It is important to note that while bifidobacteria are considered a beneficial bacterial group in Western GM profiles, their absence in the Hadza GM, combined with the alternative enrichment in "opportunistic" bacteria from Proteobacteria and Spirochaetes, cannot be considered aberrant. On the contrary, the Hadza GM likely represents a new equilibrium that is beneficial and symbiotic to the Hadza living environment. Support for the advantage of such novel GM configurations comes from the finding that GM restructuring also occurs in centenarians (Biagi et al., 2010), who are extreme examples of organismal robusticity. Additionally, these findings illustrate a need to reevaluate the standards by which we consider gut microbiota "healthy" or "unhealthy", as they are clearly context dependent.

GM diversity, as found in rural African populations and now in the Hadza, is almost certainly the ancestral state for humans. Adaptation to the post-industrialized Western lifestyle is coincident with a reduction in GM diversity, and as a result, a decline in GM stability. Diversity and stability are factors

with major health implications, particularly now that the human gastro-intestinal tract is increasingly recognized as the gateway to pathogenic, metabolic, and immunologic diseases (Blaser and Falkow, 2009). Co-speciation between host and microbiota over millions of years has shaped both sets of organisms into mutualistic supra-organisms. Dissolving that contact through sterilization and limited environmental exposure has had a drastic effect on health and immune function of modern Westernized human groups. The Hadza GM is likely an "old friend" and stable arrangement fitting their traditional hunter-gatherer lifestyle (Rook et al., 2004).

We are only just beginning to document GM diversity across populations. In our study, more than 33% of the total Hadza GM genera remain unidentified. Such taxonomic uncertainty holds exciting prospects for discovering yet-unknown microbial genetic arrangements. This finding also underscores the importance of increasing our reference phylogenies and resolving deep taxonomic relationships between bacteria by sampling a wider variety of environments and extreme ecological zones (Rinke et al., 2013).

In summary, the characterization of the Hadza GM presents a suite of unique features that suggest specific adaptation to a foraging lifestyle, which includes a large proportion of highly refractory plant foods. We expect that detailed study of the function of this GM community will expose a greater number of genetic specializations for degrading polysaccharides than what is currently found in other human populations. When viewed broadly, inconsistencies in associations among GM structure, diet, and disease belie interpretive confidence about GM phenotypes. The functional redundancy found in bacterial communities indicates that microbial activity, rather than composition, is conserved. However, the ability of novel genes to propagate through environmental transfer into common gut bacteria complicates the enterotype–function paradigm. Moreover, closely related human symbiont microorganisms have been demonstrated to differ widely in their glycan use phenotypes and corresponding genomic structures (Martens et al., 2011). Even if taxonomic similarities do exist between human populations, at finer scales their GM communities may exhibit dramatic metabolic differences tailored to suit disparate environmental constraints. With a microbiome functional assignment rate at 60% (Wu et al., 2011), these questions need to be resolved by testing GM activity using *in-vivo* techniques such as with gnotobiotic mice (Lecuit et al., 2007) or *in-vitro* techniques such as with computer controlled simulations of the large intestine (Minekus et al., 1999). Furthermore, comparative analysis between the human and great ape GM, especially with members of *Pan*, will highlight important distinctions that enabled early human ancestors to extend their dietary and ecological ranges without the need for technological buffering. Host-microbiome mutualism holds great relevance to the field of human evolution as it vastly propels the genetic landscape for adaptation well beyond somatic potential.

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CHAPTER 5

CONCLUSION

To conclude what, in three chapters, 100+ pages, and three peer reviewed publications, I have extensively researched, experimented, analyzed, and documented, merits a bit of backtracking to reestablish the original question. To quote myself in December 2011, I proposed, "a study of the tuber plant foods consumed by the Hadza hunter-gatherers of Tanzania, Africa, to assess their nutrient content and value as staple plant resources in the Hadza diet." My desired final contributions were optimistically summarized as such, "The outcome of this study will enable us to paint a more complete picture of the nutrtitional landscape of human food resources in the East African savannah," with the assumption that this deals only with food resources available to human foragers and not farmers. If I were to assess the actual outcome (unobjectively), then I have accomplished a portion of my original research goals, but with ample room for future investigations. To complete the picture of the digestibility of the Hadza tubers using *in-vitro* modeling, I have already conducted trials to simulate colonic fermentation of Hadza tubers by Hadza microbiota using the TNO *in-vitro* model of the large intestine (TIM-2). This work endeavors to answer whether Hadza microbiota do in fact work to provide the host with more metabolites for a secondary means of caloric or otherwise essential nutrient acquisition, to compensate for the overwhelming amount of indigestible material in Hadza tubers. The preliminary results so far suggest that microbial contribution to Hadza nutrition is not attributable to the quantity of metabolic products produced, but rather, the composition and timing of microbial substrate production may instead confer special health promoting attributes for the Hadza. The analysis for this forthcoming paper is underway and I anticipate publication within the year (2016).

This research had three components: 1. test the role of cooking on starch gelatinization, specifically in tubers; 2. assess the digestibility of wild East African tubers for a human consumer, informed and modeled after the diet ecology of Hadza hunter-gatherers, and quantify the relative proportion of nutrition accessible to small-intestine absorption; and 3. look at the role, specialization, and impact of the gut microbiota of the Hadza in facilitating nutrient acquisition and caloric buffering for a people still practicing a non-agrarian hunter-gatherer subsistence. The first two research phases were explored by proxy, and the third was approached directly, but using the technology, methods, and comparative references of an emerging field that is still in development. This means that more, and more precise, answers are still out there. However, I have succeeded in contributing information and evidence to support the significance of plant foods to human foragers in the tropical East African savannah-mosaic environment, which is the putative evolutionary landscape of our species. I constructed the premise for the importance, from an evolutionary perspective, of plant foods in the human diet in the introduction to this thesis, and it follows that in all the intervening text I have found that indeed African hunter-gatherers regularly eat essentially raw starchy plant foods, which are difficult to digest. However, with minimal control of fire and a highly adapted gut microbiota, these plant foods become easily consumable resources that are rapidly and readily metabolized by gut bacteria into secondary, but nutritious, host-specific metabolites. In addition, the tubers consumed by Hadza are rich in critical minerals necessary for brain development such as calcium, magnesium, iron,

potassium, and iodine, as well as potentially B_{12} vitamins that support nervous system function. Therefore, I suspect that wild tubers are an important in-land food resource that may somewhat supplant the presumed need for marine or aquatic resources by a large-brained hominin, or at least greatly ameliorate the stress of nourishing a metabolically demanding and nutritionally vulnerable organ.

While answering many initial questions about the importance of plant foods and their suitability as a staple resource throughout human evolution, there remains many unexplored areas on the topic of human digestive physiology, especially in relation to evolutionarily emergent biocultural and technological buffering systems. My broader academic goals are to help improve our understanding of hominin evolution by exploring physiological versus behavioral or technological responses to different environmental pressures as they relate to diet ecology. The dispersal of early hominins, and later archaic humans, across the globe is profoundly demonstrative of a bio-cultural marriage that has steered us on a unique evolutionary course towards co-dependence on the combined effects of our technology and our genetic heritage. While a lot of well-informed hypothesis exist about how and when early hominins became finicky omnivores with an increasingly unspecialized, yet diminishing, gut, only a few studies have set out to empirically test specific elements regarding how this could be accomplished given certain ecological and anatomical restraints. Gene polymorphisms along the human lineage that differentiate us from great apes, or even archaic humans, are powerful informants to help narrow our search for selectively advantaged phenotypes, and how, either by relaxing or increasing pressure, these came about. This in turn can lead to compelling inferences about the environment, diet, and human behavior circumscribing selective processes, which necessarily fills gaps in our knowledge where archaeological evidence is unobtainable.

The first step in framing possible future inquiries is to realize the bottlnecks that constrain our understanding of, and the actions surrounding, human evolution. From the results of my first roasting trial experiment, I concluded that probably the most significant hindrance to plant food consumption relates to comminution. In other words, humans must be able to physically reduce the particle size of their food through chewing or external processes to successfully consume and digest the food. When starch-based tuberous foods are subjected to brief thermal treatment, their parenchymateous tissues are significantly softened, while most of the interior starch remains ungelatinized, and for Hadza consumers, this is enough to make the tuber perfectly palatable. Therefore, the oral phase of digestion is one such bottleneck, and concommittant aspects of mastication include the activity of salivary amylase. Curiously, the salivary amylase gene has experienced positive selection for copy number variability among modern humans, indicating some functional significance of the alpha amylase enzyme. Whether the functional relevance under selection occurs during oral digestion or through initiation of downstream pancreatic enzyme activity and blood-glucose regulation is relatively unknown, and I intend to explore this topic in a project committed to ascertaining the physiological relevance of salivary amylase. Similarly, the functional significance and specialization of the human gut microbiome to the human host is poorly characterized, especially in non-Westernized populations.

It makes intuitive sense that the microbial population is cultivated over one's lifetime. However, recent evidence suggests that early life experiences are critical events that effectively establish a long-term scaffold for the adult microbial configuration, and that this may be somewhat immutable. Furthermore microbial communities that are identical in composition, can vary widely in functional coding and expression, leaving ample room for individual specialization or response to diet and disease. From my work with the Hadza, we know that human foragers harbor very different microbial communities from those found amongst Western human populations. Therefore, we can presume that aspects of forager life possibly require different functional configurations of the microbiota as well, which are likely tied to the activities and nutritional needs of the individual host. Specialized microbial activities that relate to diet may have allowed early humans to exploit a wider variety of resources than what we currently anticipate given our understanding of the size and absorptive limitations of the stomach and small intestine. Using both *in-vitro* and *in-silico* methods, I intend to pursue work in reconstructing rare microbial community functions and genes that may have enabled a human forager to acquire essential nutrients from the gut microbiota.

Whether human hunter-gatherers can successfully occupy and thrive in a savannah mosaic environments is self-evident. Rather it is *how* they have thrived in these environments, and still do, that captures our attention, awe and wonder. In the case of the Hadza, there is a clear and necessary dependence on local flora, particularly fibrous non-toxic tubers, and interesting concomitant adaptations. These adaptations seem to take the form of more plastic ontogentic (gut microbiome) and technological (brief roasting) developments rather than through strict inheritance of nuclear genetic traits. The implications of these findings is that humans, by virtue of their intellect and physiological non-specialization, are highly adaptable and innovative, making us the unsurprising victors in global colonization. Yet our herbivorous legacy is not so far behind us that it is surpassed in importance by our recently acquired intensive carnivorous history. Tropical modern human hunter-gatherers, as a proxy for pre-agricultural humans, beautifully exemplify the essential relationship between humans and plants. While heavily reliant on plant foods, they demonstrate the capacity for a materially and technologically minimalist existence, and yet preserve and cultivate the full spectrum of intelligence, curiosity, creativity, and sociality that are defining characteristics of our species.

ADDENDUM

Summary

Cooking is an important technology for its capacity to increase food digestibility, potentially playing a significant role in human evolution as a means to increase nutritional density of food. However, the contextual impetus for cooking is less well understood. One mode of cooking used by modern humans is to roast food directly in an open flame fire for a wide range of times. This technique may be a behavioral analogue to that of early human ancestors. Here, we provide the first experimental data on starch gelatinization patterns of USOs and starchy fruit as a result of brief fire roasting. We employed a count-based method using light microscopy and a semi-quantitative photometric strategy to observe the kinetic process of gelatinization. We find that brief, five-minute roasting over an open fire results in low levels of starch gelatinization in the parenchymal tissue, which persists even after longer roasting durations, exceeding ten minutes. However, at fifteen and twenty minutes, the majority of starch throughout each specimen is sufficiently gelatinized to enable alpha-amylase activity. These findings suggest that instances of very brief roasting, up to ten minutes, may not necessarily benefit a consumer through changes to starch structure and susceptibility to alpha-amylase activity alone.

With an initial understanding of the role of brief roasting on starch gelatinization, we then turned to the question of nutritional bioaccessibility of raw and briefly roasted wild tubers to a human consumer. Bioaccessibility is a useful measure for assessing the biological value of a particular nutrient from food, especially foods such as tubers. The wild tubers exploited by Hadza foragers in Tanzania are of interest because they are nontoxic, consumed raw or briefly roasted, and entail substantial physical barriers to consumers. In this study we elucidated the biological value of Hadza tubers by measuring the absorption of glucose through *in-vitro* digestion. We quantified digestibility using data from 24 experimental trials on four species of Hadza tuber using a dynamic *in-vitro* model that replicates digestion in the stomach and small intestine. Analysis of glucose in the input meal and output dialysate revealed the accessible glucose fraction. We also conducted assays for protein, vitamin and mineral content on whole tubers and meal fractions. Bioaccessibility of glucose varies depending on tuber species. Holding effects of chewing constant, brief roasting had negligible effects, but high intraspecific variation precludes interpretive power. Overall, Hadza tubers are very resistant to digestion, with between one to two thirds of glucose absorbed on average. Glucose absorption negatively correlated with glucose concentration of the tubers. Roasting may provide other benefits such as ease of peeling and chewing to extract edible parenchymateous tissue. A powerful factor in glucose acquisition is tuber quality, placing emphasis on the skill of the forager. Other nutrient assays yielded unexpectedly high values for protein, iron, and iodine, making tubers potentially valuable resources beyond caloric content.

To continue to evaluate the efficacy of digestion of fibrous plant foods by human consumers, we targeted the gut microbiota to explore its composition and function as an essential factor in human

digestion. Human gut microbiota directly influences health and provides an extra means of adaptive potential to different lifestyles. To explore variation in gut microbiota and to understand how these bacteria may have co-evolved with humans, here we present the first investigation of phylogenetic diversity and metabolite production of the gut microbiota from a community of human hunter-gatherers, the Hadza of Tanzania. We show that the Hadza have higher levels of microbial richness and biodiversity than Italian urban controls. Further comparisons with two rural farming African groups illustrate other features unique to Hadza that can be linked to a foraging lifestyle. These include absence of *Bifidobacterium* and differences in microbial composition between the sexes that probably reflect sexual division of labor. Furthermore, enrichment in *Prevotella*, *Treponema*, and unclassified Bacteroidetes, as well as a peculiar arrangement of Clostridiales taxa, may enhance the Hadza's ability to digest and extract valuable nutrition from fibrous plant foods.

Samenvatting

Koken speelde een belangrijke rol in de evolutie van de mens, doordat het de verteerbaarheid van voedingsmiddelen vergroot en de nutriëntendichtheid ervan kan verhogen. We begrijpen echter nog steeds weinig van de specifieke context die aanleiding gaf tot het verhitten van voedsel. Een manier van voedselbereiding die door moderne mensen gebruikt wordt, is het roosteren van voedsel, direct in de vlammen van een open vuur, gedurende kortere of langere tijd, wellicht een goede analogie voor het kookgedrag van vroegere mensachtigen. Deze studie presenteert de eerste experimenteel verkregen data met betrekking tot patronen van zetmeel gelatinisering in USO's (Underground Storage Organs: plantenwortels en knollen) en zetmeelrijke vruchten als gevolg van kort roosteren boven een open vuur. Er werd gebruik gemaakt van tellingen met behulp van microscopie en een semi-kwantitatieve fotometrische strategie om de kinetische processen van gelatinisering te observeren. Kort (vijf minuten lang) roosteren boven een open vuur blijkt te resulteren in lage niveaus van zetmeel gelatinisering van het parenchymatisch weefsel, niveaus die ook na langer roosteren, tot meer dan tien minuten, laag blijven. Bij 15 en 20 minuten roosteren blijkt het merendeel van het zetmeel in elk specimen voldoende gegelatiniseerd om alpha-amylase activiteit mogelijk te maken. Deze bevindingen suggereren dat kort roosteren (tot tien minuten) een consument geen voordeel zal opleveren in termen van veranderingen in de zetmeelstructuur en toegankelijkheid voor alpha-amylase activiteit alleen.

Met deze basiskennis van de rol van kort roosteren in gelatinisering van zetmeel is vervolgens gekeken naar de nutritionele bioaccessibiliteit van rauwe en kort geroosterde wilde knollen voor de menselijke consument. Bioaccessibiliteit is een nuttige maat voor het beoordelen van de biologische waarde van een bepaalde nutriënt in voedsel, met name in knollen en dergelijke. De wilde knollen die door de Hadza jagers-verzamelaars in Tanzania gegeten worden zijn interessant omdat ze niet giftig zijn, rauw of kort geroosterd geconsumeerd worden en nogal wat fysieke "barrières" met zich meebrengen voor de consument. In dit onderzoek probeerden we inzicht te krijgen in de biologische waarde van de Hadza knollen door het meten van de opname van glucose door middel van in-vitro digestie. Wij kwantificeerden verteerbaarheid op basis van data van 24 experimenten op vier soorten door de Hadza gebruikte knollen, gebruikmakend van een dynamisch in-vitro model dat digestie in maag en dunne darm nabootst. Analyse van de glucose in de "input"-maaltijd en het "output"-dialysaat leverde de toegankelijke glucose fractie op. We analyseerden ook eiwit, vitaminen en mineralen-samenstelling van hele knollen en gefractioneerde maaltijden. Bioaccessibiliteit van glucose verschilt per soort. Als we het effect van kauwen constant houden, blijkt kort roosteren een verwaarloosbaar effect te hebben, maar de grote variatie binnen één soort beperkt de draagwijdte van deze conclusies. In het algemeen blijken de door de Hadza geconsumeerde knollen zeer resistent tegen digestie, met gemiddeld een- tot twee-derde van de glucose opgenomen. De fractie geabsorbeerde versus input

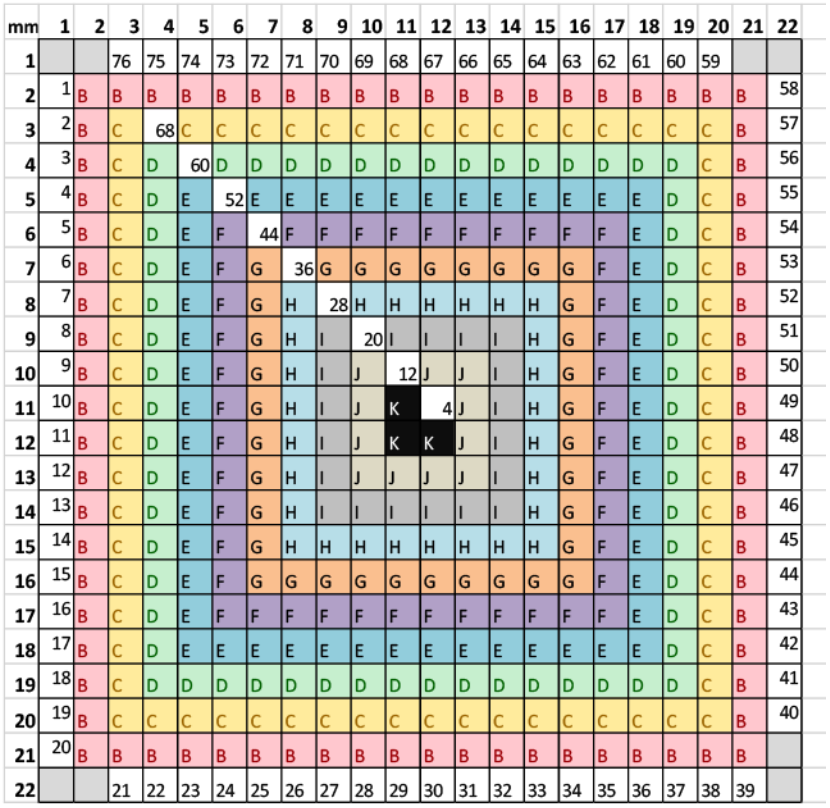
glucose correleerde negatief met de totale glucose concentratie (in vrije en gepolymeriseerde staat) in de knollen. Roosteren levert wellicht andere voordelen op, zoals gemakkelijker pellen en kauwen om het eetbare parenchymweefsel te extraheren. Een belangrijke factor in glucosevoorziening is de kwaliteit van de knol, waar de kundigheid van de individuele verzamelaar een belangrijke rol in speelt. Andere nutriënten-analyses leverden onverwacht hoge waarden voor eiwitten, ijzer en jodium, hetgeen aangeeft dat knollen wellicht niet alleen vanwege hun calorieën waardevolle voedselbronnen zijn.

Voor verdere evaluatie van de efficiëntie waarmee menselijke consumenten vezelrijk plantaardig voedsel verteren hebben we ons gericht op de microbiota van het maag-darmkanaal, ter verkenning van compositie en functie als een wezenlijke factor in de menselijke spijsvertering. De microbiota van de menselijke darm beïnvloeden rechtstreeks de menselijke gezondheid en vormen een extra aanpassingspotentieel aan verschillende leefwijzen. Als een verkennende studie van de variatie van microbiota in het maag-darmkanaal en om te begrijpen hoe de co-evolutie van deze bacteriën met mensen verlopen kan zijn, presenteren we hier het eerste onderzoek van de fylogenetische diversiteit en de metabolieten-productie van de maag-darm microbiota van een groep jagers-verzamelaars, de bovengenoemde Hadza uit Tanzania. De Hadza blijken gekenmerkt door een hogere microbiële rijkdom en biodiversiteit dan een Italiaanse stedelijke controlegroep. Verdere vergelijkingen met twee agrarische rurale groepen in Afrika illustreren andere kenmerken die uniek zijn voor de Hadza en aan een leefwijze als jager-verzamelaar gekoppeld kunnen worden: o.a. afwezigheid van *Bifidobaterium* en geslachtsgebonden verschillen in microbiële compositie die waarschijnlijk samenhangen met een geslachtsgebonden arbeidsverdeling. Bovendien zou de verrijking in *Prevotella*, *Treponema* en ongeclassificeerde Bacteroidetes soorten alsmede een ongebruikelijke samenstelling van Clostridiales taxa wellicht ook het vermogen van de Hadza kunnen vergroten om vezelrijk plantenvoedsel te verteren en er waardevolle voedingsstoffen uit te halen.

Appendix A

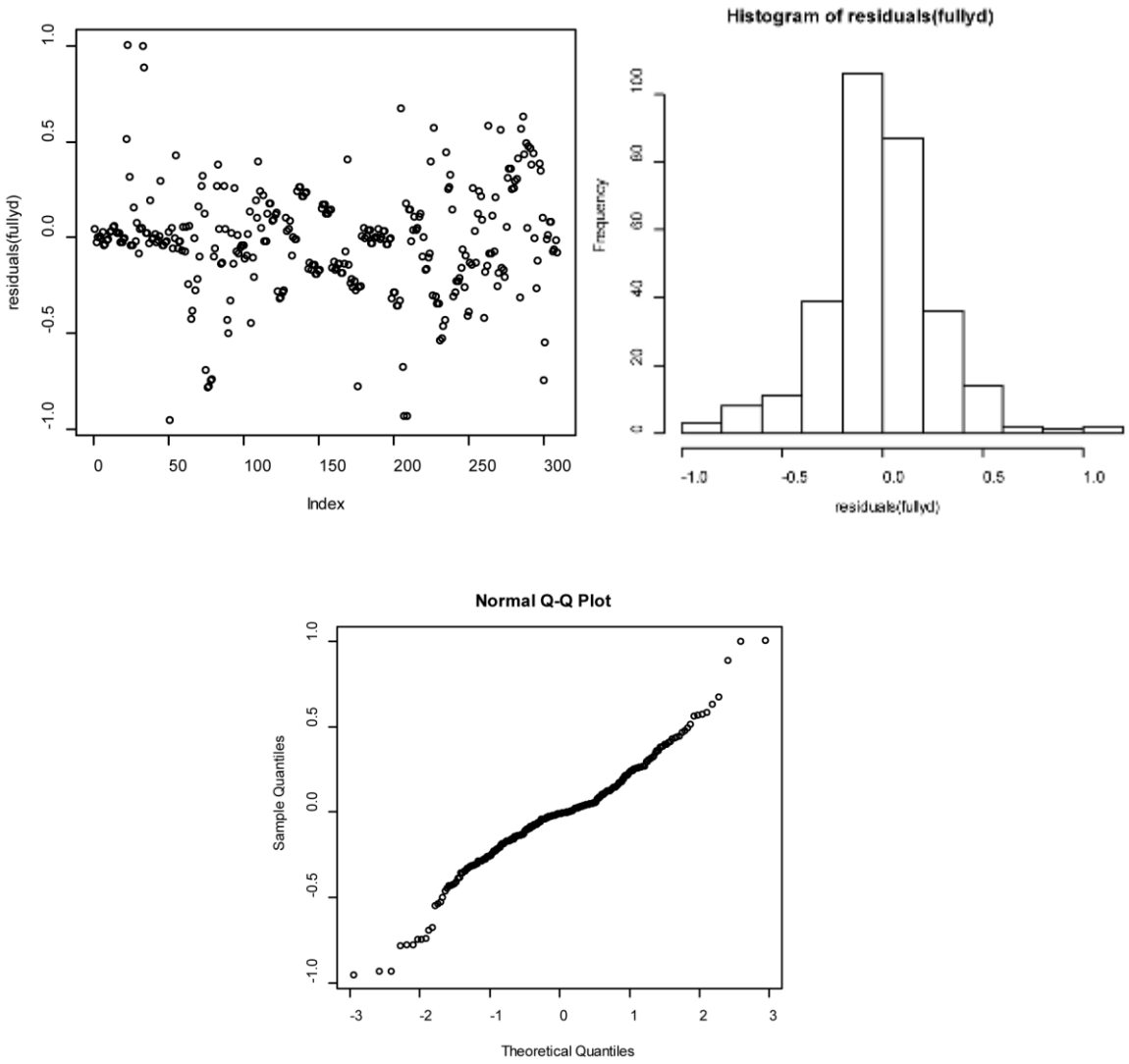
Chapter 2 supplementary information

Supplementary Figures



mm	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22
1			76	75	74	73	72	71	70	69	68	67	66	65	64	63	62	61	60	59		
2	1	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	58
3	2	B	C	68	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	57
4	3	B	C	D	60	D	D	D	D	D	D	D	D	D	D	D	D	D	D	C	B	56
5	4	B	C	D	E	52	E	E	E	E	E	E	E	E	E	E	E	E	D	C	B	55
6	5	B	C	D	E	F	44	F	F	F	F	F	F	F	F	F	F	E	D	C	B	54
7	6	B	C	D	E	F	G	36	G	G	G	G	G	G	G	G	F	E	D	C	B	53
8	7	B	C	D	E	F	G	H	28	H	H	H	H	H	H	G	F	E	D	C	B	52
9	8	B	C	D	E	F	G	H	I	20	I	I	I	I	H	G	F	E	D	C	B	51
10	9	B	C	D	E	F	G	H	I	J	12	J	J	I	H	G	F	E	D	C	B	50
11	10	B	C	D	E	F	G	H	I	J	K	4	J	I	H	G	F	E	D	C	B	49
12	11	B	C	D	E	F	G	H	I	J	K	K	J	I	H	G	F	E	D	C	B	48
13	12	B	C	D	E	F	G	H	I	J	J	J	J	I	H	G	F	E	D	C	B	47
14	13	B	C	D	E	F	G	H	I	I	I	I	I	I	H	G	F	E	D	C	B	46
15	14	B	C	D	E	F	G	H	H	H	H	H	H	H	H	G	F	E	D	C	B	45
16	15	B	C	D	E	F	G	G	G	G	G	G	G	G	G	G	F	E	D	C	B	44
17	16	B	C	D	E	F	F	F	F	F	F	F	F	F	F	F	F	E	D	C	B	43
18	17	B	C	D	E	E	E	E	E	E	E	E	E	E	E	E	E	E	D	C	B	42
19	18	B	C	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	C	B	41
20	19	B	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	C	B	40
21	20	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	B	
22			21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	

Supplementary Figure 1. Schematic showing the 22 mm cover slip area and the ten concentric squares that represent the area analyzed. The outer 2 millimeter edge was excluded. The ten concentric squares of the cover-slip area were systematically analyzed using a random coordinate generator to select a one millimeter square area for observation per concentric square. This was repeated 10 times to obtain one observation per concentric square across the whole slide area, totaling 10 observations in all per slide.



Supplementary Figure 2. Residual plots do not reveal deviations from homoscedasticity, and a histogram and Q-Q plot of residuals do not indicate any violation of normality for the generalized linear mixed effects model.

Appendix B

Chapter 3 supplementary information

Supplementary Tables

Supplementary Table 1 Summary of specimen properties and run assignment. [1]

Run 1 | Run 2 | Run 3 | Run 4 | Raw pool only | Cooked pool only

	SPECIMEN	EDIBLE	FIBER	PEEL	STATE	RUN
V. frutescens		%	%	%		
	1	31	24	45	cook	
	2	28	30	43	raw	
	3	31	32	37	cook	
	4	17	16	67	raw	
	5	23	18	59	cook	
	7	22	33	46	cook	
	8	34	14	52	cook	
	9	22	12	66	raw	
	10	19	23	57	raw	
	11	21	21	59	cook	
	12	43	31	26	raw	
E. entennulifa						
	1	50	8	42	cook	
	2	43	14	43	cook	
	3	45	16	40	cook	
	4	37	7	56	cook	
	5	51	13	36	raw	
	6	58	1	41	cook	
	7	65	1	34	raw	
	8	66	0	34	cook	
	9	32	5	64	raw	
	10	47	13	40	raw	
I. transvaalensis [2]						
	1	80	0	20	raw	
	2	76	0	24	raw	
	3	76	0	24	raw	

	4	75	0	25	raw	
	5	77	0	23	cook	
	6	76	0	24	raw	
	7	76	0	24	cook	
	8	74	0	26	cook	
	9	65	0	35	raw	
	10	76	0	24	raw	
	11	72	0	28	raw	
	12	86	0	14	cook	
	13	69	0	31	cook	
	14	66	0	34	raw	
	15	62	0	38	raw	
	16	75	0	25	cook	
	17	66	0	34	raw	
	18	80	0	20	cook	
	19	74	0	26	cook	
	20	70	0	30	raw	
	22	80	0	20	raw	
	23	74	0	26	cook	
	24	71	0	29	raw	
	25	78	0	22	cook	
	26	66	0	34	raw	
	27	70	0	30	raw	
	28	62	0	38	raw	
	29	80	0	20	cook	
	30	53	0	47	raw	
	31	72	0	28	raw	
	32	74	0	26	raw	
V. pseudolablab						
	1	56	23	21	raw	
	2	48	10	42	cook	
	3	46	17	37	raw	
	4	43	11	46	cook	
	5	46	14	40	cook	
	6	54	9	36	cook	
	7	69	11	20	raw	
	8	55	7	38	cook	
	9	61	16	23	raw	
	10	52	0	48	cook	
	11	63	14	23	raw	
	12	30	31	40	raw	
	13	58	18	24	raw	
	14	45	14	41	cook	

[1] There were six total runs per species: 2 raw, 2 cooked, 1 pooled raw, and 1 one pooled cooked. For our purposes, run numbers are effective sample numbers (n), and are referred to in lieu of specimen number in the rest of the paper for simplicity.

[2] All *I. transvaalensis* runs constitute homogenization of all tubers except for runs 3 and 4 (delineated).

Supplementary Table 2 Comparison summary of Hadza tuber nutritional values across three studies: this study (**a**), Schoeninger et al., 2001 (**b**), and Crittenden, 2009 (**c**).

a.

this study			**g kg^{-1} edible wet weight**	
specimen	**taxonomy**	**state**	**glucose**	**protein**
//ekwa 1	*V. frutescens*	Cook	11.01	21.71
//ekwa 2	*V. frutescens*	Cook	29.67	22.93
//ekwa 3	*V. frutescens*	Raw	27.36	9.42
//ekwa 4	*V. frutescens*	Raw	51.70	31.81
//ekwa 5	*V. frutescens*	Cook	17.42	45.49
//ekwa 6	*V. frutescens*	Raw	19.31	22.42
mak'alitako 1	*E. entennulifa*	Cook	46.40	29.57
mak'alitako 2	*E. entennulifa*	Cook	5.51	1.69
mak'alitako 3	*E. entennulifa*	Raw	6.14	7.67
mak'alitako 4	*E. entennulifa*	Raw	2.86	3.20
mak'alitako 5	*E. entennulifa*	Cook	9.06	18.01
mak'alitako 6	*E. entennulifa*	Raw	5.75	11.67
panjuko 1	*I. transvaalensis*	Cook	61.25	6.66
panjuko 2	*I. transvaalensis*	Cook	114.86	33.00
panjuko 3	*I. transvaalensis*	Raw	86.35	18.29
panjuko 4	*I. transvaalensis*	Raw	140.46	14.51
panjuko 5	*I. transvaalensis*	Cook	45.23	27.52
panjuko 6	*I. transvaalensis*	Raw	22.52	21.54
shumuko 1	*V. pseudolablab*	Cook	17.10	17.48
shumuko 2	*V. pseudolablab*	Cook	14.68	9.68
shumuko 3	*V. pseudolablab*	Raw	12.46	8.34
shumuko 4	*V. pseudolablab*	Raw	18.99	12.71
shumuko 5	*V. pseudolablab*	Cook	6.47	35.13
shumuko 6	*V. pseudolablab*	Raw	14.13	42.78

b.

Schoeninger et al., 2001			g 100g^{-1} edible dry weight		g kg^{-1} edible wet weight	
specimen	**taxonomy**	**% moisture**	**carbohy-drate**	**protein**	**carbohy-drate**	**protein**
//ekwa (avg)	*V. frutescens*	78	32.20	4.50	71.81	10.04
mak'alitako	*E. entennulifa*	80	42.80	6.90	86.03	13.87
panjuko	*I. transvaalensis*	86	72.20	2.30	101.80	3.24
//ekwa 1	*V. frutescens*	78	61.80	4.20	137.81	9.37
//ekwa 2	*V. frutescens*	83	32.00	4.40	53.12	7.30
//ekwa 3	*V. frutescens*	75	37.80	3.30	94.12	8.22
//ekwa 4	*V. frutescens*	77	20.10	4.20	47.24	9.87
//ekwa 5a	*V. frutescens*	76	8.20	5.60	19.84	13.55
//ekwa 5b	*V. frutescens*	76	9.90	6.80	23.96	16.46

c.

Crittenden, 2009				g 100g^{-1} peeled dry weight		g kg^{-1} peeled wet weight	
specimen	**taxonomy**	**state**	**% moisture**	**carbohy-drate**	**protein**	**carbohy-drate**	**protein**
//ekwa	*V. frutescens*	Raw	69	75.40	7.90	231.48	24.25
//ekwa	*V. frutescens*	Raw	68	88.80	4.80	285.94	15.46
//ekwa	*V. frutescens*	Raw	69	80.80	9.00	247.25	27.54
//ekwa	*V. frutescens*	Raw	70	63.20	7.10	190.86	21.44
//ekwa	*V. frutescens*	Cook	68	68.30	7.60	220.61	24.55
//ekwa	*V. frutescens*	Cook	69	82.80	10.40	259.16	32.55
//ekwa	*V. frutescens*	Cook	69	60.50	5.90	185.74	18.11
//ekwa	*V. frutescens*	Cook	69	94.70	5.10	297.36	16.01
shumuko	*V. pseudolablab*	Raw	89	62.90	3.00	71.08	3.39
shumuko	*V. pseudolablab*	Cook	91	63.10	3.60	54.27	3.10
makalita	*R. comosa*	Raw	79	60.20	5.40	124.61	11.18
makalita	*R. comosa*	Raw	79	37.40	5.90	78.17	12.33
makalita	*R. comosa*	Cook	79	54.30	5.10	112.40	10.56
matukwaya	*C. surantiaca*	Raw	87	92.60	12.40	125.01	16.74
shaehako	*V. macrorhyncha*	Raw	86	69.00	10.40	98.67	14.87

MICRONUTRIENT ASSAY METHODS

According to ISO-(N)EN-AOAC method

Analysis method

SOP-code

Technique

Description

Vitamin B1

TRIS/VIT/010

HPLC-Fluorescence

Samples are extracted in duplicate in an autoclave at 119°C for 15 minutes, using a 0.1 mol/l hydrochloric acid solution containing sodium ascorbate. The resulting extract is adjusted to pH 4.7 using an acetate buffer, and filtrated. Part of the filtrate is incubated for at least 2 hours at 37°C with phosphatase(acid), and subsequently analyzed for thiamin using reversed phase UPLC with post-column derivatisation and fluorescence detection.

Vitamin B2

TRIS/VIT/012

HPLC-Fluorescence

Samples are extracted in duplicate in an autoclave at 119°C for 15 minutes, using a 0.1 mol/l hydrochloric acid solution containing sodium ascorbate. The resulting extract is adjusted to pH 4.7 using an acetate buffer, and filtrated. Part of the filtrate is incubated for at least 2 hours at 37°C with phosphatase (acid), and subsequently analyzed for riboflavin using reversed phase UPLC with fluorescence detection.

Vitamin B3

TRIS/VIT/209

LC-MS/MS

Samples are extracted in duplicate in an autoclave at 119°C for 15 minutes, using a 0.1 mol/l hydrochloric

acid solution containing sodium ascorbate. The resulting extract is adjusted to pH 4.7 using an acetate buffer, and filtrated. Part of the filtrate is subsequently analyzed for nicotinamide and nicotinic acid using reversed phase UPLC with MS/MS detection.

Pantothenic Acid

TRIS/VIT/035

LC-MS/MS

Samples are analyzed in duplicate. First, samples are dissolved in pantothenic acid and enzymatically liberated in an extraction buffer containing papain and taka-diastase. Subsequently, the extraction process is continued in an autoclave at 119°C for 15 minutes. The resulting extract is filtrated and further diluted for analysis by reversed phase chromatography with tandem mass-spectrometry. Isotopically labeled pantothenic acid is used as internal standard.

Vitamin B6

TRIS/VIT/015

HPLC-Fluorescence

Samples are extracted in duplicate for 30 minutes, using a 5% trichloroacetic acid solution. A part of the extract is adjusted to pH 4.7 using an acetate buffer, and incubated for at least 12 hours at 37°C with phosphatase (acid), and subsequently analyzed for pyridoxal, pyridoxol and pyridoxamin using reversed phase UPLC with fluorescence detection.

Biotin

TRIS/VIT/206

LC-MS/MS

Samples are analyzed in duplicate in a series with a Quality Control sample (Infant Nutrition). Biotin is extracted using sulphuric acid (pH 4.5) at 119°C during 30 minutes. After filtration and dilution, biotin is determined using UPLC-MS/MS with isotopically labeled biotin (ring 6,6 d2) as internal standard.

Total Folate

TRIS/VIT/107

Microbiology

A liquid sample extract is added to a nutrient medium. This nutrient medium is free of the compound of interest but contains all the ingredients for the bacteria to grow. After grow of the bacteria in the sample extract grow is compared with grow of bacteria in a standard solution with a known concentration.

Vitamin B12

TRIS/VIT/065

RIA

Samples are extracted in duplicate in an autoclave at 119°C for 15 minutes, using an 0.1 M acetate buffer pH 4.6 containing 50 mg/l potassium cyanide. This procedure converts all cobalamines to the cyanocobalamine vitamer. The filtrated extract is diluted to appropriate concentrations for the competitive binding assay, and incubated for 1 hour with 57Co-cyanocobalamine, intrinsic factor, and a glutaminic acid buffer pH 4.1. To test for parallelism, four serial dilutions are being used for each of the duplicates. Subsequently, a cold activated carbon solution is added, and after vigorous mixing the solution is left for 10 minutes. The slurry is centrifuged, and the supernatant analyzed using a Wallac Wizard 1470 gamma-counter.

Vitamin C

TRIS/VIT/014

HPLC-Fluorescence

Vitamin C is extracted from the sample using trichloro acetic acid. Ascorbic acid is oxidized into dehydro-ascorbic acid which is subsequently condensed into its quinoxaline derivative using 1,2-diamino benzene. This derivative is measured using UPLC with fluorescence detection.

Vitamin A

TRIS/VIT/113

HPLC-Fluorescence

Samples are saponified in duplicate under reflux conditions for 30 minutes, using a 1.5 mol/l potassium hydroxide ethanolic solution supplemented with sodium ascorbate and disodium sulfide. After cooling at room

temperature, the mixture is extracted using diisopropyl ether, which is subsequently washed with aqueous solutions. The resulting extract is analyzed for all-trans retinol and 13-cis retinol using straight phase HPLC with fluorescence detection.

Vitamin D

TRIS/VIT/051

HPLC-DAD

Samples, to which vitamin D2 is added as internal standard, are saponified in duplicate under reflux conditions for 30 minutes, using a 1.5 mol/l potassium hydroxide ethanolic solution supplemented with sodium ascorbate and disodium sulfide. After cooling at room temperature, the mixture is extracted using diisopropyl ether, which is subsequently washed with aqueous solutions. The resulting extract is partly evaporated and redissolved in methanol. Subsequently, the vitamin D in the eluate is further purified by fractionation with straight phase chromatography. Finally, the resulting extract is analyzed by reversed phase chromatography with diode array detection, quantifying at 265 nm.

Vitamin E

TRIS/VIT/113

HPLC-Fluorescence

Samples are saponified in duplicate under reflux conditions for 30 minutes, using a 1.5 mol/l potassium hydroxide ethanolic solution supplemented with sodium ascorbate and disodium sulfide. After cooling at room temperature, the mixture is extracted using diisopropyl ether, which is subsequently washed with aqueous solutions. The resulting extract is analyzed for α-tocopherol using straight phase HPLC with fluorescence detection.

Vitamin K1

TRIS/VIT/021

HPLC-Fluorescence

Samples are dissolved in duplicate as aqueous solution, after which they are enzymatically digested using pancreatin. Subsequently, vitamin K1 is extracted using n-pentane. The extract is purified using a normal

phase SPE procedure, after which vitamin K1 is analyzed using reversed phase HPLC with post-column Zn-reduction and fluorescence detection.

Carotenoids

TRIS/VIT/058

HPLC-DAD

Samples are saponified in duplicate under reflux conditions for 30 minutes, using a 1.5 mol/l potassium hydroxide ethanolic solution supplemented with sodium ascorbate and disodium sulfide. After cooling at room temperature, the mixture is extracted using diisopropyl ether, which is subsequently washed with aqueous solutions. The resulting extract is analyzed for beta-carotene using straight phase HPLC with diode array detection.

Minerals / Trace Elements

TRIS/LSP/055

ICP-MS

For the determination of the minerals calcium, sodium, phosphorus, magnesium, sulfur and potassium and the elements iron, zinc, copper, manganese, cobalt, chromium, molybdenum and selenium, a test portion of the sample is digested with nitric acid using a digestion block. The concentration of the elements is determined by inductively coupled plasma mass spectrometry (ICP-MS) using external calibration.

Ash

TRIS/LNC/167

Incineration at 550 °C Samples are dry ashed and the residue is weighed.

Chloride and Iodide

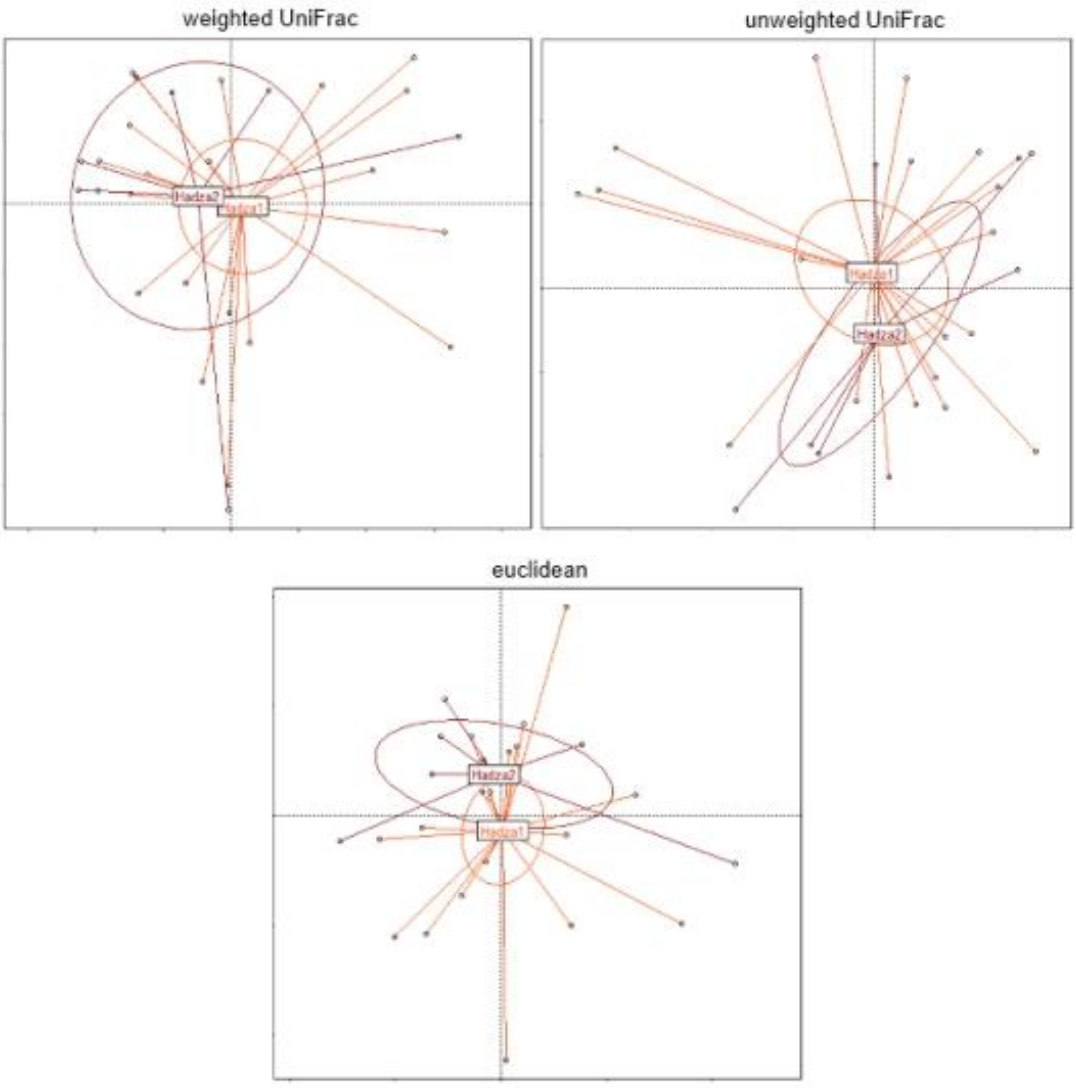
TRIS/LSP/055

ICP-MSFor the determination of Iodine and Chloride, a test portion of the sample is extracted with diluted ammonia. The concentration of Iodine and Chloride is determined by inductively coupled plasma atomic mass spectrometry (ICP-MS) using an internal standard. Quantification is performed with external calibration.

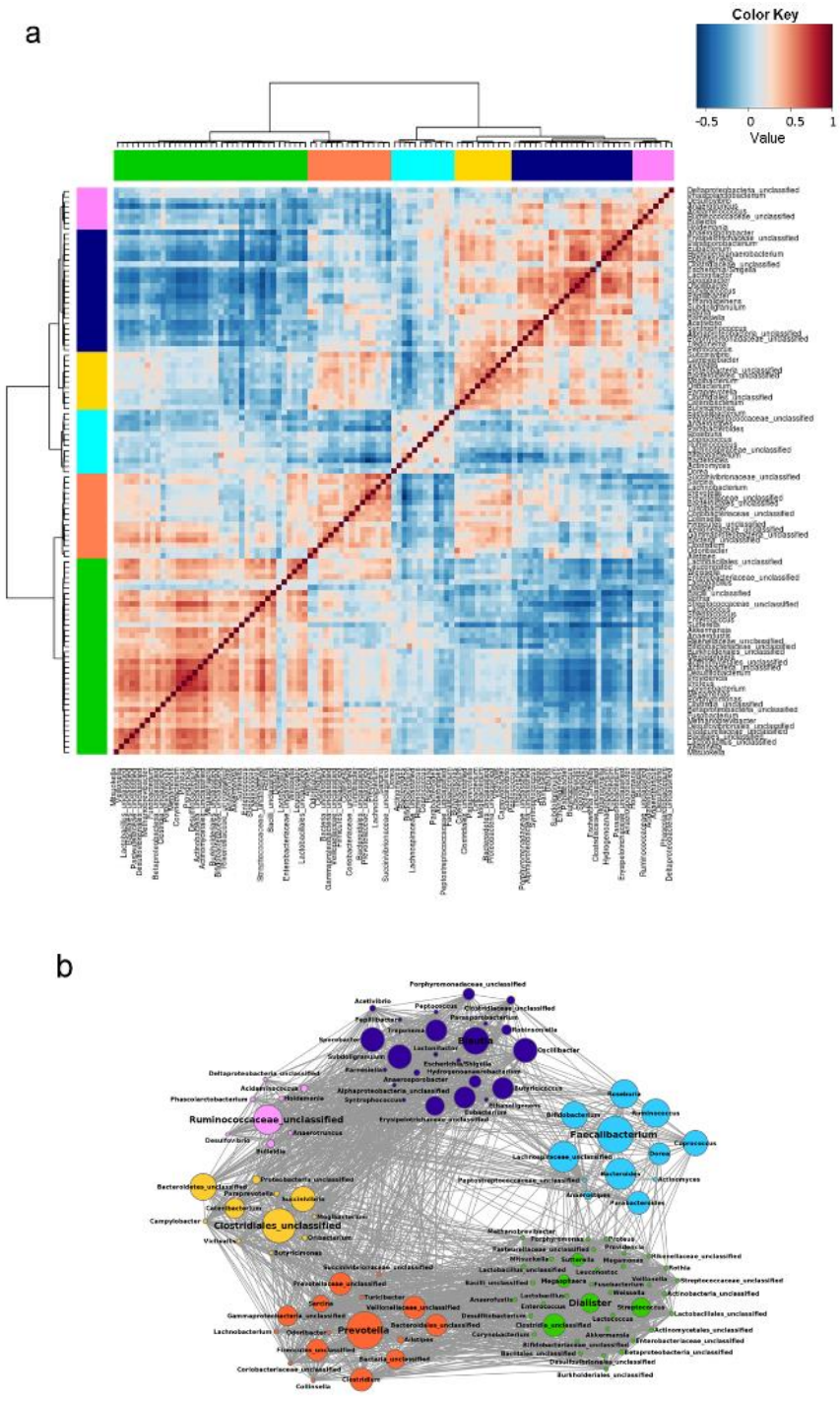
Appendix C

Chapter 4 supplementary information

Supplementary Figures



Supplementary Figure 1. Phylogenetic diversity within the Hadza gut microbial community. PCoA plots based on weighted and unweighted UniFrac distances and Euclidean distances of the genus level relative abundances are shown. Subjects are grouped according to camp provenience, Dedauko (Hadza 1) and Sengele (Hadza 2). None of the diversity metrics show significant separation of the Hadza gut microbiota by camp location.



Supplementary Figure 2. Assignments of bacterial co-abundance groups (CAGs). CAG assignment relied on a heat plot (**a**) showing Kendall correlations between genera clustered by the Spearman correlation coefficient and Ward linkage hierarchical clustering. Colors are indicative of the six identified CAGs. (**b**) Wiggum plot correlations between the six CAGs identified, circle size is representative of the genus abundance and the connections between nodes represent positive and significant Kendall correlations between genera (FDR < 0.05).

Supplementary Tables

Supplementary Table 1. qPCR quantification of *Bifidobacterium* in stool samples. For each subject, the *Bifidobacterium* 16S rRNA copy number μg^{-1} of fecal DNA is reported. BDL: Below detection limit (100 16S rRNA gene copies ng^{-1} faecal DNA)

Hadza		**Italians**	
Subject	16S rRNA gene copies ng^{-1} faecal DNA	Subject	16S rRNA gene copies ng^{-1} faecal DNA
H1	BDL	IT1	6.55E+05
H2	BDL	IT2	1.41E+06
H3	BDL	IT3	1.39E+06
H4	BDL	IT4	1.38E+04
H5	BDL	IT5	1.19E+06
H6	BDL	IT6	2.05E+05
H7	BDL	IT7	1.22E+05
H8	BDL	IT8	1.88E+05
H9	BDL	IT9	1.50E+05
H10	BDL	IT10	6.55E+04
H11	BDL	IT11	7.53E+04
H12	BDL	IT12	5.48E+05
H13	BDL	IT13	2.36E+05
H14	BDL	IT14	1.56E+05
H15	BDL	IT15	1.82E+05
H16	BDL	IT16	1.30E+05
H17	BDL		
H18	BDL		
H19	BDL		
H20	BDL		
H21	BDL		
H22	BDL		
H23	BDL		
H24	BDL		
H25	BDL		
H26	BDL		
H27	BDL		
H28	BDL		

Supplementary Table 2. Absolute and percentage based values of SCFAs in Hadza and Italian samples.

a.

HADZA	Acetic	Propionic	Butyric	Valeric
	(umol g^{-1})	(umol g^{-1})	(umol g^{-1})	(umol g^{-1})
H16	1.4	0.27	0.12	0.025
H1	2.3	0.71	0.33	0.063
H14	2.7	1.06	0.79	0.104
H2	2.3	0.67	0.69	0.119
H13	4.7	1.71	1.26	0.126
H11	4.9	1.81	1.19	0.141
H12	4.3	1.90	1.65	0.181
H10	4.8	1.38	0.71	0.108
H15	2.4	0.98	0.77	0.114
H6	3.5	1.46	0.80	0.070
H5	5.2	0.96	0.32	0.091
H4	1.8	0.52	0.33	0.096
H8	4.5	1.40	0.57	0.075
H7	4.0	1.55	0.70	0.098
H3	4.8	1.01	0.67	0.129
H9	4.3	0.98	0.44	0.063
H22	1.9	0.39	0.27	0.043
H19	2.2	0.50	0.41	0.054
H17	4.5	2.00	1.79	0.177
H21	3.4	0.71	0.48	0.063
H20	3.4	1.09	0.23	0.061
H18	2.2	0.56	0.36	0.058
H23	1.5	0.40	0.34	0.040
H25	2.2	0.87	0.67	0.082
H24	3.9	1.85	0.63	0.075
H26	0.8	0.17	0.13	0.027
H28	0.8	0.17	0.06	0.021
H27	0.8	0.17	0.11	0.021

b.

ITALIANS	Acetic	Propionic	Butyric	Valeric
	(umol g^{-1})	(umol g^{-1})	(umol g^{-1})	(umol g^{-1})
IT11	1.1	0.43	0.20	not determined
IT1	9.5	2.20	2.64	0.168
IT6	2.2	0.41	0.65	0.045
IT3	3.1	0.75	1.52	0.075
IT5	1.7	0.39	0.30	0.083
IT2	1.1	0.10	0.13	0.039
IT4	0.7	0.17	0.18	0.030
IT12	1.7	0.25	0.46	0.021
IT8	1.4	0.40	0.36	0.038
IT14	0.4	0.06	0.05	0.010
IT10	1.3	0.34	0.35	0.048
IT7	3.5	0.71	1.20	0.056
IT16	1.8	0.58	0.20	0.003
IT15	2.9	0.68	0.81	0.076
IT9	0.7	0.17	0.22	0.024
IT13	5.2	0.91	1.53	0.068

HADZA	TOTAL (µmol g^{-1})	Acetic	Propionic	Butyric	Valeric
		%	%	%	%
H16	1.83	77.23	14.56	6.82	1.39
1H	3.42	67.89	20.76	9.53	1.83
H14	4.69	58.34	22.55	16.90	2.21
H2	3.77	60.71	17.80	18.34	3.15
H13	7.80	60.23	21.96	16.19	1.62
H11	8.04	60.95	22.51	14.78	1.76
H12	8.07	53.86	23.51	20.39	2.24
H10	6.96	68.38	19.80	10.25	1.56
H15	4.31	56.67	22.81	17.89	2.64
H6	5.84	60.17	24.92	13.72	1.19
H5	6.53	79.01	14.74	4.85	1.40
H4	2.74	65.63	18.91	11.97	3.50
H8	6.57	68.95	21.24	8.67	1.14
H7	6.34	62.93	24.43	11.09	1.55
H3	6.60	72.57	15.37	10.10	1.96
H9	5.76	74.32	17.03	7.56	1.09
H22	2.63	73.07	15.01	10.27	1.65
H19	3.15	69.40	15.96	12.92	1.71
H17	8.42	52.87	23.75	21.27	2.11
H21	4.66	73.02	15.27	10.36	1.35
H20	4.81	71.35	22.55	4.83	1.27
H18	3.18	69.06	17.73	11.38	1.83
H23	2.24	65.24	17.80	15.20	1.76
H25	3.85	57.66	22.70	17.52	2.13
H24	6.43	60.27	28.81	9.76	1.16
H26	1.12	70.57	15.22	11.79	2.42
H28	1.07	76.45	16.08	5.45	2.01
H27	1.06	71.64	15.62	10.75	1.99

ITALIANS	TOTAL (µmol g^{-1})	Acetic	Propionic	Butyric	Valeric
		%	%	%	%
IT11	1.75	63.59	24.79	11.62	not determined
IT1	14.55	65.55	15.13	18.16	1.16
IT6	3.27	66.11	12.68	19.85	1.36
IT3	5.43	56.81	13.89	27.93	1.37
IT5	2.50	69.04	15.49	12.15	3.33
IT2	1.35	79.97	7.52	9.62	2.89
IT4	1.09	65.64	15.26	16.36	2.74
IT12	2.46	70.62	10.00	18.53	0.85
IT8	2.23	63.95	18.10	16.24	1.70
IT14	0.49	73.59	13.20	11.13	2.07
IT10	2.08	64.58	16.45	16.66	2.31
IT7	5.52	64.22	12.90	21.86	1.03
IT16	2.58	69.77	22.38	7.72	0.13
IT15	4.49	65.13	15.18	18.00	1.68
IT9	1.09	62.53	15.34	19.94	2.19
IT13	7.72	67.44	11.84	19.84	0.88

Supplementary Table 3. Correlations between SCFA levels and genera relative abundance. Correlations were carried out using Kendall correlation tests in R. Bacterial groups are highlighted and marked by double asterisk (**) if they are correlated, positively or negatively, with SCFA level and if they are present at 5% or more of total abundance in at least one of the two populations. Results reported in the table show that SCFAs are correspondingly enriched and depleted with certain bacterial groups that are differently represented in the Hadza and Italian GM. Propionate levels associated most strongly with bacterial genera that are enriched in Hadza and depleted in Italians whereas butyrate levels were positively associated with bacteria enriched in Italians and depleted in Hadza. These correlations inform us of the functional traits established in each community GM and support the finding that the Hadza GM community is structured around production of propionate whereas the Italian GM is better suited to butyrate production.

Acetate

taxa	tau	P	ITA	±SEM	HADZA	±SEM	P value (Kendall tau rank correlation coefficient)
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Paludibacter	-0.2699	3.55E-02	0	0	0.02	0.01	7.00E-03
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Flavisolibacter	0.2781	3.96E-02	0	0	0	0	2.90E-01
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Sarcina	0.3957	1.79E-03	0	0	0.08	0.04	3.40E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XI;Anaerococcus	0.3038	2.28E-02	0.01	0.01	0	0	6.70E-01
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XII;Acidaminobacter	0.2986	1.57E-02	0.03	0.02	0.37	0.22	1.60E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIII;Anaerovorax	0.3121	2.01E-02	0	0	0	0	9.10E-01
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Robinsoniella	0.2773	1.54E-02	0.02	0	0.86	0.2	1.30E-06
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Faecalibacterium**	-0.4367	7.24E-05	18.46	2.4	11.79	1.03	1.10E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Megasphaera	0.2836	3.02E-02	0.23	0.2	0.02	0.01	8.40E-01
Bacteria;Lentisphaerae;Lentisphaeria;Victivallales;Victivallaceae;Victivallis	0.2951	1.76E-02	0.01	0.01	0.02	0.01	7.20E-03
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Buttiauxella	-0.3482	9.06E-03	0	0	0	0	6.00E-01
Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae;Mannheimia	-0.2949	2.33E-02	0	0	0.01	0.01	6.00E-01

Valerate

taxa	tau	P	ITA	±SEM	HADZA	±SEM	P value (Kendall tau rank correlation coefficient)
Bacteria;Firmicutes;Bacilli;Lactobacillales;Lactobacillaceae;Lactobacillus	-0.2708	2.84E-02	0.02	0.01	0.06	0.04	5.10E-01
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Oxobacter	0.2882	1.97E-02	0.06	0.02	0.02	0.01	6.80E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XI;Sedimentibacter	-0.2781	3.96E-02	0	0	0	0	2.30E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Other**	-0.4651	2.12E-05	6.41	0.69	3.86	0.24	5.50E-04
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Roseburia**	-0.3741	7.77E-04	7.73	1.3	3.88	0.77	7.00E-03
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Syntrophococcus	0.2991	1.32E-02	0	0	0.07	0.02	2.50E-05
Bacteria;Firmicutes;Clostridia;Clostridiales;Other;Other**	0.2632	2.00E-02	7.37	0.79	11.13	0.56	2.20E-04

Bacteria;Firmicutes;Clostridia;Clostridiales;Peptococcaceae;Peptococcus	0.2648	3.01E-02	0.06	0.04	0.04	0.01	1.10E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Acetivibrio	0.2765	1.80E-02	0.01	0	0.4	0.07	2.70E-06
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Hydrogenoanaerobacterium	0.234	4.10E-02	0.07	0.03	0.97	0.13	9.90E-06
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Oscillibacter	0.2688	1.74E-02	1.06	0.28	3.79	0.42	1.00E-05
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Dialister	-0.3434	4.67E-03	0.09	0.04	0.06	0.02	8.70E-01
Bacteria;Firmicutes;Clostridia;Other;Other;Other	0.279	1.45E-02	0.03	0.02	0.35	0.04	9.90E-07
Bacteria;Other;Other;Other;Other;Other	0.266	1.86E-02	0.22	0.05	1.82	0.16	3.80E-09
Bacteria;Proteobacteria;Deltaproteobacteria;Other;Other;Other	0.3814	2.46E-03	0	0	0.33	0.13	2.70E-02
Bacteria;Proteobacteria;Gammaproteobacteria;Pasteurellales;Pasteurellaceae;Actinobacillus	-0.239	4.52E-02	0.02	0.01	0.11	0.05	1.10E-02

Butyrate

taxa	tau	P	ITA	±SEM	HADZA	±SEM	P value (Kendall tau rank correlation coefficient)
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Bifidobacterium**	0.2869	1.56E-02	8.13	1.5	0.02	0.01	3.20E-08
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Other	0.2949	2.33E-02	0.03	0.01	0	0	2.90E-07
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Bacteroidaceae;Bacteroides**	0.3087	6.04E-03	7.14	1.95	0.21	0.04	3.40E-08
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Other;Other	-0.2603	2.14E-02	0.07	0.02	0.79	0.11	1.50E-09
Bacteria;Bacteroidetes;Other;Other;Other;Other**	-0.248	2.87E-02	0.15	0.07	6.57	0.81	8.10E-08
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Chitinophagaceae;Flavisolibacter	-0.2781	3.96E-02	0	0	0	0	2.90E-01
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter	-0.2758	3.99E-02	0	0	0	0	1.80E-01
Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Enterococcus	0.2779	3.15E-02	0.03	0.01	0	0	1.00E-04
Bacteria;Firmicutes;Clostridia;Clostridiales;Clostridiaceae;Sarcina	-0.4578	3.04E-04	0	0	0.08	0.04	3.40E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Gracilibacteraceae;Lutispora	-0.2724	3.05E-02	0.01	0.01	0.03	0.01	9.90E-03
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XII;Acidaminobacter	-0.3298	7.62E-03	0.03	0.02	0.37	0.22	1.60E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIII;Mogibacterium	-0.2813	2.05E-02	0	0	0.04	0.01	2.80E-05
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIV;Blautia**	0.3201	4.35E-03	9.45	1.25	3.46	0.4	8.60E-08
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Dorea	0.303	7.09E-03	1.35	0.2	0.41	0.06	4.60E-07
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Marvinbryantia	-0.2869	3.37E-02	0	0	0	0	3.20E-01
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Robinsoniella	-0.3408	2.91E-03	0.02	0	0.86	0.2	1.30E-06
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Anaerotruncus	-0.2329	4.03E-02	0.07	0.02	0.32	0.04	5.20E-05
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Faecalibacterium**	0.5192	1.53E-06	18.46	2.4	11.79	1.03	1.10E-02

Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Oscillibacter	-0.2688	1.74E-02	1.06	0.28	3.79	0.42	1.00E-05
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Ruminococcus**	0.2916	9.66E-03	8.55	1.82	2.05	0.29	2.70E-03
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Other	-0.2473	3.02E-02	0.02	0.01	0.83	0.17	3.00E-07
Bacteria;Firmicutes;Erysipelotrichi;Erysipelotrichales;Erysipelotrichaceae;Other	0.3428	2.18E-03	1.49	0.33	0.7	0.16	1.30E-02
Bacteria;Lentisphaerae;Lentisphaeria;Victivallales;Victivallaceae;Victivallis	-0.3378	6.60E-03	0.01	0.01	0.02	0.01	7.20E-03
Bacteria;Proteobacteria;Alphaproteobacteria;Other;Other;Other	-0.2311	4.69E-02	0.02	0.01	0.25	0.07	1.20E-04
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Methylobacteriaceae;Other	-0.2958	2.86E-02	0	0	0	0	2.90E-01
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;Sutterella	0.3051	2.14E-02	0.03	0.02	0	0	7.90E-04
Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Campylobacteraceae;Campylo-bacter	-0.3054	1.01E-02	0	0	0.24	0.07	1.20E-06
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Buttiauxella	0.2849	3.27E-02	0	0	0	0	6.00E-01
Bacteria;Proteobacteria;Other;Other;Other;Other	-0.2917	1.08E-02	0.02	0.01	0.52	0.15	1.60E-07
Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae;Treponema	-0.2831	1.37E-02	0	0	2.5	0.72	4.40E-08

Propionate

taxa	tau	P	ITA	±SEM	HADZA	±SEM	P value (Kendall tau rank correla-tion coefficient)
Bacteria;Actinobacteria;Actinobacteria;Actinomycetales;Actinomycetaceae;Actinomyces	-0.2734	2.35E-02	0.02	0.01	0.01	0	2.20E-01
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Bifidobacterium**	-0.3609	2.35E-03	8.13	1.5	0.02	0.01	3.20E-08
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Other	-0.4469	5.87E-04	0.03	0.01	0	0	2.90E-07
Bacteria;Actinobacteria;Actinobacteria;Coriobacteriales;Coriobacteriaceae;Asaccharobacter	-0.3224	1.44E-02	0.01	0	0	0	4.40E-04
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Marinilabiaceae;Anaerophaga	0.2323	4.48E-02	0	0	0.61	0.1	4.20E-08
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Other	0.3062	7.48E-03	0.03	0.02	0.91	0.28	1.50E-06
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae;Paludibacter	0.4205	1.05E-03	0	0	0.02	0.01	7.00E-03
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Prevotella**	0.41	3.05E-04	0.38	0.26	6.23	1.11	5.20E-07
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae;Xylanibacter	0.288	2.98E-02	0	0	0	0	7.50E-02
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;Alistipes	-0.3626	3.99E-03	0.92	0.22	0	0	2.30E-08
Bacteria;Bacteroidetes;Bacteroidia;Bacteroidales;Rikenellaceae;Other	-0.2758	3.99E-02	0.03	0.02	0	0	7.60E-03
Bacteria;Bacteroidetes;Sphingobacteria;Sphingobacteriales;Other;Other	0.2942	1.22E-02	0	0	0.6	0.11	7.10E-07
Bacteria;Firmicutes;Bacilli;Lactobacillales;Enterococcaceae;Enterococcus	-0.5012	1.05E-04	0.03	0.01	0	0	1.00E-04
Bacteria;Firmicutes;Bacilli;Lactobacillales;Streptococcaceae;Lactococcus	0.2758	3.99E-02	0	0	0	0	1.80E-01
Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XI;Finegoldia	-0.3339	1.29E-02	0	0	0	0	2.30E-02

Bacteria;Firmicutes;Clostridia;Clostridiales;Incertae Sedis XIV;Blautia**	-0.2745	1.51E-02	9.45	1.25	3.46	0.4	8.60E-08
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Oribacterium	0.3911	7.31E-04	0.01	0	0.31	0.03	7.20E-07
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Other**	-0.2432	3.19E-02	6.41	0.69	3.86	0.24	5.50E-04
Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Pseudobutyrivibrio	0.3147	9.95E-03	0	0	0.04	0.01	1.10E-03
Bacteria;Firmicutes;Clostridia;Clostridiales;Peptostreptococcaceae;Other	-0.2395	3.71E-02	0.35	0.1	0.12	0.03	1.20E-02
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Acetanaerobacterium	-0.4224	1.08E-03	0.02	0.01	0	0	1.30E-04
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Anaerofilum	-0.352	7.14E-03	0.02	0	0	0	1.90E-05
Bacteria;Firmicutes;Clostridia;Clostridiales;Ruminococcaceae;Butyricicoccus	0.303	7.09E-03	0.37	0.05	2.62	0.47	1.10E-07
Bacteria;Firmicutes;Clostridia;Clostridiales;Veillonellaceae;Other	0.4573	6.15E-05	0.02	0.01	0.83	0.17	3.00E-07
Bacteria;Firmicutes;Erysipelotrichi;Erysipelotrichales;Erysipelotrichaceae;Other	-0.2973	8.29E-03	1.49	0.33	0.7	0.16	1.30E-02
Bacteria;Firmicutes;Erysipelotrichi;Erysipelotrichales;Erysipelotrichaceae;Solobacterium	-0.2672	3.98E-02	0.01	0	0.01	0.01	3.00E-03
Bacteria;Other;Other;Other;Other;Other	0.2262	4.65E-02	0.22	0.05	1.82	0.16	3.80E-09
Bacteria;Proteobacteria;Alphaproteobacteria;Other;Other;Other	0.2756	1.78E-02	0.02	0.01	0.25	0.07	1.20E-04
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;Parasutterella	-0.4107	1.70E-03	0.05	0.01	0	0	5.00E-06
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Alcaligenaceae;Sutterella	-0.3336	1.19E-02	0.03	0.02	0	0	7.90E-04
Bacteria;Proteobacteria;Betaproteobacteria;Burkholderiales;Other;Other	0.2779	3.15E-02	0	0	0.01	0.01	5.60E-02
Bacteria;Proteobacteria;Epsilonproteobacteria;Campylobacterales;Campylobacteraceae;Campylo-bacter	0.2745	2.07E-02	0	0	0.24	0.07	1.20E-06
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Aeromonadaceae;Aeromonas	0.2781	3.96E-02	0	0	0	0	2.90E-01
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Succinivibrionaceae;Ruminobacter	0.3297	9.75E-03	0	0	1.21	0.66	2.90E-02
Bacteria;Proteobacteria;Gammaproteobacteria;Aeromonadales;Succinivibrionaceae;Succinivibrio	0.3499	2.51E-03	0.02	0.02	2.77	0.68	1.10E-07
Bacteria;Proteobacteria;Gammaproteobacteria;Enterobacteriales;Enterobacteriaceae;Other	0.3172	1.60E-02	0	0	0.01	0.01	4.80E-02
Bacteria;Proteobacteria;Other;Other;Other;Other	0.2426	3.40E-02	0.02	0.01	0.52	0.15	1.60E-07
Bacteria;Spirochaetes;Spirochaetes;Spirochaetales;Spirochaetaceae;Treponema	0.3092	7.11E-03	0	0	2.5	0.72	4.40E-08
Bacteria;Verrucomicrobia;Verrucomicrobiae;Verrucomicrobiales;Verrucomicrobiaceae;Akkerman-sia	-0.3355	1.19E-02	0.13	0.07	0	0	7.90E-04

Curriculum Vitae

The author of this dissertation was born and raised in North Texas, USA. She received her Bachelor of Arts from Boston University in 2006 in Physical Anthropology, with a Minor in Biology, focusing on neuroscience and endocrinology. In 2008, she enrolled at Texas State University in San Marcos, Texas, to continue graduate education towards her masters in Biological Anthropology, under the advising of Kerrie Lewis Graham, finishing in August 2010. In the Fall of 2011, Stephanie began the PhD program at the Max Planck Institute for Evolutionary Anthropology within the independent research group led by Amanda Henry on Plant Foods in Hominin Dietary Ecology. During the time in Leipzig, Stephanie initiated projects that focused on Hadza women's foraging and consumption of wild tubers in East Africa, building towards future field work in collaboration with and under direction of Alyssa Crittenden at the University of Nevada, Las Vegas. The analysis of Hadza tuber nutrition and gut microbiota were accomplished in collaboration with Koen Venema and Marco Candela respectively, which provided a means of nutritional and molecular laboratory training for Stephanie. Continuing education opportunities in Leipzig were numerous, and included training in starch and phytolith extraction and analysis, collagen extraction for isotope analysis, and programming and statistical computation in R. Off-site training included a Kiswahili language beginner course in Zanzibar, TZ, in-vitro gastro-intestinal digestion and fermentation modeling at TNO in Zeist, NL, beginning bioinformatics community data analysis in Bologna, IT, and procedures for DNA extraction, amplification, quantification, and sequencing at LMAMR in Norman, OK. Throughout the PhD program, Stephanie gave numerous contributed and invited presentations. In 2013, she was awarded the student poster presentation prize at the European Society for the study of Human Evolution (ESHE) in Vienna, and in 2014, the Juan Comas student presentation prize for her podium presentation at the American Association of Physical Anthropologists (AAPA) in Calgary. Publication of the Hadza gut microbiome paper gained considerable attention in the popular press, and Stephanie was invited to contribute a small piece in a double feature, called "Innovations In", on the gut microbiome for the March 2015 issues of *Scientific American* and *Nature*. Peer reviewed publications of the papers that comprise the chapters of this dissertation were published in *Ethnoarchaeology, American Journal of Physical Anthropology,* and *Nature Communications* respectively. Continuing research on the Hadza gut microbiota resulted in a second successful publication on this microbial community, Rampelli et al. 2015 in *Current Biology*, which Stephanie co-authored and completed in tandem with completion of this dissertation work. The journal featured this latter publication on their June 29, 2015 issue cover (see image on the following page). Stephanie currently lives in Norman, OK, USA to pursue postdoctoral research with the Laboratories of Molecular Anthropology and Microbiome Research (LMAMR).

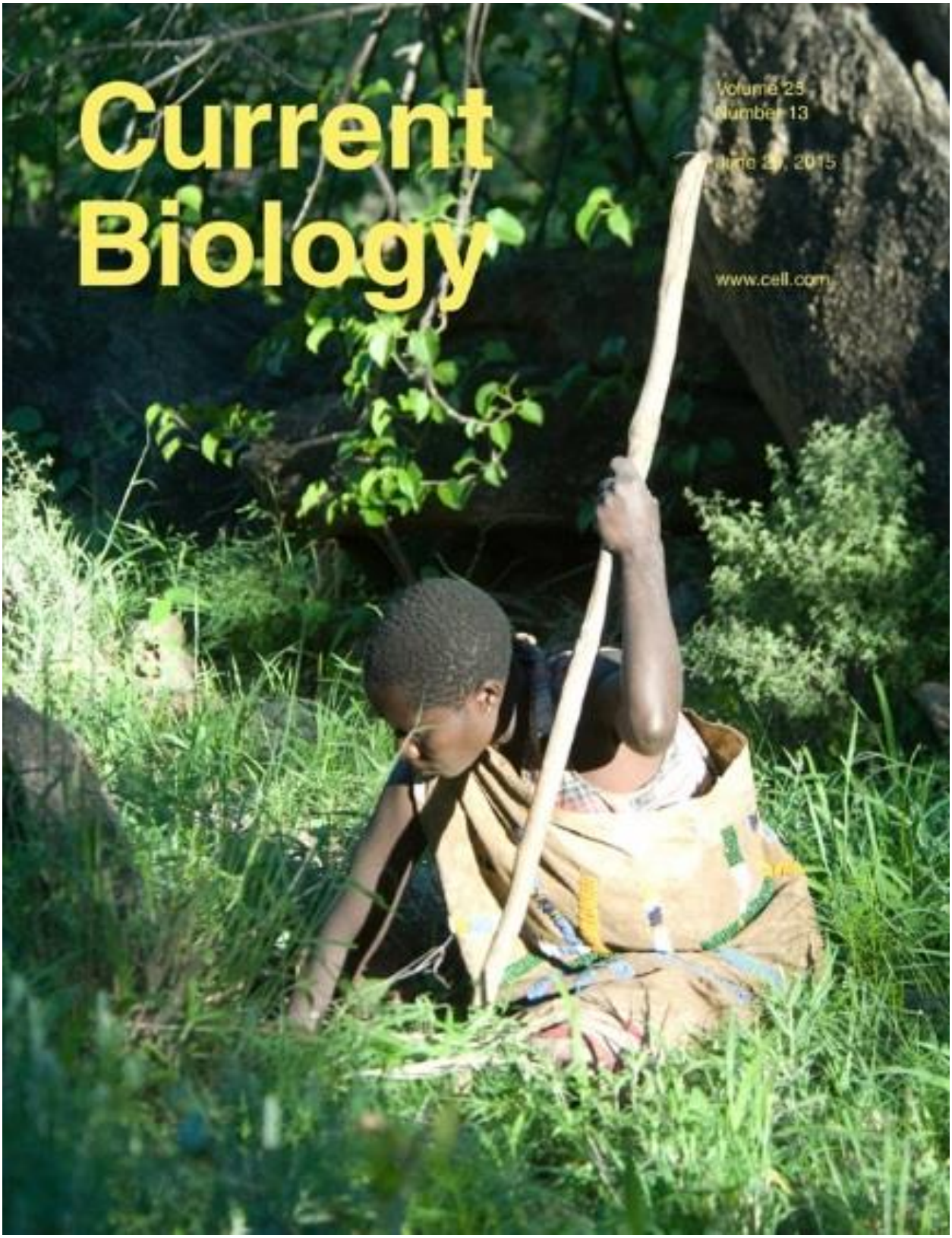


Image 13. Cover feature for June 2015 issue of Current Biology of a Hadza woman digging for wild tubers near Lake Eyasi in Northern Tanzania. Photo taken by Mika Peterson while assisting the author with field research in Tanzania, January 2013.