STABLE CARBON ISOTOPE ANALYSIS AND MAIZE-STALK BEER DIET IN RATS: IMPLICATIONS FOR THE ORIGINS OF MAIZE

by

MARIA CECILIA CANAL
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ABSTRACT

Maize is one of the world's most important staple crops but theories explaining the ancestry of maize are focused mostly on domestication as it relates to food for human consumption. Much research was conducted on the wild ancestor of maize; current trends support teosinte as the ancestor of maize; but the question that remains unexplained is why people initially cultivated teosinte considering the plant has so little yield? Smalley and Blake (2003), elaborating on a concept proposed by Iltis (2000), explored this question. Iltis argued that the ancestor of maize was first domesticated for its sugar content. Building on this idea, Smalley and Blake suggested also the possibility of making alcoholic beverages. This suggestion that the ancestor of maize was selected for its sugar content changes the focus of early maize research. Maize is found in the archaeological record at 5400 B.P. but was not yet a staple food crop. Researchers must consider alternate uses early Mesoamerican people had for this plant. The production of alcohol from the sugary maize stalk is an example of an alternate use for maize. In an attempt to understand what occurred between the initial appearance of maize as a crop and its use as a staple food source, researchers have been studying the plant's C_4 photosynthetic pathway and its impact on bone chemistry. The carbon isotope signature in human bone resulting from the consumption of maize is quite different depending on whether the maize is eaten directly as food or first converted to alcohol before being consumed. This study tests the hypothesis that C_4 carbon from maize-stalk beer leaves a signature in bone collagen. Rats were used in a feeding experiment to determine if a diet with a significant component of maize-stalk beer would elevate the stable carbon isotope ratios in the consumers. Results of this experiment showed that maize-stalk sugar converted to alcohol did not raise the stable carbon isotope ratio measured in the rats. This suggests that archaeologists must look for raised stable carbon isotope values in apatite, rather that collagen, if they are to detect maize alcohol use in ancient populations.

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INTRODUCTION

Maize is one of the world's most important staple crops but theories explaining the ancestry of maize are focused mostly on domestication as it relates to food for human consumption. For more than 100 years, archaeologists, geneticists and botanists have studied the origin of maize and many theories have been proposed regarding how this crop evolved (Bennetzen et al. 2001; Gallinat et al 1984; Piperno and Pearsall 1998, Pope et al 2001). However, the most important aspects of this issue remain unexplained. As Mangelsdorf et al. (1964:538) explain, "a living wild form of corn has never been discovered, despite the extensive searches for it which have been carried on in various parts of the hemisphere." Much research has been conducted on the wild ancestor of maize and current trends support the teosinte hypothesis proposed by Beadle in 1939 (Beadle 1980), a geneticist (Doebley 2004:39-40). The teosinte hypothesis suggests that the wild annual grass, teosinte, is the sole progenitor of maize (Beadle 1980; Doebley 2004:40). Benz (2006:9) defines teosinte as an English term adapted from the Nahuat "tecintli" (good or evil grain) used widely to refer to the seven taxa of wild grasses that are closely related to maize. One of the seven taxa, *Zea mays* ssp. *parvigumis*, exhibits a close genetic relationship with maize and because of this evidence, teosinte is regarded as the ancestor of maize (Doebley 2004:39; Matsuoka et al. 2002).

The physical differences between teosinte and maize are striking. Teosinte is a tall thin grass with many branches of tasselled spikes that produce small ears, approximately 10 cm long, with two rows of seeds (Iltis 2006:31-33). The yield from the teosinte plant is minimal compared to the maize plant that produces large ears containing as many as 16 rows of seeds (see Figure 3-15A-F in Iltis 2006:40). If teosinte is in fact the wild ancestor of maize, the question that remains unexplained is why people initially cultivated teosinte considering the plant has so little yield? Another aspect to consider is that, based on ethnographic accounts and archaeological data, teosinte was known both as a "starvation" and an unpalatable food by early peoples of

Mesoamerica (Beadle 1980; Coe 1994:33; Flannery 1973:290). Consequently, this leads to the important question, why were people cultivating teosinte if it was so difficult to eat?

This question was explored by John Smalley and Michael Blake (Smalley and Blake 2003) who elaborate on a concept proposed by Hugh H. Iltis (2000). Iltis (2000:36) argued that the ancestor of maize was first domesticated not for its grain but for its sugar content. Building on this idea, Smalley and Blake (2003:675) suggested, "that the social importance of alcohol production was a precipitating factor in *Zea's* early and rapid spread." This suggestion that the ancestor of maize was selected not for its grain parts, but for its sugar content, changes the focus of early maize research. Until now, researchers focused on maize's evolved characteristics, and not the initial ones such as small, hard kernels, small cobs and sweet stalks (Smalley and Blake 2003:675). As maize is currently an important staple crop, it is difficult for researchers today to focus on its origin as something different, a source of sugar or alcohol. However, when considering the initial plant characteristics noted above, maize ears as a "staple crop" become less important compared to the stalk, because it is the stalk that offers a more easily accessible source of sugar.

Considering this new perspective, the question then becomes: what types of archaeological data would be most useful for illustrating the importance of the sugary teosinte or maize stalk for early peoples? There is paleoenthnobotanical evidence showing that domesticated maize (*Zea mays*) with cobs was present in Guila Naquitz Cave in Oaxaca, Mexico at least 5400 carbon-14 years B.P. (Piperno and Flannery 2001:2102). However, isotopic data suggests that maize did not become a dietary staple in many parts of Mesoamerica until 2500 years later (Smalley and Blake 2003:684). Since maize is found in the archaeological record at 5400 B.P. but was not yet a staple food crop, researchers must consider the range of alternate uses that early Mesoamerican people had for this plant. The production of alcohol from the sugary maize stalk is an example of an alternate use for maize.

There are archaeological residues and ethnographic evidence for the use of alcohol, such as *chicha* (beer made from sprouted maize), in communal drinking, feasting, as well as in other political and social activities (Dietler 1990:362; Hastorf 1999; Hastorf and Johannessen 1994; Jennings et al. 2005; Mandelbaum 1965; Marshall 1979; Moore 1989; Ubelaker et al. 1995). Recent research by Jennings et al. (2005) discusses the archaeological evidence regarding the production and consumption of alcoholic beverages by ancient populations around the world. Adams (2004) and Jennings et al. (2005) note that archaeological research on feasting has tended to focus on the political aspects of feasting. This focus on feasting as a political event "...can obscure the labour and resources committed to growing, harvesting, and processing the food and drink that were consumed on these occasions" (Jennings et al. 2005:275). For Mesoamerica, Bruman (2000) describes the arious types of alcoholic beverages that can be made from plants and fruits indigenous to Mexico. Of particular interest for this thesis is the production of "corn-stalk" wine[1], which he argues was one of the beverages that people consumed prior to the introduction of sugarcane to aboriginal America (Bruman 2000:57). According to Bruman (2000:57) many explorers and chroniclers attested to the economic importance of maize-stalk syrup and that some tribes who made syrup, may have known the process of diluting it with water and allowing it to ferment to produce an alcoholic drink. In summary, the archaeological and ethnohistoric evidence indicates that maize was present by 5400 years B.P. and that at least by the time of Columbus the plant was used to produce alcoholic beverages as maize-stalk beer (Piperno and Flannery 2001, Bruman 2000).

In an attempt to understand what occurred between the initial appearance of maize as a crop and its use as a staple food source, researchers have been turning their attention to the study of the plant's C_4 photosynthetic pathway and its impact on bone chemistry (Tykot 2006:132). The carbon isotope signature in human bone resulting from the consumption of maize may be

[1] Although Bruman (2000) uses the term "cornstalk, the more common term used today is maize, so I will use maize throughout. Also, although technically fermented maize stalk juice is a "wine", I refer to it as beer, after Smalley and Blake (2003).

quite different depending on whether the maize is eaten directly as food or first converted to alcohol before being consumed. The basic hypothesis this thesis is trying to test is that C_4 carbon from maize-stalk beer does not leave a signature in bone collagen. If Mesoamerican people were cultivating maize and consuming it as an alcoholic beverage, it may not be reflected in stable carbon isotope ratios present in bone collagen (the preserved organic parts of bone). The reason for this is the fact that the portion of the diet that gets routed to the bone collagen comes from protein, not fat or carbohydrates (Ambrose and Norr 1993:2; Chisholm, personal communication; Smalley and Blake 2003:684; Ubelaker et al. 1995). Building on the evidence provided by the experiments conducted by Ambrose and Norr (1993:27-28) and the literature survey by Chisholm et al (1982) I suggest that in general, alcohol does not build protein, because it contains mostly calories, vitamins and very little protein. Because this has never been tested experimentally, I designed an experiment to measure the effects of a maize alcohol diet on collagen production in rats.

Rats were chosen as a proxi for human consumers, because they breed rapidly, can be easily handled with some practice, and can be housed in large numbers in a relatively confined area. Moreover, being a small animal, it is both economical and practical to use large numbers in an experiment. Much is now known about their physiology, anatomy, genetics and behaviour which suggests that meaningful results can be obtained from rats that, if interpreted with care, can be extrapolated to humans (Waynford and Flecknell, 1992).

In the following section, I present the theories and methods for stable isotope analysis and for alcohol administration in rats, as well as the experimental design to determine if C_4 from maize-stalk beer leaves any traces in bone collagen. In the third section I explain the procedures and methods used in this experiment. In the final section I discuss the results, the implications and conclusions of this study and its archaeological importance.

BACKGROUND

Importance of Maize today:

Maize has become one of the most important grain crops in the world, but it was not always a staple food crop. Tykot and Staller (2002:667) argue that western scholars tend to overemphasize the importance that maize played in early diets. Maize was an unknown product in the Old World before 1492 (Mangelsdorf et al. 1964:538; Mangelsdorf 1974:1). Spaniards made the first European historical reference to maize on November 5, 1492. They were exploring the island of Cuba and then reported to Christopher Columbus that they had found "a sort of grain they called *maiz* which was well tasted, bak'd, dry'd, and made into flour" (Mangelsdorf 1974:1).

The Native Americans had, independently from other areas of the world, developed food production. By the time of Columbus, Native Americans had a vast knowledge of many kinds of plants, which they used for subsistence, ritual activities, as well as medicinal purposes (Mangelsdorf 1974:1). Included among these many plants was maize. By the time of the Spanish conquest, maize was the most important staple crop in Mesoamerica. Columbus brought maize back to Spain, and from there it quickly spread throughout Europe, to North Africa, the Middle East, India and China.

Maize has remained an important staple crop in the Americas, and all over the world. As Mangelsdorf (1974:2) explains, a crop of maize matures somewhere in the world every month of the year. It grows in a diverse range of environments from 58° N latitude, in Canada and Russia, to 40° S latitude, in the southern hemisphere. Fields of maize grow below sea level in the Caspian plain and at altitudes of more than 12,000 feet (more than 3600 meters) in the Peruvian Andes. Maize is grown in regions with less than ten inches of annual rainfall in the semiarid plains of Russia and regions of more than 400 inches of rainfall on the Pacific Coast of Colombia. Today, maize is grown in every suitable agricultural region of the globe (Mangelsdorf 1974:2). By 1974, maize was grown on 119,770,684 hectares (Ha) and produced an annual grain crop of nearly

306,287,347 metric tonnes. By 2005, maize was grown on 147,017,069 hectares around the world and produced 692,034,184 metric tonnes (FAO 2005). Maize has much higher yield per hectare (47,072 tonnes/Ha) compared with wheat (28980 tonnes/Ha), rye (22670 tonnes/Ha) and barley (24484 tonnes/Ha) (FAO 2005). By current standards, maize is easy to grow and is inexpensive to purchase. Because of this, it has become the dominant food and main source of dietary energy and limited protein for underprivileged segments of society around the world.

Cultivating and consuming maize with beans and squashes, has long been recognized to provide an excellent diet. Maize supplies carbohydrates, small amounts of protein, and fat while beans are the principal source of protein, essential amino acids and vitamins. Squashes are important in supplying additional calories as well as Vitamin A and fat (in the seeds) (Mangelsdorf 1974:1).

The Ancestor of Maize – Teosinte

The most widely agreed-upon candidate for the wild ancestor of maize is a perennial grass named *teosinte* (Doebley 2004:41). Teosinte is the name for a group of large grasses of the genus *Zea* that inhabits Central and South America. There are five species of teosinte known today: *Zea perennis, Zea luxurians, Zea nicaraguensis, Zea diploperenni's,* and *Zea mays.* The species *Zea mays* is divided into four subspecies: ssp. *huehuetenangensis*, ssp. *mays*, ssp. *mexicana*, and ssp. *parviglumis. Zea mays* ssp. *mays*, (maize or corn) is the only domesticated taxon in the genus Zea, and is derived directly from *Zea mays* ssp. *parviglumis.*(Matsuoka et al. 2002: 6080) However, the processes that led early people to interact with teosinte, harvest and utilize the seeds, leaves and stalks, and engage in the transportation or trade of the plant into new areas require more research (Blake 2006:55). As Tykot and Staller (2002:667) note, "our understanding of the phylogeny, origins, chronology, and routes of dispersal of maize remains incomplete."

Spread and Timing of Maize

A new study on the genetics of *Zea mays* by Matsuoka et al. (2002) indicates that all modern maize evolved from teosinte (*Zea mays* ssp. *parviglumis*) which originated in the Río Balsas drainage of West Mexico. From there it spread out into new habitats, from eastern Canada to northern Chile (Matsuoka et al. 2002: 6080). Independent domestications were proposed for maize, based on morphological and genetic diversity (Gallinat 1988) however, this diversity can be explained by a single domestication and subsequent diversification (Matsuoka et al. 2002: 6080). From an early diversification in the Mexican highlands, two lineages are proposed for the dispersal of maize throughout the Americas (Blake 2006:55-59). One path leads from western and northern Mexico, into the southwestern U.S. and north to Canada. The second path can be traced from the Mexican Highlands to the western and southern Lowlands of Mexico, into Guatemala, the Caribbean Islands, the Lowlands of South America and the Andes Mountains (Matsuoka et al. 2002: 6084).

Researchers who study early maize have begun to focus their attention on the direct dating of maize macroremains using Accelerated Mass Spectrometry (AMS) radiocarbon dating (Blake 2006:56). Dating the early spread of maize begins with the 5400 B.P. date from Guilá Naquitz Cave, Oaxaca (Piperno and Flannery 2001:2102) and San Marcos Cave in the Tehuacan Valley (4700 ± 110 B.P.) (Benz and Iltis 1990; Benz and Long 2000). Blake (2006:55-59) summarizes the most recent data relating to the initial spread of *Zea mays* from this region. The South to North progression of maize dates suggests that maize spread slowly northward from its homeland in the Río Balsas drainage of West Mexico to Tamaulipas (Romero's Cave 3903± 50 B.P.), into Chihuahua (Cerro Juanaqueña 2980 ± 50 B.P.) and into the American Southwest (Fresnel Shelter, New Mexico 2945 ± 55 B.P.) where it then eventually spread into eastern North America. Matsuoka et al. (2002:6083) suggests that it is possible to estimate the date of the origin of maize as a single event using DNA microsatellite data. Microsatellite data utilizes a large number of loci

to construct the entire genome instead of focussing on a single gene region (Benz 2006:13; Matsuoka et al. 2002:6080). According to Matsuoka et al. (2002:6083) teosinte, ssp. p*arviglumis* and Mexican maize have an average divergence date of 9188 B.P (with a 95% confidence interval between 5689 – 13093 B.P.) which occurs more than 3500 years before maize was incorporated into the Tehuacan Cave deposits (Benz 2006:13) and is consistent with the archaeological estimates that crop domestication in Mesoamerica did not precede 10,000 B.P. (Matsuoka et al. 2002: 6083).

Considering these early dates for maize manipulation in Mesoamerica, it is important to understand how people were utilizing maize. One of the ways to determine relationships between people and plants is to test stable isotope ratios in bone collagen to determine how people were consuming maize. Smalley and Blake (2003:684) summarize published data on stable carbon isotope analysis of 622 individual human remains recovered from numerous South American and Mesoamerican sites. The pattern that emerges indicates that stable carbon isotope ratios increased, suggesting increasing reliance on maize in the diet, from the first appearance of maize in Central Mexico to the time of the Spanish Conquest. In most regions, with few exceptions, the shift to higher stable carbon ratios, reflecting significant maize consumption in the diet, did not occur until after 3000 years ago. As Smalley and Blake (2003:684) suggest, moderate to high stable carbon ratios are represented by values greater than –15.0‰. When stable carbon ratios have values greater than –15.5‰ in non-marine environments, individuals are consuming a significant percentage of their diet from C_4 plants, such as maize. If maize has been present in the archaeological record (as seen in micro and macro botanical remains) for 5000 years, yet not generally a dietary staple until after 3000 B.P. (Blake 2006:66) when higher stable carbon ratios are recorded, then it is logical to think of an alternate explanation for the first use of maize.

THEORY AND METHODS OF ISOTOPE ANALYSIS

Carbon, present in the atmosphere as CO_2, is incorporated into plant tissues by photosynthesis. Plants can be divided into three different categories. One category, plants that use the Calvin-Benson or C_3 photosynthesis pathway, generates a three-carbon molecule (phosphoglyceric acid) as the first photosynthesis intermediate. The second category is plants that use the C_4, or Hatch-Slack pathway, which incorporates the CO_2 carbon into a molecule that contains four carbon atoms (oxaloacetate) as its first intermediate product (Chisholm 1989:12). The third group, "plants utilizing the Crassulacean Acid Metabolism (CAM) for carbon dioxide fixation have an isotopic content similar to C_4 plants" (Vogel and van der Merwe 1977:239) when growing in conditions that favour C_4 plants.

Examples of the Calvin-Benson or C_3 plants include most of the flowering plants, trees and shrubs, and most of the temperate zone grasses. Only ten plant families represent the Hatch-Slack or C4 plants. The most interesting for us, because they are part of human diets, are maize, millet, sorghums, cane sugar and chenopods. The Crassulacean Acid Metabolism (CAM) plants include the pineapple and various cacti, some of which may be used as a food source (Chisholm 1989:12).

As the photosynthetic pathways diverge chemically they produce different degrees of isotopic fractionation. This has been utilized to classify species as being C_3, C_4 or CAM. Isotopic fractionation is ..." the selection for or against one or more isotopes of an element during the course of a chemical or physical reaction. As a result there is a change in the relative concentration of the isotopes involved in the reaction" (Chisholm 1989:12). As Chisholm and Koike (1996:201) note, "...the values for meat are only slightly displaced from those of the foods that the animals eat, which may allows us to average meat and plants from the same food chains together to form human's alternative food groups." It is worth mentioning here that when marine

or terrestrial herbivores eat plants, their metabolism selects and recombines plant chemicals, resulting in further fractionation of the carbon isotopes (Chisholm 1989:13).

Isotope ratio measurements are reported using the delta notation (for example $\delta^{13}C$, $\delta^{15}N$) relative to international standards and are expressed in parts per mil (‰) (Tykot and Staller 2002: 669). Modern C_3 plants have average values of about –26.5 ‰ whereas modern C_4 plants have averages of about –12.5 ‰. This separation of 14‰ allows for discrimination between group averages. CAM plants, such as pineapples, agave and cacti, also produce high stable carbon ratios (Blake 2006:66) and according to Tykot (2006:132), can switch between C_4 and C_3 pathways depending upon both their environment and geographic location.

The analysis of stable isotopes of carbon from preserved bone has been used to study past diets and past environments (Ambrose and Norr 1993; Burger and van der Merwe 1990; Chisholm and Koike 1996; Tykot and Staller 2002; Ubelaker et al. 1995; van der Merwe et al 1993; Vogel and van der Merwe 1977). Stable carbon isotope analysis is particularly useful in New World dietary studies (Tykot and Staller 2002; Ubelaker et al. 1995) because maize is often the only C_4 plant contributing significantly to past human diets (Tykot and Staller 2002: 670).

The current trend for researchers interested in ancient diet involves both carbon and nitrogen isotope analysis (DeNiro and Epstein 1981; Tykot 2006:134). Stable isotope analysis of nitrogen is a clear indicator of "...the effects of climate and environment on both plant and animal values and trophic level increases in both terrestrial and marine ecosystems." As Ubelaker et al. (1995:404) discuss, stable isotope analysis of nitrogen is useful in the study of past human diet with regard to individuals who have more positive $\delta^{15}N$ values when compared to adults and older children. In general, nitrogen isotope ratios increase 2 to 3‰ with each trophic level so terrestrial plants and animals have much lower values than fish and mammals from freshwater or marine environments. The nitrogen value of maize ($\delta^{15}N$) is 2-3‰ while freshwater and marine mammals and fish have a $\delta^{15}N$ value of 9-18‰ (see Figure 10-2 in Tykot 2006:134).

Using stable carbon and nitrogen isotopes for diet reconstruction is based on the assumption that you are what you eat. It is assumed that the carbon isotopic composition in animal tissues is a direct and constant function of the diet (Ambrose and Norr 1993:1). As previously mentioned, collagen in consumers is made from the protein portion of the diet, not from fats or carbohydrates (Ambrose and Norr 1993:27-28). Ambrose and Norr (1993:27-28) demonstrate that the value of rat collagen largely reflects dietary protein, but it is poorly correlated with the whole diet when the isotopic composition of protein and non-protein components differ considerably. Therefore carbon in dietary proteins is routed mainly to collagen (Chisholm et al 1982), rather than scrambled with that in carbohydrates and lipids. Another source of information comes from apatite (found mostly in bone and tooth enamel) which is the inorganic portion of bone and that reflects whole diet. Bone apatite carbonate provides the most accurate measure of the energy portion of the diet (Ambrose and Norr 1993:27-8) while the protein of the diet is routed to the bone collagen. For this thesis, I am interested in the reconstruction of diet by measuring the ratios of carbon-13 to carbon-12 and nitrogen in the collagen (protein portion) of rat bone[2].

CONSUMPTION OF MAIZE PRODUCTS AND CARBON RATIOS

Both *chicha* (maize beer) and maize-stalk beer do not build up much protein, but mostly calories and vitamins (Chisholm, personal communication; Smalley and Blake 2003:684). Thus, consumption of maize beer or maize-stalk beer may not have produced higher stable carbon isotope ratios in human bone collagen. It is important to distinguish the differences between *Chicha* and maize-stalk beer. *Chicha* has two common recipes that produce a similar product (Jennings et al. 2005:278-279). One recipe involves the grinding of maize kernels into flour, which is then masticated with saliva. The second recipe is made with sprouted maize kernels, which are

[2] A later study will examine the $\delta^{13}C$ values in the apatite portion of the rat samples.

then ground into flour (Bruman 2000:40-41). The next step in both of these recipes involves adding water and bringing the mixture to a low boil. At this stage the liquid is transferred to jars to allow fermentation. (see Jennings et al. 2005 for a thorough description). In contrast, maize-stalk beer, is made with the sweet juice from the stalk. This process involves steaming maize stalks until they become soft and opaque green-brown in colour. The stalk is then pressed or squeezed, much like sugar cane, to remove the juice out of the stalk. The juice is then placed in a container with a narrow neck and spout until the liquid ferments (John Smalley, personal communication, 2004). The key to both processes is the fermentation of the maize sugar into alcohol. In order to test the idea that alcohol made from maize will not leave a C_4 signature in bone collagen, maize-stalk beer was brewed to conduct a feeding experiment with rats. Chicha was not used in this experiment because its isotopic reading would be identical to maize-stalk beer. Future experiments, however, will include *chicha*, particularly recipes that might have a higher protein component.

Making Maize-stalk Beer

The maize was harvested in late September 2004. Only the maize stalk is needed to make beer but because the modern emphasis is on the maize ears, stalks were more difficult to purchase. Ralph's Farm Market, on the Fraser Highway in Langley, British Columbia had a small section of their farm, which they planned to turn into a maize maze for visitors. However, the area set aside for the maze was too small and the maize plants were left to grow. The maize plants were approximately 1.82 m (6ft) in height and were a mixture of various types of maize. Using a small knife, 41 kg of maize plant were harvested from the farm. To prepare the stalks for storage, the other parts of the plant were removed (leaves, cobs and seeds) and the stalks were cut into quarters to fit into plastic freezer bags. The stalks were kept in two home freezers until the beer making process began in mid- October. Freezing the stalks (and, later on, the beer) was done in

order to stop all bacterial growth and chemical reactions. Freezing would not have had any impact on the chemical composition of the stalks or beer (Chisholm, personal communication).

John Smalley had made maize-stalk beer in the past and was able to explain the methods used to extract the liquid from the maize stalk. For the first batch, ~21 kg of maize stalk were defrosted overnight and then cut into small pieces (about 10 cm long). The maize stalks were then steamed in 12 litre pots filled with 500 ml of water, for about 20 minutes or until the color of the stalk changed from bright green to an opaque green/brown. The steamed stalks were placed in a manual grape press in order to remove the juice from the stalks. It took a lot of arm strength to get all of the juice out of every batch. From the 21 kg of stalk, 12 kg of juice were obtained and 9 kg of pressed stalk were left. A beer and wine hydrometer was placed in the juice to test the amount of sugar present. The first test showed that with the amount of sugar present, after the fermentation process, the beer would have an alcohol content of 3%. The relatively low amount of sugar recorded from this liquid is likely a result of harvesting the maize late in the growing season when most of the sugar had already migrated from the stalk into the cobs (John Smalley and Michael Blake, personal communication, 2005).

The 12 kg of juice were transported to the Department of Anthropology and Sociology at UBC for fermentation. Commercial yeast (*Saccraromyces bayanus*, 5 g) for wine/beer making was then added to the juice and left to ferment for about 4 days in a clean plastic bucket covered with a sheet of plastic as a lid. After 4 days the juice fermentation was complete and the alcoholic content of the beer was measured at 3%. The beer was bottled into 12, 1 litre plastic bottles, the kind used to store homemade beer. The 12 litres of homemade maize-stalk beer were stored in a home freezer until needed. As the experiment progressed, more beer was needed but maize stalk was not available for harvest. The first batch was fermented a second time with the addition of commercial maize sugar to increase the alcoholic percentage to a measurement of 12%. The beer

was then left to ferment for another 10 days. As the maize sugar is also a C_4 product, no potential impact was envisioned by this decision.

A second batch of beer was made with a different method in late May, using the remaining ~21 kg of frozen stalk. The stalks were defrosted overnight and cut in 10 cm long pieces. Then, all the stalk pieces were placed in a container (able to retain the heat), and boiling water was added until all the stalks were covered. As stalks tend to float, a weight was put on top of the stalks to keep them submerged. This "tea" was left to infuse for 2 hours, then, 12 litres of liquid were extracted and deposited into a large glass bottle for fermenting. The extraction process involved a long plastic tube, which was connected to a copper pipe. Ice was packed around the copper pipe and served to cool the hot "tea" as it was extracted. Once the "tea" reached the large glass bottle it was left to cool. In order to reach the desired 12% alcohol content maize sugar was added to the juice.

The juice was stored in a large glass bottle, and 5 grams of yeast (*Saccraromyces bayanus*) was added to the juice. The bottle was stored in a room at 24°C. The glass bottle was covered with a dark cloth in order to keep the light out. In the spout of the bottle, a pump was added, allowing the gasses to escape and also preventing any contamination from the outside environment. The fermentation for an alcoholic content of 12% took 10 days.

EXPERIMENT DESIGN

The main purpose of this experiment is to determine if C_4 carbon from maize-stalk beer leaves any discernable traces in rat bone collagen. To do this, rats and not mice were used as consumers of maize-stalk beer, because rats are larger and thus provide more bone collagen than mice. Bone collagen, tooth, hair and muscle samples were taken from second generation rats raised on the same diets as their mothers. The second generation rats were selected because their diet had been controlled from the prenatal to postnatal stage. Consumers from the second

generation were alive for about 70 days or until they reached between 275-300 grams, in order to even out irregularities in diet, the effects of weaning and sexual dimorphism.

The rats were divided in three groups: Group 1, Group 2 and Group 3. This experiment analyzed 12 individuals in Group 1, 10 individuals in Group 2 and 12 individuals in Group 3.

The special diet material for Group 1 (C_4 solid diet) and Group 3 (C_3 solid diet) was standard rat chow made by the company Test Diets Inc. (PMI ® Nutrition International rodent diet #5012). The Test Diet Inc. formula #5012 supplies complete life-cycle nutrition specifically designed to support reproduction, lactation, growth and maintenance of rats. It is low in cholesterol content, with an increased level of unsaturated fatty acids compared to other rodent diets. Some changes were required from the original formula because of the C_4 ingredients that would have affected the carbon ratios. As seen in Table 1, for Group 1 (C_4 solid diet), all other sources with C_4-like values, except maize were removed from the original formula and replaced with C_3 ingredients. The amount of maize present in the formula was 70%. For Group 3 (C_3 solid diet), all sources of C_4 were removed from the diet and exchanged for 100% C_3 ingredients. The Maize-stalk Beer diet of Group 2 was a custom made powdered diet (Dyets Inc. Formula #710341), which was mixed with water and maize-stalk beer. For this powdered diet, maize oil was replaced with soybean oil, and the alcohol from the maize-stalk beer was the only source of C_4. It is important here to state that the UBC Animal Care Centre did not allow for a pure (100%) maize diet, or a pure maize-stalk beer diet because of the nutritional concerns for the rats. Vitamins and minerals had to be added to the diet in order to ensure a well balance diet. In order to confront to these requirements, the diets could not be pure C_4 (Group 1 diet) or only maize-stalk beer (Group 2), supplements of vitamins and minerals were added to the diets of all groups.

Table 1. Composition and Diet of Experiment Groups

Group 1: (C_4 solid diet)	**Group 2:** (Maize-stalk Beer diet)	**Group 3:** (C_3 solid diet)
Generation one: Pregnant mother **Generation Two**: 12 pups **Diet**: 70% C_4 plant based (pure maize) + 30% C_3 plant based + water	**Generation One**: Pregnant mother **Generation Two**: 10 pups **Diet**: Custom made diet #710341 (C_3) + maize-stalk beer (C_4). No other source of liquid.	**Generation One**: Pregnant mother **Generation Two**: 12 pups **Diet**: C_3 plant based + water

EXPERIMENTING WITH RATS

Three pregnant rats[3] arrived in the Department of Psychology[4], on May 5th, 2005. The rats were housed individually and each was given a specific diet. The mother of Group 1 (C_4 solid diet) was fed the C_4 diet (70% of maize + C3 + vitamins and minerals) and water. The mother of Group 2 (Maize-stalk Beer Diet) was fed the custom made liquid diet of C_3 + maize-stalk beer (the only C_4 source) with no other liquid. The mother of Group 3 (C_3 solid diet) was fed a 100% C_3 diet (no C_4 or maize) and water.

The mother of Group 1 gave birth on May 6, 2005 to 10 males and 4 females. The pups remained with their mother until May 9th, 2005, when 2 males were culled. The remaining litter of 12 consisted of 8 males and 4 females. The mother of Group 2 gave birth on May 12th, 2005 to 14 pups, 8 males and 6 females. Five males and 5 females were kept, and were fed maize-stalk beer once they reached 25 days of age. The mother of Group 3 gave birth on May 12th, 2005 to 16 pups, 4 males, 11 females and one stillborn. Four males and 8 females were selected.

[3] Sprague-Dawley: A strain of albino rats developed by the Sprague-Dawley Animal Company, widely used in experimental work because of their calmness and ease of handling (NDI foundation 2006)

[4] The feeding experiment took place in UBC Department of Psychology, Room #4310. Facilities in this department were ideal to conduct the experiment, as they are currently working in etho-experimental studies of rodent defensive behaviour and learning.

Animal Care During the Experiment

The cage of Group 1 was changed every 2 days and food and water were added when needed. The mother and pups were moved to a bigger cage when the pups were 10 days old. When the pups were weaned they were pair-housed according to sex. The four females were divided into 2 cages while the 8 males were paired into 4 cages.

The cage of Group 2 was also changed every 2 days but their feeding routine was different. The liquid diet was administered every morning. The amount of liquid diet given changed from 100 ml per day of the non-alcoholic diet (consumed by the mother during lactation) to 1200 ml per day just prior to the pups being weaned because the pups were now drinking. In accordance with the UBC Animal Care Committee, the maize-stalk beer was kept out of the diet until the pups were 25 days old and then alcohol was gradually added. Once the pups were weaned, they were housed according to sex. There were four cages; Cage 1 held 2 females, Cage 2 held 3 females, Cage 3 held 3 males and Cage 4 held 2 males.

The cage of Group 3 was changed every 2 days with food and water added when needed. The mother and pups were moved to a bigger cage when the pups were 11 days old. Once the pups were weaned, they were pair-housed according to sex. There were 2 cages that held 2 males each and 4 cages that held 2 females each.

Once the second generation rats had reached 275-300 grams, the experiment was completed. Following the UBC Ethics Committee procedures for animal care and treatment, the second generation rats were euthanized (see Appendix 2 - UBC Ethics Committee Approval Document). There are many methods of euthanasia. In selecting a method, the prime requisite is that it must be carried out humanely, causing only the absolute minimum amount of anxiety and pain to the animal. The methods commonly used for rats include: an overdose of anaesthetic, carbon dioxide, decapitation (guillotine), cervical dislocation and microwaves. While selecting a

method, it is important to take into account the purpose for which the animal is being killed (Waynford and Flecknell, 1992).

The method that was chosen was an overdose of carbon dioxide (CO_2). This method was selected in part because the laboratory had the equipment in place, because it was less intrusive and because it would not interfere with the carbon isotope analysis. As the pups were born on different days, the euthanasia of the second generation took place on different dates. The euthanasia was carried out for Group 1 on July 11th, 2005, and on July 19th, 2005 for Group 2 and Group 3. In the "Euthanasia room," CO_2 was pumped into the cage for at least 5 minutes; the rat's heartbeat was monitored to ensure they were not longer alive. After the euthanasia, Group 1 remained in a freezer in the Department of Psychology for 9 days while Group 2 and Group 3 were transported to the Department of Anthropology and Sociology after only one day in the Psychology Department's freezer.

The mothers were donated to another project in the Psychology Department at UBC to be used as surrogate mothers. Before donating them, hair samples were taken for analysis (for a future experiment).

Methods of Alcohol Administration in Rat Populations

Joanne Weinberg (1984:261-2; Weinberg, personal communication, 2003) proposes four main methods to expose pregnant rodents to alcohol. These include; injection, intubation, alcohol in the drinking water or, adding alcohol to a liquid diet. Both injection and intubation have the advantage that a controlled dose of alcohol can be administered and high blood alcohol levels can be obtained. The major criticism of these two methods is that both require a great deal of handling of the pregnant female and involve a fair amount of stress for the rat. Since prenatal stress in itself can affect hormonal, physiological and behavioural responses to rodent offspring, these may not be the best methods. Also, an increase in fetal deaths and/or resorptions, reduced

litter weights, and retarded postnatal growth has been observed using these methods (Gallo and Weinberg 1982; Weinberg 1984:261).

Placing alcohol in the drinking water and providing this as the only source of fluid is regarded as the simplest method of administrating alcohol. The majority of rodents, however, will not consume alcohol voluntarily, and if it is placed pure in the drinking water, they will reduce their fluid intake. With water intake suppressed, the amount of alcohol ingested and therefore blood alcohol levels are also reduced. While using this technique, lower body weights, retarded growth, deficient skeletal and muscle development, and delayed eye opening have been observed (Weinberg 1984:261).

The fourth option involves adding alcohol to a liquid diet. A liquid diet is based on, casein, enriched with methionine and cystine sucrose, and oil suspension mixture formulated to meet or to exceed the nutritional requirements needed (Lieber and DeCarli 1982: 523-26). This liquid diet is then mixed with alcohol and water in necessary quantities. Based on the condition that this is the only source of nutrition, this form of administering alcohol to pregnant rats appears to be a more effective method. This method results in greater and more consistent intake of alcohol, and more consistently elevated blood alcohol levels. Furthermore, it is less stressful to the pregnant rat than injection or intubation. Several concerns have been raised in relation to this method. One is that animals consume greater amounts of water with a liquid diet than they would with a pelleted diet. It is possible that increased fluid intake could affect water balance and/or kidney function, and thus contributed to fetal distress. The use of a semi-purified diet formulated specifically for rodents, and tailored to meet the needs of the particular experiment overcomes some of the problems that may occur (Weinberg 1984:261-2). Many liquid diets for rodents have been produced over the years; however, the most commonly used in rodent labs throughout North America is the Lieber-deCarli Liquid Diet.

The Liquid Diet

Prior to the Lieber-deCarli diet, alcohol had been commonly administered to animals as part of their drinking water. With this technique, however, alcohol intake is insufficient to result in sustained appreciable levels of alcohol in the blood. This low intake results from a natural aversion by many animals for alcohol which was overcome by the feeding of alcohol through exclusive liquid diets. (Lieber and deCarli 1982).

The composition of the Lieber-deCarli liquid diet formula is based on amino acid, sucrose, and oil suspension mixtures formulated to meet or to exceed the nutritional requirements needed. The adequacy of the diet was illustrated by the fact that when fed *ad libitum*, this type of diet promoted growth (in a way) comparable to that of commonly acceptable commercial diets. However, when alcohol was brought into the diet, spontaneous food consumption decreased, but it was nevertheless sufficient to ensure continued growth of the animals. To avoid the need for expensive amino acids, the original formula was changed to substitute the amino acids with casein, enriched with methionine and cystine (Lieber and deCarli, 1982:523-26).

The Lieber-deCarli liquid diet has three variants; The High Protein variant of the diet is useful for conditions such as gestation and lactation that require increased protein consumption (Lieber and deCarli 1982:529-30). The ingredients of the High Protein of the Lieber-deCarli liquid diets are shown in Table 2.

Due to the need for high nutrition during pregnancy and lactation, the High Protein variant was used in this experiment. The diet was custom made by Dyets Inc. in the USA. To meet the requirements of the experiment, the maize oil was replaced by soy oil and an increment of the other oils already present in the formula (olive and safflower) in order to control the amount of C_4 in the liquid diet shown in Table 3. By doing this, the only C_4 the rats were exposed to was obtained from the maize-stalk beer, not from the liquid diet.

Table 2. Lieber-deCarli Liquid Diet

Ingredients	Grams/L
Casein	57.6
L-cystine	0.65
DL-methionine	0.4
Maize Oil	2.5
Olive Oil	8.4
Safflower oil	2.7
Dextrin-maltose	155.6 **
Vitamin Mix	2.5
Salt mix	8.75
Choline bitartrate	0.53
Sodium Carrageenate	2
Total Grams	**241.63**

** in the alcohol formula, replaced by 66.0 g of dextrin-maltose and 50 g of alcohol

Table 3. Custom made Liquid diet

Ingredients	Grams/L
Casein	57.6
L-cystine	0.65
DL-methionine	0.4
Soy bean Oil	2.5
Olive Oil	8.4
Safflower oil	2.7
Dextrin-maltose	66
Vitamin Mix	2.75
Mineral mix	8.75
Choline bitartrate	0.53
Sodium Carrageenate	2
Xanthan Gum	3
Total Grams	**163.03**

To make 1 Litre of Liquid Diet. Mix 163 grams of Dyets #710341 with 530 ml of maize stalk beer (12% alcohol) & complete with cold water to one litre and mix for 30 second in a blender.

Alcohol interacts with nutrition in many ways, and often, nutrition in animal experiments is overlooked, even when there is a need of nutritional control. When alcohol is added to an animal diet, the animal may modify its nutritional intake because alcohol has a high energy value (7.1 kcal/g) and may displace other foods in the diet. Calories in alcohol are not associated with vitamins, minerals, proteins or other essential nutrients. These calories are called "empty calories" and can result in nutritional deficiencies. Alcohol also has an anorexigenic effect, and may compromise nutrient intake. It is well known that pregnant or lactating females, whose nutritional requirements are greater than non-lactating, can be in a state of nutritional deficiency when being fed alcohol in large quantities (Weinberg 1984:262).

As mentioned above, food intake and nutrient intake is invariably reduced in animals and humans consuming alcohol in large quantities. Investigators working with animal models have begun to include a control group, which is "pair-fed" to the alcohol, in order to control for this reduced intake. Each animal in the control group receives control diet (with carbohydrates isocalorically substituted for alcohol) in the amount consumed by its partner in the alcoholic diet on the previous day. The inclusion of a pair fed group enables the investigator to separate effects due to pharmacologic actions of alcohol from those related by alcohol malnutrition (Weinberg 1984:265). Although alcohol passes freely from the maternal circulation into the breast milk, and from there to the nursing infant, maternal under-nutrition can reduce the amount of milk available and thus compromise offspring nutritional status. Alcohol can also directly affect milk secretion, and this could reduce offspring weight gain and harm nutritional status (Weinberg, 1984:266).

As the objective of this experiment is to find out if C_4 from maize stalk beer leaves any traces in the bone collagen, and not the relationship between nutrition and alcohol, a "pair-fed" group was not used in this experiment. However, the nutritional status of both mothers and pups was monitored during the whole experiment to ensure that they were not under-nourished in any way. Weight gain during the experiment was a very important issue to take in consideration. In order to monitor this, the rats were weighed every week to ensure weight gain. Appendix 1 illustrates the weight gain for individual rats in every diet group complied with the standards outlined by the UBC Animal Care Centre.

EXPERIMENTAL PROCEDURES

The euthanized rats were separated by diet and by cage, and were stored from mid July until mid-September in a freezer in the Anthropology Department. The 34 individuals were then transferred from the freezer to a fridge and stored for 24 hours. Under the supervision of Dr. Chisholm, four samples were taken from each individual; a long bone sample from the hind leg, a portion of muscle from the hind leg, hair, and 2-4 teeth. In order to have samples for future study, a single hind leg was removed for this experiment leaving the rest of the body intact for long-term storage. The long bone samples were manually cleaned of flesh, tendons, cartilage, and marrow. Soluble lipids (fat) were then removed by soaking the bone in acetone (CH_3COCH_3) until the bone was clean. The final step, to ensure collagen purification, involved a demineralization of the bone using Hydrochloric Acid (HCl). Collagen was then solubulized by heating the sample in water at pH3; it was then filtered, condensed by evaporation and freeze-dried (Ambrose and Norr 1993:18-19; Chisholm, personal communication, 2005, Chisholm and Blake 2006:162). The collagen samples where then sent to the Isotopic lab in the Department of Earth and Ocean Science at UBC for stable carbon and nitrogen isotope analysis. The muscle material was manually removed from the bone samples, processed with acetone (CH_3COCH_3) in order to remove all the soluble lipids, and then transferred to a mortar and pestle for grinding. The crushing and grinding of the muscle material involved adding more acetone when needed and then it was filtered through a thin paper filter and placed in vials for long-term storage. Also placed in long-term storage for future studies were 34 hair samples and 2-4 teeth taken from each of the individuals. Hair samples were also taken from the 3 mothers prior to their transfer to another experiment. These samples in long-term storage could be utilized, but not limited to, experiments on bone apatite, tooth enamel, or collagen tests of hair and muscle. If necessary, the experiment on bone collagen discussed below could be replicated because the 34 individuals remain in long-term storage.

EXPECTATIONS OF THE EXPERIMENT

Considering the results of the experiments conducted by Ambrose and Norr (1993) and the Stalk-Sugar Hypothesis of Smalley and Blake (2203), it was expected that the diet of Group 2 (maize-stalk beer diet) would not fall in the C_4 range of the stable carbon isotope analysis because maize-stalk beer builds calories and vitamins, not protein. As we know, collagen is formed from the protein portion of the diet not from calories received from carbohydrates or fats. So, although the maize-stalk beer was brewed from parts (stalks) of the maize plant (C_4), when it is consumed in the form of an alcohol, it may not deposit in the collagen as an expected C_4 value. However, the Group 1 diet was designed as a 70% C_4 diet with the expectation that when consumed it would produce a typical C_4 value (greater than -15‰) comparable with the studies by Blake (2006:66); Chisholm (1989:13); Smalley and Blake (2003:684); Tykot (2006:132); Tykot and Staller (2002:669) and Ubelaker et al. (1995:404). As a further test, the Group 3 diet (C_3 solid diet) was expected to demonstrate values within the typical range (-26.5‰ to –16‰) of a diet that is derived from C_3 sources. While the central goal of this thesis focuses on the consumption of maize-stalk beer and its stable carbon isotopic value, the diets created for Groups 1 and 3 provide the necessary parameters to measure the values of Group 2. It was expected that Group 2 (maize-stalk beer diet) would cluster closer to the individuals of Group 3 (C_3 solid diet) rather than the individuals in Group 1 (C_4 solid diet) because the maize-stalk beer should not be recognized in the collagen as a C_4 value.

RESULTS

Collagen was extracted from the bone samples using well-established laboratory procedures described above (see Ambrose and Norr 1993:18-19; Brian Chisholm, personal communication, 2005) and results from the stable isotope of carbon and nitrogen analysis were received. As the samples were divided in diet, cage number and gender (when applicable); so were

the results. Tables 4, 5 and 6 present the results of the isotopic analysis of stable carbon and nitrogen. Table 4, the results from Group 1 (C_4 solid diet), shows a mean value of $\delta^{13}C$ -14.88‰, which is consistent with the expectations for individuals consuming a moderate to high C_4 diet (i.e., is greater than -15‰). The mean for the $\delta^{15}N$ value is 4.25‰, which is consistent with the expected values of a individual consuming maize (C_4) or C_3 leguminous plants (see Figure 10-2 in Tykot 2006:134; Ubelaker et al 1995:404).

Table 4. Results of Isotopic Analysis from Group 1 (C_4 solid diet)

Sample	C_4 solid diet	$\delta^{15}N$	$\delta^{13}C$
1	Cage 2(F) Bone	4.56	-14.42
2	Cage 1(F) Bone	4.47	-14.16
3	Cage 3 Bone	4.41	-14.62
4	Cage 1 Bone	4.25	-15.13
5	Cage 3 Bone	4.32	-14.96
6	Cage 1(F) Bone	4.56	-14.76
7	Cage 4 Bone	4.14	-15.85
8	Cage 2(F) Bone	4.52	-14.50
9	Cage 1 Bone	4.24	-15.24
10	Cage 2 Bone	4.01	-14.92
11	Cage 4 Bone	4.15	-14.90
12	Cage 2 Bone	4.47	-15.11
Mean		**4.34**	**-14.88**
Range		**4.01 – 4.56**	**-14.16 to -15.85**

The results from the Group 3 diet can be seen in Table 5, and provide a mean $\delta^{13}C$ value of –22.14‰, which falls within the expected range of individuals who consume C_3 plants with little to no maize (-26.5‰ to -16‰). The nitrogen values from this diet, with a mean of 2.72 are again the expected values for individuals consuming either maize (C_4) or C_3 leguminous plants. While the carbon values from Group 1 and Group 3 diets indicate that the individuals are on C_4 or C_3 diet respectively, the nitrogen values shows that both groups were consuming plants or terrestrial fauna, rather than fish or mammals.

Table 5. Results of Isotopic Analysis from Group 3 (C_3 solid diet)

Sample	C_3 solid diet	$\delta^{15}N$	$\delta^{13}C$
16	Cage 4 Bone	2.61	-22.49
17	Cage 5 Bone	2.54	-22.36
18	Cage 2 Bone	2.57	-23.62
19	Cage 2 Bone	2.45	-21.98
20	Cage 1 Bone	2.68	-21.95
21	Cage 6 Bone	2.91	-22.17
22	Cage 4 Bone	2.92	-22.35
23	Cage 6 Bone	2.73	-21.94
24	Cage 3 Bone	2.70	-21.76
25	Cage 5 Bone	2.85	-21.41
26	Cage 1 Bone	2.54	-22.08
27	Cage 3 Bone	3.08	-22.18
Mean		**2.74**	**-22.14**
Range		**2.45 – 3.08**	**-21.41 to –23.62**

Finally, the results from Group 2 (maize-stalk beer diet) can be seen in Table 6, and produced interesting yet not unexpected results. The mean $\delta^{13}C$ value of -18.80‰ which falls within the expected -20‰ to -15‰ range, but clearly when interpreting the -18‰ value ($\delta^{13}C$) the isotopic values for the powder portion of the diet must be considered. The isotopic value ($\delta^{13}C$) for the powder portion if the diet was -18.17‰. It is important to note that although the C_3 portion of the diet was minimal compared to the C_4 portion, the results for $\delta^{13}C$ show a stronger correlation to a C_3 value. The mean for the $\delta^{15}N$ value of 8.51‰ is consistent with the expected values of individuals consuming C_3 based terrestrial fauna.

The diets were also analysed to understand what influence the diets isotopic values may have had on the rat collagen, see Table 7. As mentioned above, pure maize and a pure maize-stalk beer diet did not fulfill the nutritional needs outlined by the UBC Animal Care Centre. Due to the fact that vitamins and minerals were added to all the Groups' diets, it was necessary to test the $\delta^{13}C$ and $\delta^{15}N$ values of the diets in order to incorporate these values into the bone collagen results (see Table 7 and Figure 1). It was important to test each diet to see if the added vitamins or minerals influenced the carbon or nitrogen values in the food. As the diets were served as the only source of food for the rats, the carbon and nitrogen values in each diet served as indicators of the

diets the rats were consuming and what values could be expected for each group. As mentioned before, modern C_3 plants have average values of about –26.5‰ whereas modern C_4 plants have averages of about –12.5‰. This separation of 14‰ allows for discrimination between group averages (Chisholm 1989:13).

Table 6. Results of Isotopic Analysis from Group 2 (Maize-stalk beer diet)

Sample	Maize-stalk beer diet	$\delta^{15}N$	$\delta^{13}C$
29	Cage 2 Bone	8.80	-18.55
30	Cage 1 Bone	8.56	-18.69
31	Cage 4 Bone	8.34	-18.85
32	Cage 4 Bone	8.17	-19.66
33	Cage 3 Bone	8.20	-18.69
34	Cage 1 Bone	8.49	-18.51
35	Cage 2 Bone	8.72	-18.34
36	Cage 2 Bone	8.69	-19.20
37	Cage 3 Bone	8.50	-18.88
38	Cage 3 Bone	8.64	-18.60
Mean		**8.51**	**-18.80**
Range		**8.17 – 8.80**	**-18.34 to –19.66**

Table 7. Results of the Isotopic Analysis of the Diets

Sample	$\delta^{15}N$	$\delta^{13}C$
Group 1 diet (C_4 solid diet)	1.902	-15.74
Group 2 Powder diet formula #710341 (C_3)	5.546	-18.17
Group 3 diet (C_3 solid diet)	1.606	-25.09

The C_4 solid diet consisted of 70% C_4 and 30% C_3 which may account for why the diet returned a δ13C value of -15.74‰. This value clusters near the average value of -12.5‰ for C_4 plants; but the value has been affected by the C_3 component in the diet. The C_3 solid diet $\delta^{13}C$ value of -25.09‰ is consistent with the average of -26.5‰ for modern C_3 plants. The powder diet formula #710341 has a $\delta^{13}C$ value of -18.17‰, which falls within the 14‰ separation between the C_3 and C_4 averages. This $\delta^{13}C$ value was not expected because the diet was designed with only C_3 ingredients to which the maize-stalk beer was added. This is of interest because in this experiment, the diets were controlled and the isotopic analysis of this diet could be tested, but in an archaeological sample, the diets can only be postulated.

Figure 1 shows the distribution of the stable carbon isotope ratios ($\delta^{13}C$ and $\delta^{15}N$) for all of the individual rats sampled, as well as for the three diet samples tested. The three diets are grouped according to their isotopic ratio measurements. The values of the food are also added to the graph. The figure shows that the isotopic reading for the rats is very similar to that of the foods. The results for the individual rats in Group 2 (C_3 powder diet + C_4 from maize-stalk beer) who consumed the maize-stalk beer diet only reflect the C_3 value of the original powder diet. As expected, the maize-stalk beer (as a C_4 value) is virtually invisible in the values measured for Group 2, which clusters closer to a C_3 reading than that of a C_4.

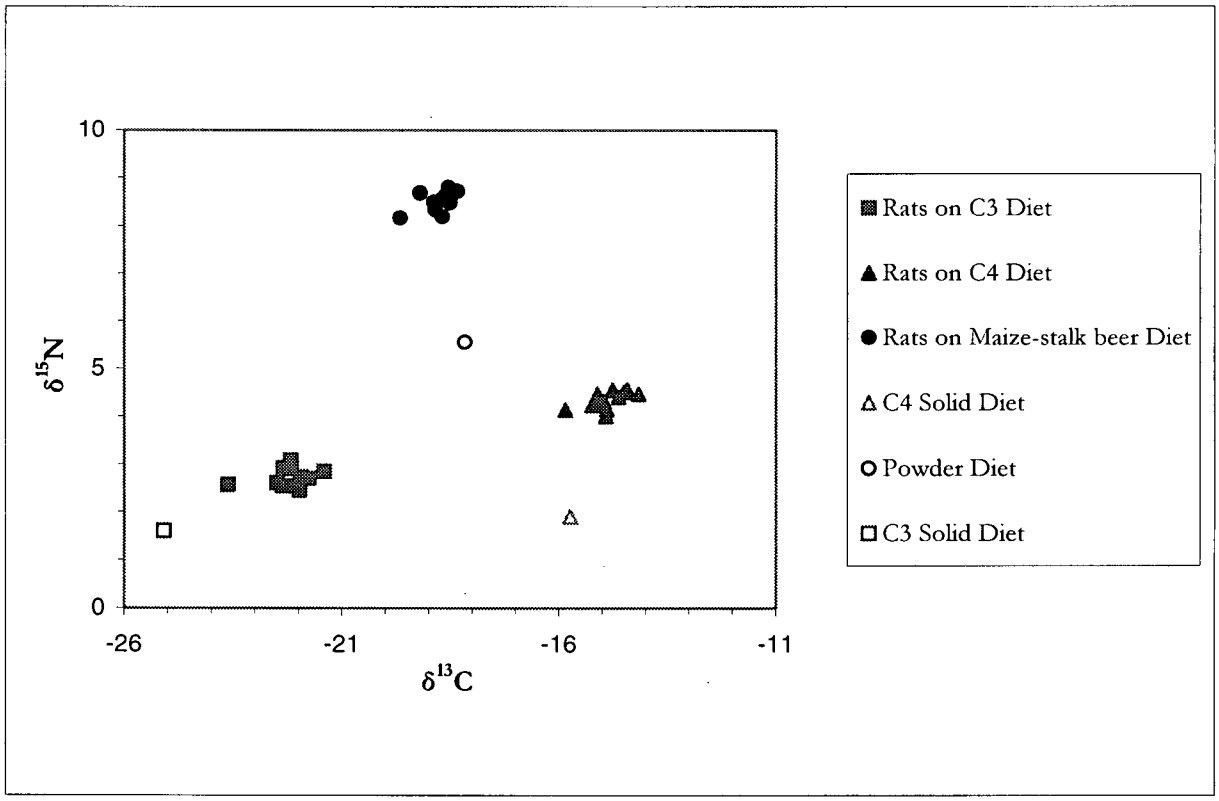

Figure 1. Distribution of Stable Carbon Isotope Ratios for Individual Rats

The results of this experiment support two important ideas. First, they support the hypothesis that maize consumed in the form of alcohol (beer) does not leave any measurable traces in bone collagen. Second, this experiment supports the idea noted by Ambrose and Norr (1993:27-28) and Chisholm et al. (1982) that protein in consumers is built from the protein

portion of the diet, not from fats or carbohydrates. Thus, the carbon isotopes that make up dietary protein are directly reflected in the carbon isotopes found in the bone collagen. The content of protein in the maize-stalk beer is insignificant as it contains mostly sugars and water (Smalley and Blake 2003:684). This seems to be the reason why the maize-stalk beer did not show up in the rats bone collagen analysis.

CONCLUSION

Being able to demonstrate that consuming C_4 plants (maize) as an alcoholic beverage does not show in carbon isotope analysis of bone collagen is a very important step in archaeological research on the origin and spread of early maize. For some years now, archaeologists have been trying to understand the reasons why the first Mesoamericans started to cultivate maize, and the focus has generally been on the yield from the cobs rather than the other parts of the plant. However, if we can separate ourselves from the importance maize has as a cereal in today's world, and focus on other uses maize may have had in the past, this perspective can help us understand the reason for its first domestication and spread throughout the Americas. Research is beginning to consider other potential uses for the maize crop in the past. Iltis's (2000) suggestion that teosinte was first cultivated for its sweet stalk and tender ears; and Smalley and Blake's (2003) proposition that the stalks were used to make alcoholic beverages are viable alternatives. As mentioned previously, maize was first domesticated in the Río Balsas region of southwestern Mexico before 6,000 B.P. (Benz 1999, 2006; Matsuoka et al, 2002, Smalley and Blake 2003:678). Paleoethnobotanical evidence shows the presence of domesticated *Zea* at least as early as 5,400 B.P. in Mexico (Piperno and Flannery 2001:2102) and isotopic data suggests that maize did not become a dietary staple until 2500 years later. To explain this "isotopic gap" in maize use, Smalley and Blake (2003:678) proposed the "Stalk-Sugar Hypothesis", in which they argue that the extraction of stalk juice – as a sweetener and possibly for making alcohol - may have been the key

factor in the domestication of *Zea.* I agree with Smalley, Blake and Iltis that this different way of approaching the subject can yield some new light on the origin of maize use. I also agree in the advantages of not focusing on the tendency that Western scholars have in overemphasizing maize's dietary importance. As Tykot and Staller (2002: 43) mentioned, in South America, at least, maize was used more as a vegetable than as a cereal.

With regard to isotopic analysis, Smalley and Blake (2003) suggest the possibility that the practice of converting maize to alcoholic beverages may explain low stable carbon isotope values during the "isotopic gap". They also explained "consumption of beer made from the juice would not necessarily have produced higher stable carbon isotope ratios in human bone collagen" (Smalley and Blake 2003:686). The average $\delta^{13}C$ value (of –18.80‰) obtained from Group 2 offers validation of the idea that beer made from maize products does not produce a strong C_4 reading in bone collagen as predicted by Ambrose and Norr (1993) and Smalley and Blake (2003). As this experiment has shown, maize in the form of an alcoholic beverage does not exhibit a C_4 value in stable carbon isotope analysis in bone collagen. These results should serve as a caution for future investigations in stable carbon isotopic analysis on bone collagen. It appears that the importance of maize present in the diet of ancient peoples of Mesoamerica, is measurable through carbon isotope analysis of collagen only if people were eating maiz rather than "drinking" it. (Ambrose and Norr 1993; Chisholm 1989; Farnsworth et al 1985; Tykot and Staller 2002; Uberlaker et al 1995; Vogel and van der Merwe 1977 among others) The approach to overcoming the problem of invisible maize beer consumption is to also analyze the stable carbon isotope values of apatite in bone and tooth enamel. These are proven to reflect the whole diet (Tykot 2006:131-2). Future analysis of collagen and apatite from ancient peoples who were known to have consumed C_4 plant beverages such as *chicha* could be helpful in better understanding this process and the necessary precautions to be taken when using stable carbon isotope analysis among (e.g., The Wari brew master and Cerro Baul in Southern Peru, Moseley et al. 2006)

The possibility of other uses of early maize should be taken into consideration when searching for archaeological evidence. As Smalley and Blake (2003:682) mentioned, maize stalk uses can be inferred both by direct and indirect archaeological evidence. The presence of maize stalk and maize stalk quids[5] in dry caves in Archaic Period and later deposits is so far the only direct evidence (see Smalley and Blake 2003:682-684 for a thorough description). Sources of indirect evidence are isotopic analysis in human bone samples (apatite in bone and tooth enamel, as well as collagen and nitrogen); the presence of pottery or any other type of containers that may have worked as part of the beverage preparation/storage/consumption process (large ceramic jars suitable for brewing as reported by Ubelaker et al. 1995:409) and artefacts that may have been use as presses to extract the juice of the stalk. Chemical studies on ceramic vessels to identify function and uses of the container can also be used as evidence. It is important to use as many tools as archaeologists have available today to help understand maize domestication and early spread. The results obtained in this research, do not prove that the consumption of maize as an alcoholic beverage happened at the beginning of maize domestication, but this research demonstrates that Smalley and Blake's and Iltis's hypotheses are viable and that this "alternate" use of maize may have been one of the original ones.

The next step in research that will follow from this project is to analyze the stable carbon isotope values of the remaining samples of muscle, hair, specially the apatite in both bone and teeth. Once these other values are known, we will have a better understanding of the differential pathways of carbon from diet vs. carbon from alcohol consumption in ancient peoples. This, I hope, will lead to new knowledge about the role of maize alcohol in the ancient cultures of Latin America and ultimately shed light on the questions of the origin and spread of maize in antiquity.

[5] The mass of plant fibre that was chewed and expectorated after sucking out the sweet juice (Smalley and Blake 2003:682).

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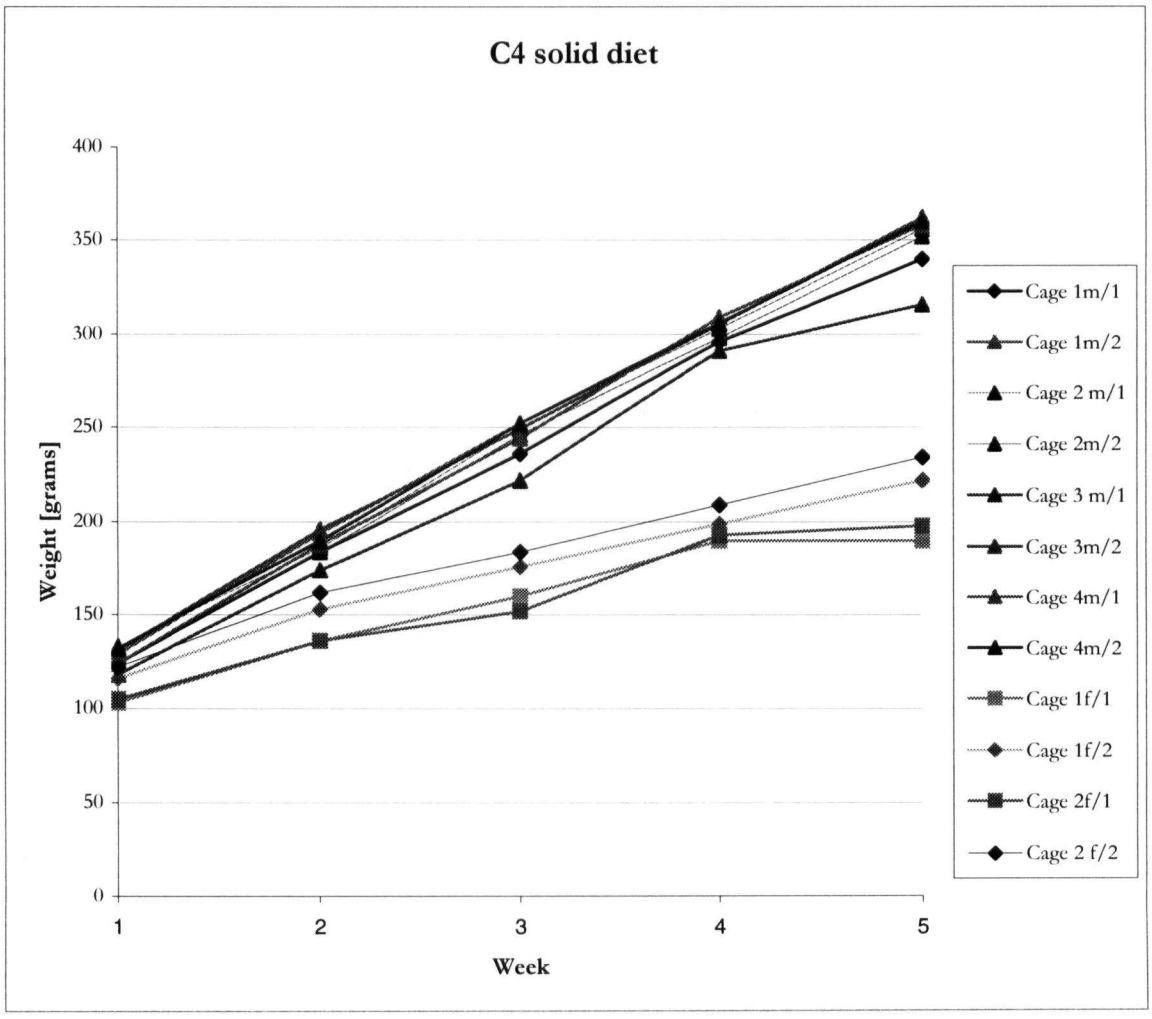
APPENDIX 1 – Rats weight's gain

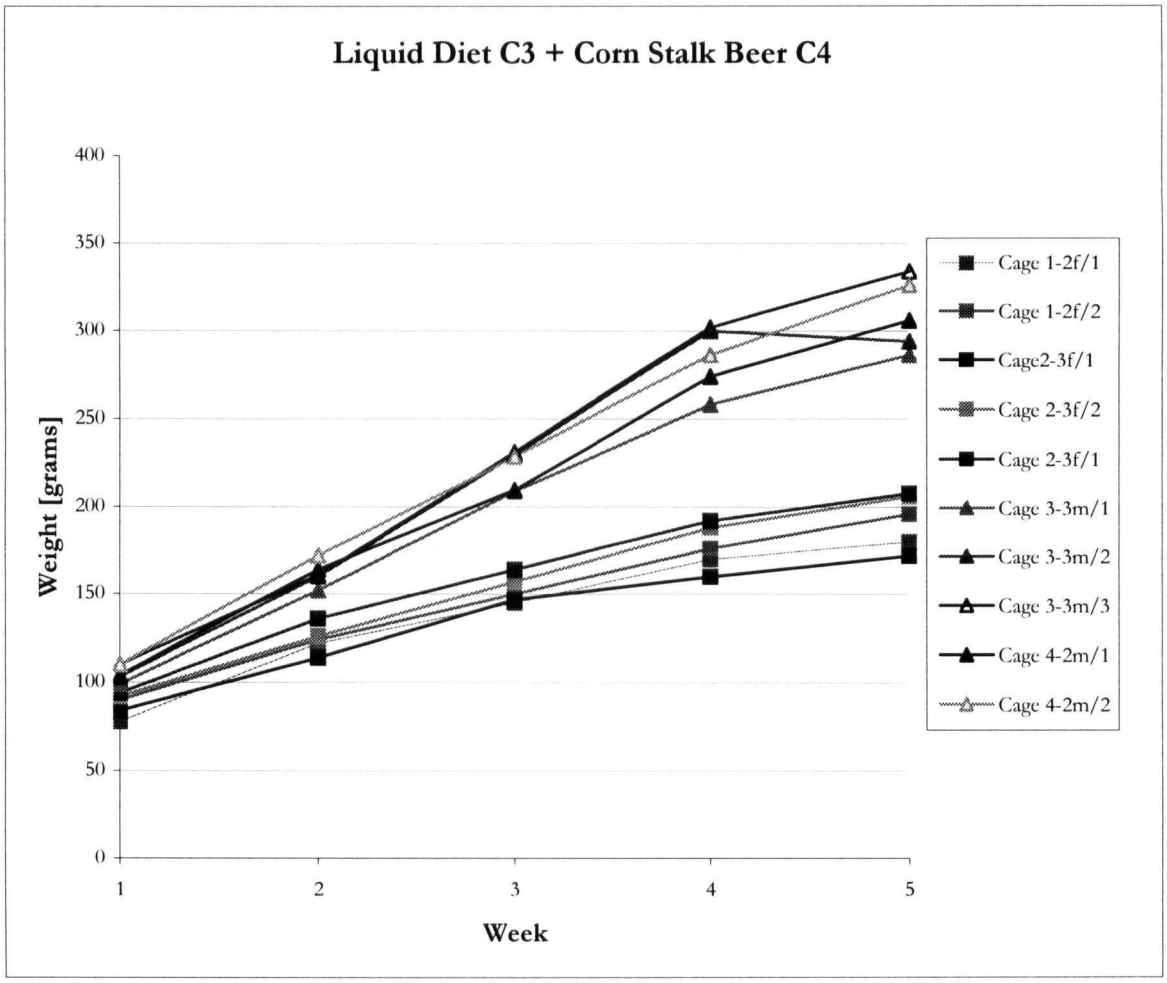
C_4 solid diet

	Jun-08	Jun-15	Jun-22	Jun-29	Jul-06
	124	184	236	296	340
Cage 1 m	129	194	252	306	360
	125	184	246	298	352
Cage 2 m	133	186	250	303	356
	118	174	222	291	316
Cage 3 m	131	196	249	306	362
	124	188	244	309	358
Cage 4 m	132	190	252	306	360
	103	136	160	190	190
Cage 1 f	116	153	176	199	222
	105	136	152	193	198
Cage 2 f	122	162	184	209	234



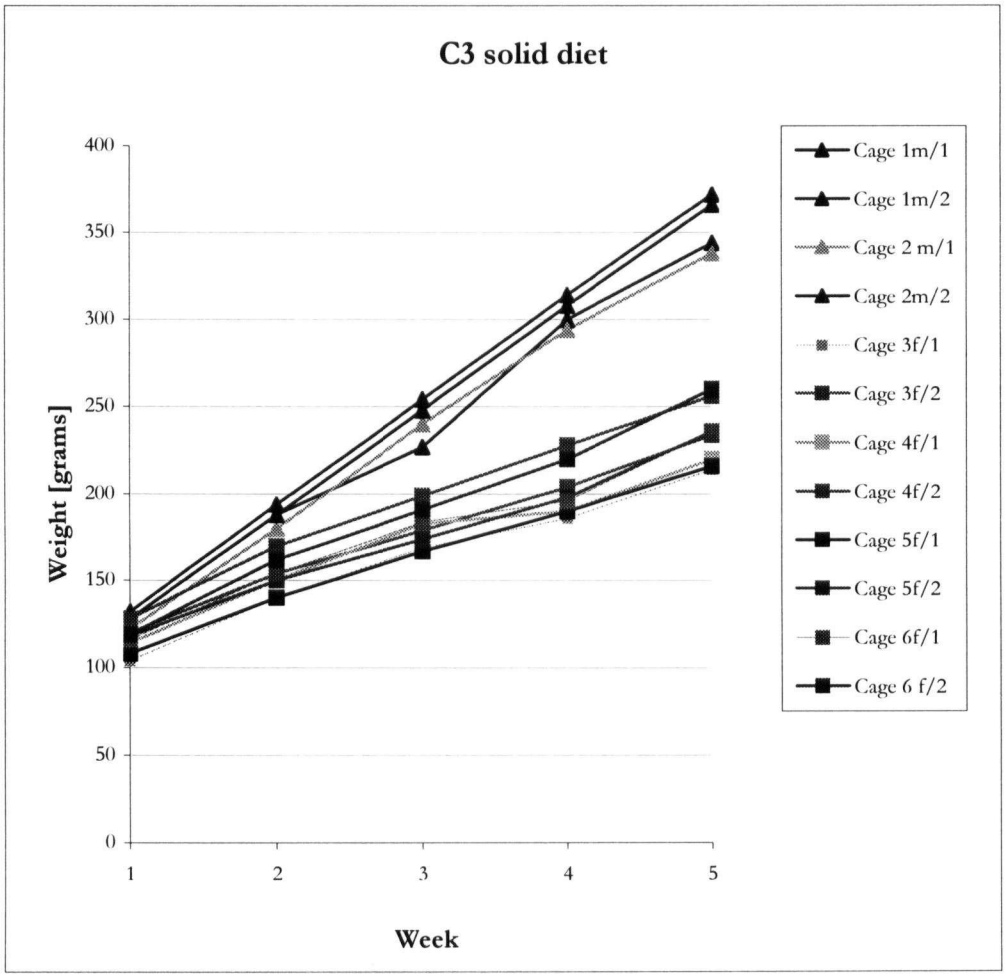
Maize-stalk beer diet

	Jun-15	Jun-22	Jun-29	Jul-06	Jul-13
	78	122	145	170	180
Cage 1 2f	90	124	150	176	196
	84	114	147	160	172
	92	126	157	188	206
Cage 2 3f	94	136	164	192	208
	99	152	209	258	286
	103	160	230	300	294
Cage 3 3m	110	161	232	302	334
	104	164	210	274	306
Cage 4 2m	110	172	229	286	326



C3 solid diet

	Jun-15	Jun-22	Jun-29	Jul-06	Jul-13
	128	188	227	300	344
Cage 1 m	132	194	254	314	372
	122	180	240	294	338
Cage 2 m	128	188	248	308	366
	104	140	169	186	214
Cage 3 f	120	154	179	204	234
	114	150	183	190	220
Cage 4 f	128	170	199	228	256
	108	140	167	190	216
Cage 5 f	118	150	174	198	236
	116	154	184	196	236
Cage 6 f	119	162	191	220	260



APPENDIX 2. UBC Ethics Committee Approval Document

The University of British Columbia

Animal Care Certificate

Application Number: A04-1035

Investigator or Course Director: Michael T.M. Blake

Department: Anthropology & Sociology

Animals Approved: Rats 39

Start Date: **2005-1-15** Approval Date: 2005-2-22

Funding Sources:

Funding Agency:	Unfunded Research
Funding Title:	Stable Isotope Analysis of Maize Beer Consumption
Unfunded title:	Stable Isotope Analysis of Maize Beer Consumption

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility

Office of Research Services and Administration
102, Agronomy Road, Vancouver, V6T 1Z3
Phone: 604-827-5111 Fax: 604-822-5093