

## ANTHROPOLOGY

## Early isotopic evidence for maize as a staple grain in the Americas

Douglas J. Kennett<sup>1\*†</sup>, Keith M. Prufer<sup>2,3\*†</sup>, Brendan J. Culleton<sup>4</sup>, Richard J. George<sup>5</sup>, Mark Robinson<sup>6</sup>, Willa R. Trask<sup>7</sup>, Gina M. Buckley<sup>5</sup>, Emily Moes<sup>2</sup>, Emily J. Kate<sup>5</sup>, Thomas K. Harper<sup>5</sup>, Lexi O'Donnell<sup>8</sup>, Erin E. Ray<sup>2</sup>, Ethan C. Hill<sup>2</sup>, Asia Alsgaard<sup>2</sup>, Christopher Merriman<sup>2</sup>, Clayton Meredith<sup>9</sup>, Heather J. H. Edgar<sup>2</sup>, Jaime J. Awe<sup>10</sup>, Said M. Gutierrez<sup>11</sup>

Maize is a cultigen of global economic importance, but when it first became a staple grain in the Americas, was unknown and contested. Here, we report direct isotopic dietary evidence from 52 radiocarbon-dated human skeletons from two remarkably well-preserved rock-shelter contexts in the Maya Mountains of Belize spanning the past 10,000 years. Individuals dating before ~4700 calendar years before present (cal B.P.) show no clear evidence for the consumption of maize. Evidence for substantial maize consumption (~30% of total diet) appears in some individuals between 4700 and 4000 cal B.P. Isotopic evidence after 4000 cal B.P. indicates that maize became a persistently used staple grain comparable in dietary significance to later maize agriculturalists in the region (>70% of total diet). These data provide the earliest definitive evidence for maize as a staple grain in the Americas.

## INTRODUCTION

Maize is a staple grain of global dietary importance (1), shaping ecosystems, landscapes, cultures, fire regimes, and biodiversity. However, the early adoption of this crop as a staple (here defined as >25% of total diet) is unknown because of the dearth of archeological sites in the Americas containing early skeletal assemblages needed to directly assess its dietary contribution. Here, we use stable isotope evidence from an unparalleled time series of human skeletal samples, excavated from two rock shelters in Belize spanning the past 10,000 years, to reconstruct the dietary importance of maize through time. We demonstrate that maize became a significant dietary staple for some individuals starting between 4700 and 4000 calendar years before present (cal B.P.) and a persistently used staple grain by 4000 cal B.P. (>70% of total diet). The demographic and environmental impact of this transition had far-reaching consequences for the people of Mesoamerica that have now been amplified on a global scale, particularly in attaining food security in the context of increasingly volatile global climate change.

Maize was domesticated from teosinte (*Zea mays* spp. *parviglumus*), a wild grass growing in the lower reaches of the Balsas River Valley of southwestern Mexico (Fig. 1), and molecular clock estimates suggest that it was domesticated in the Early Holocene (~9000 cal B.P.) (2). Microfossils (phytoliths and starch) confirm the use of maize in the Balsas by ~8700 cal B.P. (3). Microfossil data also support widespread dispersal of this important domesticate through the lowland

neotropics by at least ~7000 cal B.P. (4). However, the initial dietary significance of maize and the process and timing of its adoption as a staple carbohydrate in the human diet remain unclear. Two rock shelters in Belize are the only known contexts in the Americas containing human skeletal material deposited persistently over the past 10,000 years and during the transition to maize-based food production. Bone preservation in these dry rock shelters provides an unparalleled opportunity to study dietary change associated with the introduction of maize into the region and its subsequent increasing economic and dietary importance across the New World.

Early maize cobs (~6250 cal B.P.) from Guilá Naquitz in the Mexican highlands are small and have only two seed rows (5). Ancient DNA data from the earliest Tehuacán Valley cobs (5300–4950 cal B.P.) indicate a mixture of modern maize-type alleles, controlling stalk and inflorescence architecture, and ancestral teosinte alleles, controlling ear shattering and starch biosynthesis (6, 7). In addition, paleoethnobotanical, paleoecological, and paleogenomic data from South America suggest that maize was dispersed by foraging populations, possibly in a semidomesticated state, as early as ~7000 cal B.P. (8). These observations have led some to speculate that domesticated maize was initially of little dietary importance but instead spread because of the sugar content of stalk juice, enhancing its social significance as a fermented beverage (9). Selection for larger, multirow cobs by ~4300 cal B.P. (10), along with paleoecological studies indicating increased burning, forest clearance, and erosion associated with maize pollen (11), suggests the increasing importance of maize as a staple crop, but even its dietary significance at that time remains elusive.

Stable isotopes from radiocarbon (<sup>14</sup>C)-dated archeological bone collagen ( $\delta^{13}\text{C}_{\text{collagen}}$  and  $\delta^{15}\text{N}_{\text{collagen}}$ ) combined with bone apatite ( $\delta^{13}\text{C}_{\text{apatite}}$ ) provide a powerful measure of the dietary significance of maize in the Americas.  $\delta^{13}\text{C}_{\text{collagen}}$  is a proxy for the primary sources of protein in an individual's diet. Variation results primarily from the isotopic composition of animals and the plants they consume and how the primary producer plants metabolize carbon during photosynthesis. The lowland tropics of Mesoamerica are dominated by plants using the C<sub>3</sub> (Calvin-Benson) photosynthetic pathway with distinctively negative  $\delta^{13}\text{C}$  values [~-26.5 per mil (‰)] (12). Maize was

<sup>1</sup>Department of Anthropology, University of California, Santa Barbara, CA 93106, USA.

<sup>2</sup>Department Anthropology, University of New Mexico, Albuquerque, NM 87131, USA.

<sup>3</sup>Center for Stable Isotopes, University of New Mexico, Albuquerque, NM 87106, USA.

<sup>4</sup>Institutes of Energy and the Environment, The Pennsylvania State University, University Park, PA 16802, USA.

<sup>5</sup>Department of Anthropology, The Pennsylvania State University, University Park, PA 16802, USA.

<sup>6</sup>Department of Archaeology, Exeter Univ. Exeter, UK.

<sup>7</sup>Central Identification Laboratory, Defense POW/MIA Accounting Agency, Joint Base Pearl Harbor-Hickam, Hawaii, 96853 USA.

<sup>8</sup>Department of Sociology and Anthropology, University of Mississippi, University, MS 38677, USA.

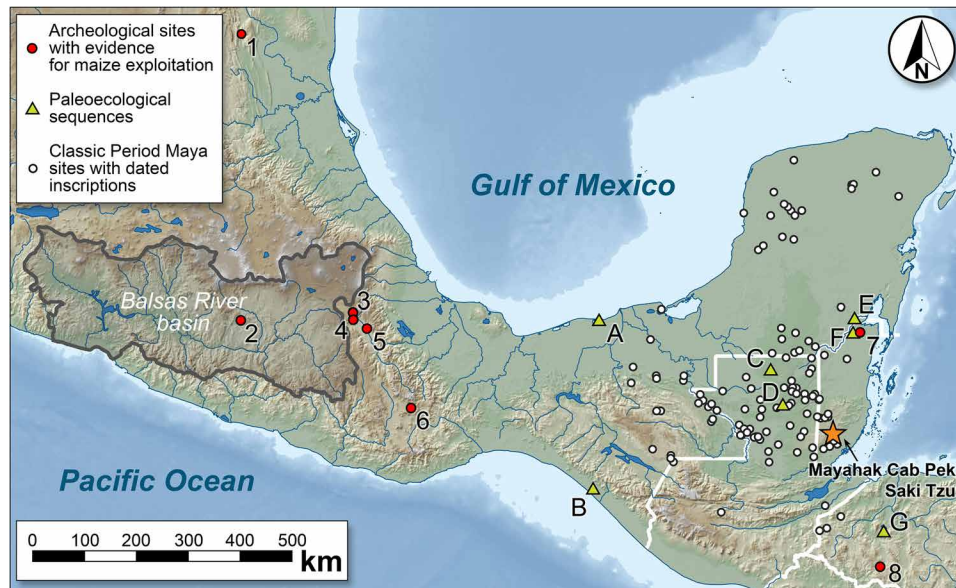
<sup>9</sup>New Mexico BioPark Society, 903 10th Street SW, Albuquerque, NM 87102, USA.

<sup>10</sup>Department of Anthropology, Northern Arizona University, Flagstaff, AZ 86001, USA.

<sup>11</sup>Ya'axché Conservation Trust, Punta Gorda Town, Belize.

\*Corresponding author. Email: kennett@anth.ucsb.edu (D.J.K.); kmp@unm.edu (K.M.P.)

†These authors contributed equally to this work.



**Fig. 1. Map showing the location of MHCP and ST relative to archeological sites in Mesoamerica with early maize, Classic Period Maya sites with dated inscriptions, and paleoenvironmental sequences.** Paleoecological sequences are shown as green triangles as follows: (A) San Andres, Tabasco (36); (B) SOC05-2 (19); (C) Puerto Arturo (11); (D) Peten Lakes region (57); (E) Cob Swamp (38); (F) Pulltrowser Swamp (38); and (G) Lake Yojoa (58). Archeological sites with early maize are shown as red dots: (1) Ocampo Caves (59), (2) Xihuatoxtla (3), (3) El Riego (60), (4) San Marcos (60), (5) Coxcatlán (60), (6) Guilá Naquitx (5), and (7) Caye Coco (25). Map was produced in ArcGIS 10.4, with all subsequent layout and design performed in Photoshop CC 14.2.

the primary plant domesticated in the lowland neotropics, and it uses the  $C_4$  (Hatch-Slack) photosynthetic pathway with distinctively more positive isotopic values ( $\sim -9.3\text{‰}$ ) (13). Isotopically, the increasing dietary importance of this domesticate should be evident with more positive  $\delta^{13}\text{C}$  ratios. The consumption of marine resources can complicate the interpretation of  $\delta^{13}\text{C}$  data by mimicking the consumption of  $C_4$  plants if they are not evaluated in conjunction with nitrogen isotope data. Differences in nitrogen isotope values ( $\delta^{15}\text{N}$ ) are sensitive to the trophic position of plants and animals consumed and are used to distinguish between terrestrial and aquatic food consumption (14). Herbivores are enriched by 3 to 7‰ relative to the plants eaten, and carnivores are enriched by 3 to 5‰ compared to the animals eaten. Marine mammals and fish are generally enriched by 5 to 10‰ relative to the terrestrial mammals. However, fish from coral reef ecosystems have high  $\delta^{13}\text{C}_{\text{collagen}}$  and low  $\delta^{15}\text{N}_{\text{collagen}}$  values that overlap isotopically with maize (15). In addition,  $\delta^{13}\text{C}_{\text{collagen}}$  is strongly biased to the protein component of the diet, but  $\delta^{13}\text{C}_{\text{apatite}}$  generally reflects the whole diet (carbohydrates, lipids, and proteins) (16). Maize kernels contain 8 to 11% protein and 73% carbohydrate, but maize stalk juice is largely composed of carbohydrates and sugar. Therefore, the consumption of fermented beverages from carbohydrate-rich and protein-poor sugary stalk juice (e.g., maize beer or chicha) would be more likely to be visible in  $\delta^{13}\text{C}_{\text{apatite}}$  and not in  $\delta^{13}\text{C}_{\text{collagen}}$  (17).

Human skeletal material in the Americas is relatively rare before  $\sim 3000$  cal B.P., and the decomposition or contamination of bone collagen and apatite in the humid lowland neotropics has largely limited stable carbon and nitrogen isotope studies of skeletal material dating before this time. Until our work, the handful of samples available in the lowland neotropics were too poorly preserved to yield reliable results or were from earlier studies that analyzed unpurified collagen that was not directly radiocarbon-dated to verify age (18). One of these studies on the Pacific coast of southern Mexico provides a potentially important time series of samples spanning the

last 4500 years (17). The two earliest samples in that study (from contexts thought to be 4500 and 4000 years old) have  $\delta^{13}\text{C}_{\text{collagen}}$  and  $\delta^{15}\text{N}_{\text{collagen}}$  consistent with significant maize consumption, a result compatible with evidence for the intensification of maize farming in the region during this time (19). However, individuals from contexts thought to date to between 3400 and 2900 cal B.P. showed less dietary reliance on maize. In addition, resources from an adjacent estuary (e.g., shrimp and crabs) were shown to overlap isotopically with maize and confounded interpretation of these data (17). The absence of a clear maize signature in individuals dating between 3400 and 2900 cal B.P. led Blake and colleagues (9) to hypothesize that maize was being consumed as a beverage fermented from stalk juice, but this has not been tested directly with  $\delta^{13}\text{C}_{\text{apatite}}$  measurements. Overall, chronological and interpretive difficulties in the dataset obscure the dietary importance of maize through time, and the earliest skeletal samples in this time series do not predate the introduction of maize to the region ( $\sim 6500$  cal B.P.) (19).

Stable isotope dietary studies in the Mesoamerican neotropics are most extensive in the Maya lowlands, where emphasis has been placed on variability during the Preclassic (3000–1700 cal B.P.) (20) and Classic (1700–1000 cal B.P.) (21, 22) Periods. These studies show strong and increasing reliance on maize after 3000 cal B.P. (23), age- and sex-dependent differences in maize consumption (24), greater access to meat and maize in elite populations (24), and regional variability in the access to maize and marine foods (22). All of the skeletal materials analyzed in these studies postdate the introduction of maize to the region by  $\sim 6500$  cal B.P. (25) and its intensification after 4300 cal B.P. (10, 11), so dietary variability before and during the transition to maize-based food production is unknown. To evaluate dietary change through time, we obtained a stable isotope dietary data transect from 52 directly radiocarbon-dated human skeletons spanning the past 10,000 years from two remarkably well-preserved rock-shelter sites, Mayahak Cab Pek (MHCP) and Saki Tzul (ST), located in the Maya Mountains of Belize.

**MHCP and ST**

MHCP and ST are rock shelters located in an interior valley of the Maya Mountains in the Bladen Nature Reserve (BNR) [16°29'28.98 N, 88°54'37.42 W; 430 m above sea level (masl); Fig. 1], a protected wilderness area where there has been minimal modern human disturbance of archeological sites. The Classic Period Maya settlement of Ek Xux sits in a valley between these two rock shelters, and the much larger Classic Period center of Muklebal Tzul is located only 3 km away. Tikal, Caracol, and 63 other major Classic Period population centers with dated inscriptions occur within 200 km of these rock shelters, 13 with comparative baseline  $\delta^{13}\text{C}_{\text{collagen}}$ ,  $\delta^{15}\text{N}_{\text{collagen}}$ , and  $\delta^{13}\text{C}_{\text{apatite}}$  data from agriculturalists consuming maize (22).

The terrain in the Maya Mountains is rugged (~400 to 1000 masl), and MHCP and ST are positioned above active floodplains at ~430 masl along the Bladen Branch of the Monkey River and the Ek Xux Creek, respectively. Neotropical broadleaf forest predominates in the region and provides a range of edible tree fruits, nuts, and seeds. Palms are sources of fiber, thatch, and food (26), most notably the Cohune (*Attalea cohune*) palm found in high-density stands that produce a rich source of nuts/oils, fronds used for roofing, and large palm hearts historically used as a famine food (27). Most of the edible plants in this environment use the  $\text{C}_3$  photosynthetic pathway with the exception of Amaranthaceae, with pollen common in Pleistocene-age sediments (28). Trace amounts of Amaranthaceae pollen has been used as a disturbance indicator in Holocene paleoecological sequences (11). The most notable large prey animals in this region are three deer species (white-tailed, *Odocoileus virginianus* and brocket deer *Mazama americana* and *Mazama pandora*), tapir (*Tapirus bairdii*), and two peccary species (*Tayassu pecari* and *Pecari tajacu*). These animals are largely herbivores and can exploit the interface of the  $\text{C}_3$  tropical forest and areas of disturbance (29). The Monkey River provides freshwater mollusks (*Pachychilus* spp.), crabs, and small fish. Overall, protein and carbohydrate availability are dispersed, relatively low density, and seasonally modulated in neotropical forests and would not support concentrated human populations without agriculture (26).

Within this forested environment, MHCP is formed by an east-facing 20-m-high limestone outcrop that creates a 26-m-wide and 6-m-deep rock shelter. Dry sediments and limited root activity inside the dripline (~160  $\text{m}^2$ ) have favored the preservation of bone and carbonized plant materials (30). One 2.5 m-by-2.5 m excavation trench (figs. S1A and S2) in the center of the rock shelter revealed a ~2.8-m sequence of cultural midden and mortuary deposits. The lowest stratigraphic units (G to K; fig. S2) are organic-rich (silt to silty loam) and contain debris from the limestone cliff outcrop, igneous flaked stone tools of local origin (choppers and hammer stones), large chert bifaces (Lowe points) (30), and animal, riverine shellfish (*Pachychilus* spp.), and human remains. These deposits do not contain pottery and date between 12,000 and 6000 cal B.P. The upper portion of the sequence (units A to D) is composed of alternating layers of organic-rich rocky sediment and a dense *Pachychilus* spp. midden. These deposits date after ~3000 cal B.P. and contain pottery fragments, flaked stone chert, and igneous tools and the remains of mammals, birds, and reptiles. Stratigraphic units E and F are transitional between the ceramic and preceramic units and date between 6000 and 3000 cal B.P. and contain *Pachychilus* midden deposits.

ST formed below another sheer limestone cliff face that is located 1.4 km to the northeast of MHCP across the Bladen River. The shelter sits 70 m above the river, and it is less than 300 m away from the

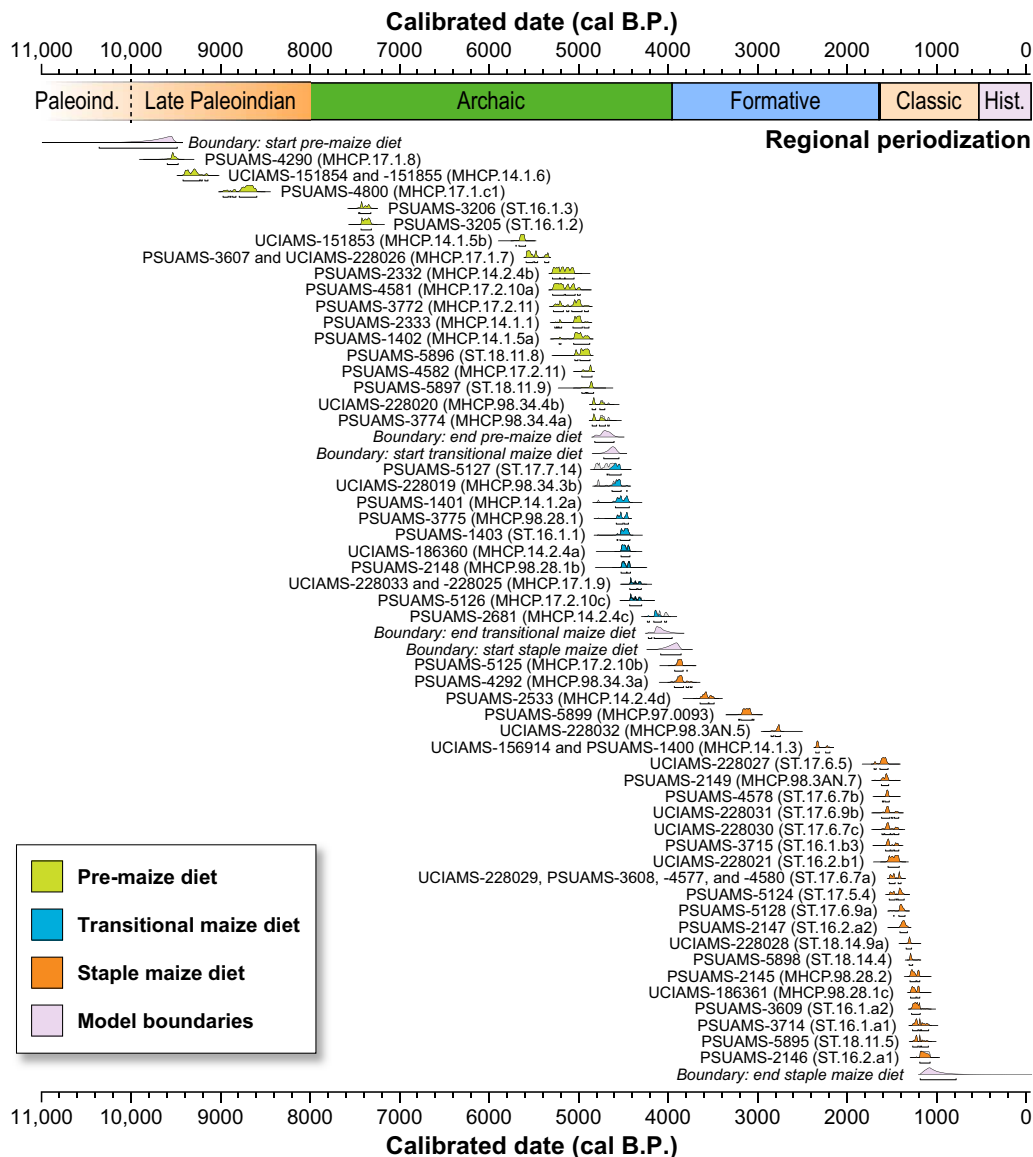
Classic Period Maya center of Ek Xux. It is larger (145 m long and 8 to 15 m wide) than MHCP and has ~1700  $\text{m}^2$  of dry sediments inside the dripline. Multiple excavation units (figs. S1B and S3) reveal a parallel sequence to MHCP spanning the past 12,500 years. Artifact density is high in the upper ceramic-bearing strata containing high concentrations of animal bone, burned wood, and disarticulated human remains. Two dense *Pachychilus* lenses (>70% shell) occur just below these mixed deposits. The preceramic deposits dating to the Middle Holocene are dominated by dark midden sediments and high concentrations of *Pachychilus* shells and contain stone tools, bone, carbonized plant material, and human skeletal material. The Early Holocene sediments change to a relatively compact light gray silt and contain lower, but consistent, concentrations of *Pachychilus* shells, stone tools, carbonized plant material, and human burials.

Human remains at both rock shelters reflect similar burial practices and include both primary and secondary burials (section S1). Isolated elements were recovered from many stratigraphic levels, and while some are the result of intrusive disturbance into earlier contexts, others are intentional deposits of individual skeletal elements or multiple elements. Although a wide range of burial practices are reflected in this long mortuary transect, burials in varying degrees of flexure were the most common and are found in all time periods. In total, 63 inhumations have been excavated from both rock shelters, not including isolated remains. Here, we report data for 52 individuals represented by 32 adults, 4 juveniles, 13 infants, and 3 indeterminate age (see dataset S1 and section S1 for details).

We obtained  $\delta^{13}\text{C}_{\text{collagen}}$ ,  $\delta^{15}\text{N}_{\text{collagen}}$ , and  $\delta^{13}\text{C}_{\text{apatite}}$  data and accelerator mass spectrometer (AMS) radiocarbon ( $^{14}\text{C}$ ) dates for 30 individuals from MHCP and 22 individuals from ST (Fig. 2 and dataset S2; also see Materials and Methods and section S2 for methodological details and our standards for quality control). Both males ( $n = 13$ ) and females ( $n = 12$ ) are represented in the sample of adults; however, the sex for 27 individuals could not be determined because they were incomplete skeletons or too young. Infants under the age of 3 were included in the analysis, but we subtracted 2‰ from  $\delta^{15}\text{N}_{\text{collagen}}$  values to offset well-documented enrichment associated with nursing (31).  $\delta^{13}\text{C}_{\text{collagen}}$  in infants largely reflects the mother's diet, but some enrichment can occur. However, we did not correct for  $\delta^{13}\text{C}_{\text{collagen}}$  because it is highly variable and less than 1‰. Juveniles over the age of 3 rarely show  $\delta^{15}\text{N}_{\text{collagen}}$  or  $\delta^{13}\text{C}_{\text{collagen}}$  enrichment associated with nursing (31). The most ancient individuals in this dataset come from the lower cultural strata at MHCP and date to between 9600 and 8600 cal B.P. (dataset S1). Two other individuals date earlier than 6000 cal B.P., and the remainder was persistently buried at these locations until ~1000 cal B.P., with a possible hiatus between 3200 and 2700 cal B.P.

**RESULTS**

In this study, we group skeletal remains into three chronological categories based on  $\delta^{13}\text{C}_{\text{collagen}}$ ,  $\delta^{13}\text{C}_{\text{apatite}}$ , and  $\delta^{13}\text{C}_{\text{enamel}}$  data that are presented below: pre-maize diet (9600–4700 cal B.P.), transitional maize diet (4700–4000 cal B.P.), and staple maize diet (4000–1000 cal B.P.). Statistical significance between temporal periods was determined using a one-way analysis of variance (ANOVA) and Tukey's post hoc test with significant differences observed among  $\delta^{13}\text{C}_{\text{collagen}}$  ( $n = 47$ ; ANOVA;  $F_{2,44} = 290.0$ ,  $P < 0.001$ ,  $\eta^2 = 0.929$ ,  $\beta = 0$ ) and  $\delta^{13}\text{C}_{\text{apatite}}$  ( $n = 34$ ; ANOVA;  $F_{2,39} = 66.2$ ,  $P < 0.001$ ,  $\eta^2 = 0.772$ ,  $\beta = 0$ ). All post hoc results were corroborated using nonoverlapping bias-corrected



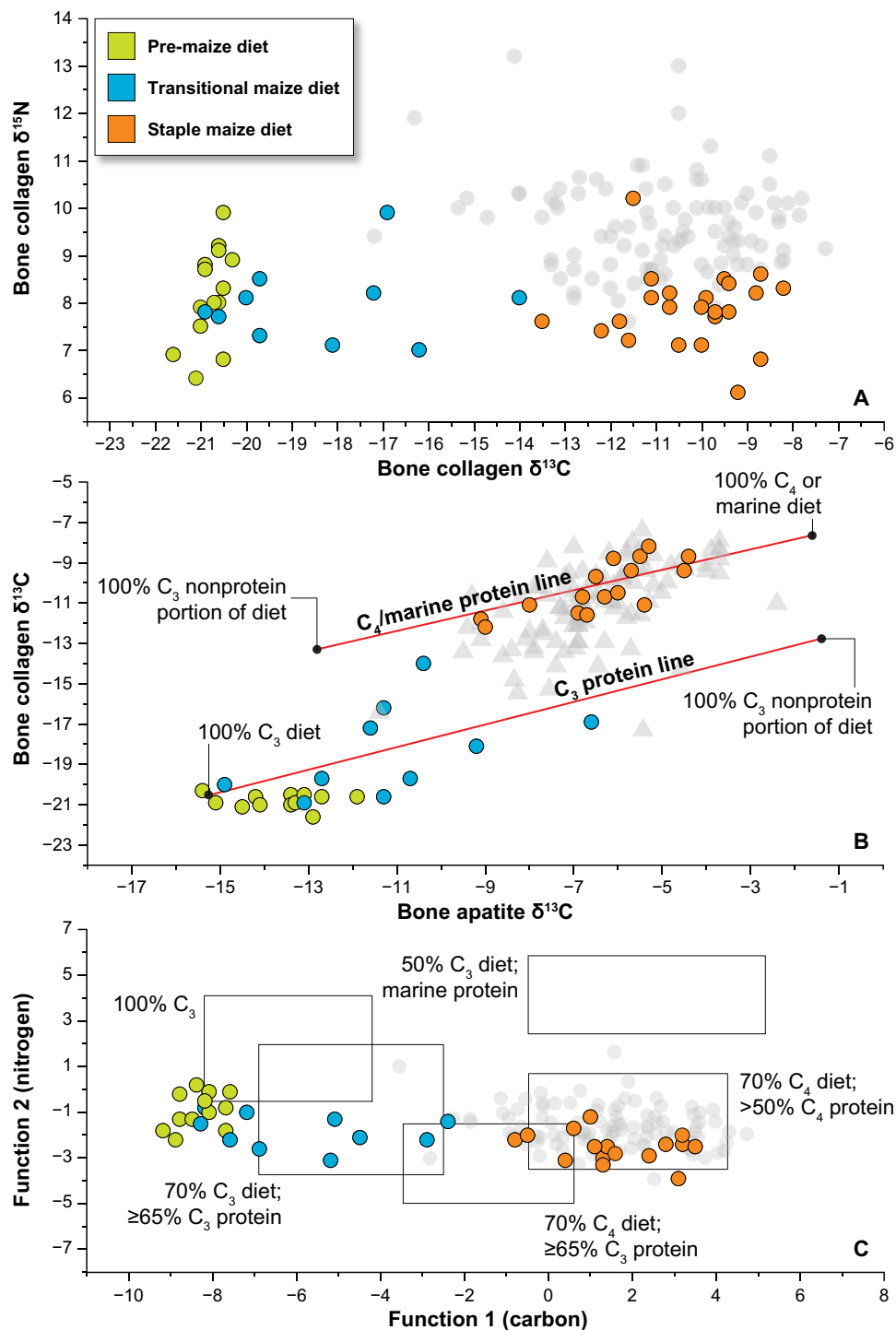
**Fig. 2. Directly radiocarbon-dated skeletal material from MHCP and ST grouped into primary dietary phases.** (A) Pre-maize diet (9600–4700 cal B.P.), (B) transitional maize diet (4700–4000 cal B.P.), and (C) staple maize diet (4000–1000 cal B.P.). For more details on the skeletal sample in this study, see section S1 and age model parameters in section S2. The radiocarbon plot produced in OxCal 4.2 with subsequent layout and design was performed in Illustrator CC 17.1.

and accelerated (BCa) confidence intervals (95% CI) with 1000 bootstrap replicates and verified with permutation test. We evaluated changes in  $\delta^{13}\text{C}$  using a linear mixing model to estimate the dietary contribution of maize (%) based on its distinctive  $\text{C}_4$  photosynthetic pathway (32). We also appraise diet based on a carbon isotope model (33) and a multivariate isotope model (22), both calibrated with controlled feeding studies. We also use a large sample of Classic Period (1750–1000 cal B.P.) individuals with demonstrated staple maize diets as a comparative baseline to track changes in maize consumption through time (22).

### Pre-maize diet (9600–4700 cal B.P.)

$\delta^{13}\text{C}_{\text{collagen}}$  values ( $n = 14$ ) in the earliest individuals (older than 4700 cal B.P.) range between  $-21.6$  and  $-20.3\text{‰}$  (mean =  $-20.8\text{‰}$ ; SD = 0.3) and indicate minimal or no  $\text{C}_4$  plant consumption (Fig. 3A). When combined with nitrogen isotopes ( $n = 14$ ;  $\delta^{15}\text{N}_{\text{collagen}}$ , 6.4

to 9.9‰), these measurements are consistent with a population consuming  $\text{C}_3$  plants and terrestrial animals from lowland tropical environments. The  $\delta^{15}\text{N}_{\text{collagen}}$  values are, on average,  $\sim 3$  to 6‰ higher than published values for herbivores in the region (*O. virginianus* and *M. Americana*) (34). Carbon isotope enrichment in these early humans indicates that the consumption of marine foods is not evident. We cannot rule out small dietary contributions of aquatic resources such as small gastropods (*Pachychilus* spp.), which are abundant throughout the middle sequence and overlap isotopically with  $\text{C}_3$  plants (34). Other domesticates such as squash (*Cucurbita* spp.) or manioc (*Manihot esculenta*) also cannot be ruled out because they also use the  $\text{C}_3$  photosynthetic pathway and overlap isotopically with wild plant foods found in neotropical forests.  $\delta^{13}\text{C}_{\text{collagen}}$  values may not be sensitive to  $\text{C}_4$  plant consumption, especially if only carbohydrate-rich portions of the plant were consumed (e.g., sugary stalk juices) (4, 9).



**Fig. 3. Isotopic values from MHCP and ST plotted relative to data from Classic Period maize agriculturalists from across the Maya lowlands.** (22) (A)  $\delta^{13}\text{C}_{\text{collagen}}$  versus  $\delta^{15}\text{N}_{\text{collagen}}$ . (B)  $\delta^{13}\text{C}_{\text{collagen}}$  versus  $\delta^{13}\text{C}_{\text{apatite}}$  plotted against dietary regression lines from experimental feeding studies (33). (C) Function 1 versus function 2 discriminant analysis plotted against data from experimental feeding studies (35). Data analysis in R, with subsequent layout and design performed in Illustrator CC 17.1.

We measured  $\delta^{13}\text{C}_{\text{apatite}}$  to evaluate total diet (proteins, lipids, and carbohydrates) and to determine whether  $C_4$  plants were a substantial part of the total diet.  $\delta^{13}\text{C}_{\text{apatite}}$  values ( $n = 15$ ) range between  $-15.4$  and  $-11.9\text{‰}$  (mean =  $-13.6$ ; SD = 1.0), and comparable results were found in four paired  $\delta^{13}\text{C}_{\text{enamel}}$  samples (dataset S1). A simple linear mixing model (32) of  $\delta^{13}\text{C}_{\text{apatite}}$  indicates a  $C_4$  contribution to total

diet between 0 and 21% (average = 10.5%; SD = 6.1). Therefore, we cannot rule out minimal consumption of  $C_4$  plants (e.g., Amaranthaceae or maize), particularly in the case of three individuals (MHCP.14.1.6 and MHCP.17.1.8, and ST.18.11.9) with values between 16 and 21%  $C_4$  dietary contribution coming from  $C_4$  carbohydrate-rich source(s). However,  $\delta^{13}\text{C}_{\text{apatite}}$  and  $\delta^{13}\text{C}_{\text{collagen}}$  plotted

against  $C_3$  and  $C_4$  protein regression lines and calibrated with published archeological and experimental data (33) show that these individuals still cluster on the  $C_3$  protein line, indicating a close to pure  $C_3$  diet (combined protein and carbohydrates; Fig. 3B), but with small-scale variations in resource consumption. Discriminant function analysis (F1 and F2) in a multivariate model including  $\delta^{13}C_{\text{apatite}}$ ,  $\delta^{13}C_{\text{collagen}}$ , and  $\delta^{15}N_{\text{collagen}}$  (35) shows clustering of samples within the 100%  $C_3$  diet space (Fig. 3C).

### Transitional maize diet (4700–4000 cal B.P.)

Carbon isotopic enrichment consistent with an increase in  $C_4$  consumption occurs in some individuals after 4700 cal B.P., but diets are highly varied ( $n = 10$ , mean =  $-18.3\%$ ; SD = 2.2) for  $\sim 700$  years. The age profile of this sample (including seven individuals younger than 3 years of age) contributes to this variability, but the nursing signature shows that some mothers were consuming substantial amounts of  $C_4$  plants.  $\delta^{13}C_{\text{collagen}}$  values are significantly different from the pre-maize dietary values, indicating that individuals were consuming a protein-rich  $C_4$  plant, like maize (8 to 11% protein by kernel weight), or consuming animals eating  $C_4$  plants ( $P < 0.01$ ). There is no statistical difference between  $\delta^{15}N_{\text{collagen}}$  isotopes (7.0 to 9.9‰;  $P = 0.576$ ), and these values do not suggest a major change in the source of protein (e.g., aquatic foods). Therefore, the enrichment in the  $\delta^{13}C_{\text{collagen}}$  in some individuals reflects a significant increase in the dietary importance of  $C_4$  plants or animals consuming  $C_4$  plants.

$\delta^{13}C_{\text{apatite}}$  values are consistent with an increase in the dietary importance of  $C_4$  plant consumption, most certainly maize, ranging between  $-14.9$  and  $-6.6\%$  ( $n = 10$ ; mean =  $-11.2\%$ ; SD = 2.3;  $P = 0.04$ ). This translates to  $C_4$  plant consumption averaging 25.8% (SD = 14.1) of total diet based on a simple linear mixing model (32). Bivariate plots of  $\delta^{13}C_{\text{apatite}}$  and  $\delta^{13}C_{\text{collagen}}$  show separation of some individuals in this group from the pre-maize diet along the  $C_3$  protein line, indicating an increase in  $C_4$  protein and carbohydrate consumption consistent with increased maize consumption (Fig. 3B) (33). Discriminant functions (F1 and F2) in the multivariate model show the majority of transitional farmers in the 30%  $C_4$  diet space ( $\geq 65\%$   $C_3$  protein; Fig. 3C). Three individuals dating within this interval have isotopic values that are indistinguishable from pre-maize diets and highlight the variability during this transitional period. Permutation tests show low retest reliability in the mean comparisons; however, the observed difference and simulation absolute-threshold differences suggest that transitional maize diets are more enriched than the simulated baselines (figs. S4 to S6). This largely results from high variability and small sample size of this group.

### Staple maize diet (4000–1000 cal B.P.)

$\delta^{13}C_{\text{collagen}}$  values for individuals dating after 4000 cal B.P. ( $n = 23$ ) range between  $-13.5$  and  $-8.2\%$  and overlap with values for staple maize diets during the Classic Maya Period (Fig. 3A). Comparisons indicate that these values are significantly different from pre- and transitional maize diets ( $P < 0.001$ ).  $\delta^{15}N_{\text{collagen}}$  values (6.1 to 10.2‰) are comparable to earlier populations. There is no evidence for the consumption of marine fish from pelagic environments (low  $\delta^{15}N_{\text{collagen}}$  isotope values), but we cannot rule out the possibility of some coral reef fish in the diet obtained via trade (22). However, the bones of these animals are largely absent in associated middens, even though we have used techniques designed to recover small bone samples (e.g., 200- $\mu\text{m}$  mesh sieves). Overall, the patterns indicate the consumption of forest-dwelling herbivores and heavy reliance on maize.

$\delta^{13}C_{\text{apatite}}$  values range between  $-11.9$  and  $-4.4\%$  (mean =  $-6.7\%$ ; SD = 1.9) and are consistent with 21 to 68% (mean = 53.7%; SD = 11.7) of total dietary carbon coming from a  $C_4$  source with significant differences between pre-maize and transitional diets ( $P < 0.001$ ). Bivariate plots of  $\delta^{13}C_{\text{apatite}}$  and  $\delta^{13}C_{\text{collagen}}$  fall on or near the  $C_4$ /marine protein line and are consistent with staple maize diets from the southern Maya lowlands (Fig. 3B) (22). Discriminant function analysis shows all of the post-4000 cal B.P. samples overlapping with individuals from the southern Maya lowlands in the 70%  $C_4$  range and with  $>50\%$  of dietary protein coming from  $C_4$  plants or animals eating  $C_4$  plants (Fig. 3C). Permutation results support all post hoc differences tested with staple maize diets (figs. S4 to S6).

## DISCUSSION

MHCP and ST are the only archeological deposits in the Americas that contain human skeletal material deposited persistently over the past 10,000 years and the only sites in the lowland neotropics that span the transition to maize-based food production. Preservation of bone organics in these dry rock shelters provides an unparalleled opportunity to study dietary change associated with the introduction of maize into the region and its subsequent development as a food staple of increasing economic and dietary importance. Edible plants of economic value using the  $C_4$  photosynthetic pathway are rare in the neotropical lowlands, and this makes stable carbon and nitrogen isotopic analysis a powerful tool for tracking the dietary importance of maize through time.

Isotopic evidence in individuals dating between 9600 and 4700 cal B.P. is consistent with dietary dependence on plants and animals from a  $C_3$ -dominated neotropical forest.  $\delta^{13}C_{\text{collagen}}$  values indicate that minimal  $C_4$  plant consumption and  $\delta^{15}N_{\text{collagen}}$  are consistent with the consumption of forest-dwelling herbivores (e.g., white-tailed and brocket deer). We cannot completely rule out minimal consumption of a carbohydrate-rich  $C_4$  plant source (e.g., Amaranthaceae or maize) based on slightly elevated  $\delta^{13}C_{\text{apatite}}$  values in some individuals based on a linear mixing model (32). Evidence for maize cultivation in the Maya lowlands first appears at  $\sim 6500$  cal B.P. (25) at about the same time that it appears along the Pacific Coast of Mexico (SOC05-2) (19) and Mexico's Gulf Coast lowlands (36), and it is possible that after this time, maize was adopted in the region for its sugary stalk. However, when  $\delta^{13}C_{\text{collagen}}$ ,  $\delta^{13}C_{\text{apatite}}$ , and  $\delta^{15}N_{\text{collagen}}$  are considered together and evaluated against data from controlled feeding studies (33, 35), all individuals dating before 4700 cal B.P. are consistent with 100% consumption of  $C_3$  plants and animals consuming  $C_3$  plants. These data are also consistent with the early dietary importance of neotropical trees, particularly cohune palm (27), and tubers in early foraging economies in the neotropical lowlands. Use of these rock shelters until  $\sim 5600$  cal B.P. appears to be persistent but episodic, suggesting low-density populations exploiting a resource-poor neotropical forest (26).

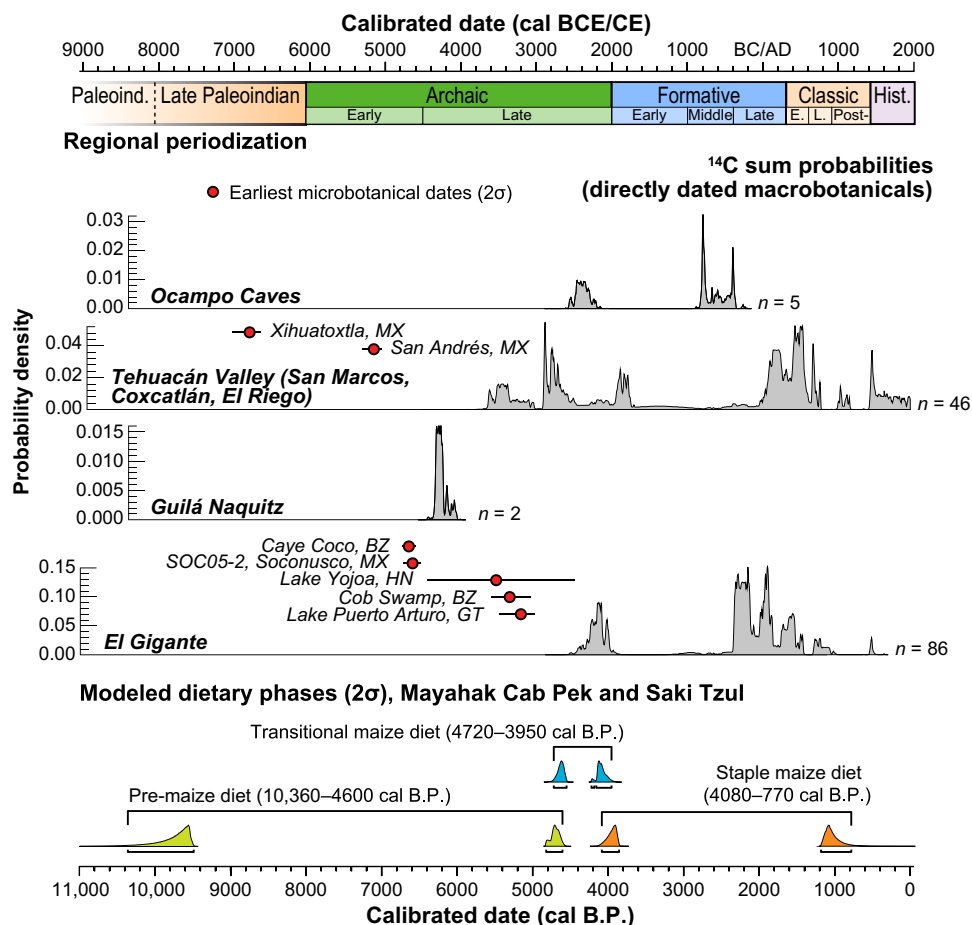
The first evidence for a significant dietary contribution of maize as a staple (defined here as  $>25\%$  of total diet) occurs between 4700 and 4000 cal B.P. During this transitional interval, there is a clear increase in both  $\delta^{13}C_{\text{collagen}}$  and  $\delta^{13}C_{\text{apatite}}$  in most individuals consistent with increased maize consumption. However, three individuals overlap isotopically with pre-maize diets, indicating variable reliance on maize during this period. Higher  $\delta^{13}C_{\text{collagen}}$  and  $\delta^{13}C_{\text{apatite}}$  indicate that by this time, maize protein and carbohydrates (kernels) were being consumed. We cannot rule out the consumption of glucose-rich

stalk juice, and it is likely that both stalks and kernels were used in beverage preparations. Our data partially overlap isotopically with an early population from South America (Pacopampa, Peru) (37) with higher  $\delta^{13}\text{C}_{\text{apatite}}$  values that possibly indicate maize beer consumption, but those  $\delta^{13}\text{C}_{\text{collagen}}$  values are not as  $^{13}\text{C}$ -enriched as the MHCP and ST individuals. The isotopic data during this transitional interval are inconsistent with the hypothesis that maize was solely used for its sugary stalk in a beverage (17). Overall, our multivariate model of  $\delta^{15}\text{N}_{\text{collagen}}$ ,  $\delta^{13}\text{C}_{\text{collagen}}$ , and  $\delta^{13}\text{C}_{\text{apatite}}$  indicates that maize provided about 30% of total diet (Fig. 3C). These data are consistent with evidence for increased forest burning and clearing, maize cultivation, and erosion across the Maya lowlands, suggesting a greater commitment to maize farming between 4500 and 4000 cal B.P. (11, 38). Increases in maize productivity may have resulted from the development and/or the introduction of new landraces (10) or technological innovation (e.g., nixtamalization) (39). Increasing dietary dependence on maize as a staple grain in this region also coincides with the widespread adoption of maize throughout Mesoamerica (Fig. 4) and its diffusion into the United States by ~4100 cal B.P. (40).

Individuals in our sample dating after 4000 cal B.P. overlap isotopically with a large dataset from the Classic Period (1750–1000 cal B.P.)

Maya populations (22). In this larger dataset, elites were more enriched isotopically than commoners, and our samples overlap more with the commoner population, indicating equal amounts of  $\text{C}_3$  and  $\text{C}_4$  foods and relatively high in  $\text{C}_4$  protein sources like maize or maize-fed animals. A persistent contribution of reef fish is largely discounted because of the interior position of most sites, and this would certainly be the case for MHCP and ST located at least a 2-day walk from the coast and where marine food residues have not been identified in associated archeofaunal assemblages. These data point to a strong commitment to maize-based food production combined with the continued exploitation of foods from the neotropical forest that may have involved more sophisticated forest management systems (4). The dietary dependence on maize is consistent with greater investments in surplus agricultural production, and the resulting deforestation, soil degradation, and erosion as populations increased in size and aggregation (41).

Our results suggest that maize-based food production and dietary dependency on maize came to form the economic basis for these developments but did not stimulate them immediately. Increasing dietary dependence on maize between 4700 and 4000 cal B.P. precedes archeological evidence for the earliest pottery-making agricultural



**Fig. 4. Dietary phases at MHCP and ST plotted relative to sum probability distributions of directly radiocarbon-dated maize from the Gulf Coast of Mexico (Ocampo Caves, Tamaulipas) (59), the highlands of Mexico (Tehuacán and Oaxaca) (5, 60), and nearby Honduras (10). The earliest radiocarbon dates associated with microbotanical evidence for maize in the Balsas region (Xihuatotxtla) (3), Mexico’s Gulf Coast (San Andrés) (36), and the Maya region (SOC05-2, Caye Coco, Lake Yojoa, Cob Swamp, and Lake Puerto Arturo) (9, 10, 12, 13, 16) are also shown. Summed probability distributions and dietary phases produced in OxCal 4.2 with subsequent layout and design were performed in Illustrator CC 17.1.**

villages in the Maya region by over 1000 years (ca. 3100–3000 cal B.P.) (23, 25). It remains unclear whether the transition to maize-based food production and the associated dietary changes resulted from the influx of a new population into the Maya lowlands. Linguistic data suggest that Proto-Maya diversified out of the western Guatemalan Highlands likely around 4200 cal B.P. as Huastecan speakers moved northeastward and then eventually settled along the Gulf Coast of Mexico, followed shortly after by diversification of Yucatec speakers into the Maya lowlands (42). Proto-Mayan contained words for maize planting, harvesting, and processing of maize, minimally indicating some cultivation of the domesticate (43). Given uncertainties associated with glottochronology and its partial reliance on archeological data, it is plausible that increases in the dietary reliance of maize co-occurred with language dispersal and population movements. Before the arrival of Yucatec speakers, the lowlands were inhabited by unknown non-Maya populations who we show were consuming increasing amounts of maize as new people and new varieties of maize were moving into the region. It has been suggested that they may have spoken a language typologically similar to lower Central American Xinkan, Lenkan, or Tol based on limited phonemic evidence (43). The term for maize in Xinkan derives from a very early, possibly Proto-Mayan root, suggesting that Xinkan foragers adopted the word after contact with early Maya farmers in the Guatemalan Highlands (44). The linguistic evidence also hints at the complex demographic history in the region. We now know that the earliest colonists in North and South America were genetically distinct from modern Maya populations (45), but it remains unclear how long these ancestral colonizing populations persisted in the region before being replaced or admixed. Coinciding with the transition to agriculture and the emergence of societal complexity leading to the Classic Period, the dietary shifts evident in our dataset therefore provide tantalizing evidence for the origins of Maya people in the lowland neotropics.

MHCP and ST are highly significant because they are the only archeological sites in the Americas with a relatively continuous diachronic sample of human skeletal material spanning the past 10,000 years. Therefore, the stable isotope analysis of these directly radiocarbon-dated individuals provides an unprecedented view of dietary changes during the transition from foraging to farming in the Americas. In the lowland neotropics, these data complement genetic and paleobotanical evidence for the domestication and spread of maize after ~9000 cal B.P. Paleoecological records in the Maya region suggest that the initial introduction of maize, in its earliest form, occurred between ~6500 and 5500 cal B.P. (25), but maize had little dietary impact as a staple before 4700 cal B.P. Consumption increased after this time, and it became a persistent dietary staple by 4000 cal B.P. Comparable isotopic studies indicate the adoption of maize as a staple elsewhere in Mesoamerica by ~3000 cal B.P. (9), in South America no earlier than 3500 cal B.P. (46), and in North America later than 2500 cal B.P. (47, 48). Ultimately, this transition contributed to the expansion and aggregation of populations, increases in social inequality, and major environmental transformations.

## MATERIALS AND METHODS

### Community engagement and bioethics statement

Permits for field research in Belize and permissions to export and conduct direct dating and isotopic analyses of ancient human remains were issued by the Institute of Archaeology, National Institute of Culture and History, Belize, with additional permits to conduct

fieldwork in the BNR issued by the Forest Department (FD), Belize. They are the legal entities responsible for permitting research. Research permit applications were also formally reviewed and supported by our local collaborator, the Ya'axché Conservation Trust (Ya'axché), a Belizean conservation nongovernmental organization strongly committed to preservation of environmental diversity and heritage. Ya'axché co-manages the BNR with the Belize FD. Ya'axché is largely staffed and administered by members of local communities, some of whom are park rangers working alongside our archeological team conducting fieldwork with other members of local communities. From 2016 to 2020, the results of our field and laboratory studies were presented to members of local communities through events organized by Ya'axché and as a condition of archeological permits and in mutual support of our ongoing collaboration. The results of both field and laboratory studies have been presented annually (2014 and 2016–2019) at the Belize Archaeology Symposium, a public conference attended by members of many diverse communities in Belize.

### Stable carbon and nitrogen isotopes

Carbon and nitrogen isotope ratios were measured on extracted and purified bone collagen or the amino acids that comprise bone collagen. Bone collagen was extracted and purified using a modified Longin method with ultrafiltration (49). Samples (200 to 400 mg) were demineralized for 24 to 36 hours in 0.5 N HCl at 5°C, followed by a brief (<1 hour) alkali bath in 0.1 N NaOH at room temperature to remove humates. The residue was rinsed to neutrality in multiple changes of H<sub>2</sub>O (18.2 megohm per cm) and then gelatinized for 12 hours at 60°C in 0.01 N HCl. The resulting gelatin was lyophilized and weighed to determine percent yield as a first evaluation of the degree of bone collagen preservation. Rehydrated gelatin solution was pipetted into precleaned Centriprep ultrafilters (retaining >30-kDa molecular weight gelatin) and centrifuged three times for 20 min, diluted with H<sub>2</sub>O (18.2 megohm per cm), and centrifuged three more times for 20 min to desalt the solution.

In most instances, bone collagen samples were too poorly preserved for ultrafiltration, and amino acids that comprise bone collagen were extracted from bone samples and pretreated using a modified XAD process (50). The sample gelatin was hydrolyzed in 2 ml of 6 N HCl for 24 hours at 110°C. Supelco ENVI-Chrom SPE (solid-phase extraction; Sigma-Aldrich) columns with 0.45- $\mu$ m polyvinylidene difluoride filters were equilibrated with 50 ml of 6 N HCl, and the washings were discarded. Two milliliters of collagen hydrolyzed as HCl was pipetted onto the SPE column and driven with an additional 10 ml of 6 N HCl dropwise with a syringe into a 20-mm culture tube. The hydrolyzate was lastly dried into a viscous syrup by passing UHP (ultra-high purity) N<sub>2</sub> gas over the sample heated at 50°C for ~12 hours. The isotope ratios of extracted amino acids may not be directly comparable to collagen ratios, but the differences are minor (50).

Carbon and nitrogen concentrations and stable isotope ratios of the collagen or amino acid samples were measured at the Yale Analytical and Stable Isotope Center with a Costech elemental analyzer (ECS 4010) and Thermo DELTAPlus analyzer. Sample quality was evaluated by % crude gelatin yield, %C, %N, and C/N ratios. C/N ratios ranging between 3 and 3.5 indicated good collagen or amino acid preservation (49).

### Radiocarbon dating

Bone collagen or individual amino acids were directly dated at the Pennsylvania State University (PSU) AMS radiocarbon dating facility.



Samples (Ultrafiltration, ~2.1 mg; XAD, ~3.5 mg) were combusted for 3 hours at 900°C in vacuum-sealed quartz tubes with CuO and Ag wires. Sample CO<sub>2</sub> was reduced to graphite at 550°C using H<sub>2</sub> and an Fe catalyst, with reaction water drawn off with Mg(ClO<sub>4</sub>)<sub>2</sub> (49).

Graphite samples were pressed into targets in Al boats and loaded on a target wheel, and <sup>14</sup>C measurements were made on a modified National Electronics Corporation (NEC) compact spectrometer with a 0.5-MV accelerator (NEC 1.5SDH-1). The <sup>14</sup>C ages were corrected for mass-dependent fractionation, with δ<sup>13</sup>C values measured on the AMS (51) and compared with samples of Pleistocene whale bone (backgrounds, 48,000 <sup>14</sup>C B.P.), late Holocene bison bone (~1850 <sup>14</sup>C B.P.), late 1800s CE cow bone, and OX-2 oxalic acid standards for normalization. All calibrated <sup>14</sup>C ages, probability distributions, and phase boundaries (section S2 and dataset S2) were computed using OxCal version 4.3 (52) with the IntCal13 northern hemisphere curve (53).

### Stable carbon isotopes (apatite)

The preparation for carbonate analysis in bone was conducted in the Human Paleoecology and Isotope Geochemistry Laboratory at the PSU using a modified version of procedures outlined in (54). A 2.5 × 2.5 cm fragment of bone shaft was cleaned using a mechanical drill so that the outer layer of the bone cortex and all trabecular bone were removed from the sample. Using an agate mortar and pestle, bone samples were ground into a fine powder. For each sample, 50 to 100 mg of bone powder were reacted in 2 ml of 2% bleach (NaOCl) for 24 hours at room temperature with vented capped vials and then rinsed three times in H<sub>2</sub>O (18.2 megohm per cm) or until the sample reached a neutral pH. Following the bleach treatment, samples were reacted in 2 ml of 0.1 M acetic acid (CH<sub>3</sub>COOH) for 24 hours at room temperature with vented capped vials (55) and then rinsed again to a neutral pH with water (18.2 megohm per cm). The samples were then dried overnight at 60°C and subsequently analyzed at the Center for Stable Isotopes, University of New Mexico by continuous-flow isotope ratio mass spectrometry using a GasBench device coupled to a Thermo Fisher Scientific Delta V Plus isotope ratio mass spectrometer. The results are reported using the delta notation measured against VPDB (Vienna Pee Dee Belemnite). Reproducibility was better than 0.1‰ for both δ<sup>13</sup>C based on repeats of a laboratory standard (Carrara Marble). The laboratory standard is calibrated versus National Institute of Standards and Technology (NBS)-19, for which the δ<sup>13</sup>C is 1.95‰.

### Statistical analysis

Statistical analyses were performed in SPSS 25.0 (IBM SPSS, Chicago, IL, USA) and R software. The *P* value threshold for statistical significance was 0.05 for all tests. A priori power analysis (1-β) and effect size test (η<sup>2</sup>) were performed for the primary computational results. Individual burials were categorized into three groups: pre-maize diets, transitional maize diets, and staple maize diets. Normality was evaluated using the Shapiro-Wilk test, and homogeneity of variance was assessed using Levene's test. A one-way ANOVA was applied to compare differences followed by Tukey's post hoc test and Dunnett's T3 post hoc test in the cases of heterogeneity of variance. Statistical differences were corroborated via nonoverlapping BCa confidence intervals (95% CI) with 1000 bootstrap replicates. We evaluated the post hoc results using permutation tests with 1000 randomized iterations and resampling in R (figs. S4 to S6). Plots were created in R using ggplot2. Descriptive statistics are available in dataset S1.

### FTIR quality control

Analysis of carbonate (CO<sub>3</sub>) in biogenic hydroxyapatite [Ca<sub>10</sub>(PO<sub>4</sub>)<sub>6</sub>(OH)<sub>2</sub>] was first tested for diagenetic alteration using Fourier transform infrared (FTIR) spectroscopy at the Materials Characterization Laboratory at the PSU using a Bruker Vertex 70v FTIR spectrometer with a DiaMax attenuated total reflection accessory. Each spectrum was the result of 100 scans for mid-range IR (4000 to 400 cm<sup>-1</sup>) with a spectral resolution of 4 cm<sup>-1</sup>. Corrected baselines for the spectra were calculated by adding the heights of the absorptions and then dividing by the height of the minimum between them (56). A new background was created for each sample run. To determine the degree of apatite recrystallization, the crystallinity index or infrared splitting factor was calculated using the height of the absorption bands at 603 and 565 cm<sup>-1</sup> divided by the height of the valley between them at ~595 cm<sup>-1</sup>. All but two measured sample spectra had a crystallinity index less than 3.8, which indicated a well-preserved biogenic signal, and remained in this study for analysis. While the ratio of the absorption peak height at 1415 cm<sup>-1</sup> (CO<sub>3</sub>) and 1035 cm<sup>-1</sup> (PO<sub>4</sub>) indicates degraded carbonate material, all sample spectra are consistent with expected C/P ratios observed in archeological bone (56), and none of the spectra indicate CaCO<sub>3</sub> contaminant absorption at 710 cm<sup>-1</sup> (figs. S7 to S10).

### SUPPLEMENTARY MATERIALS

Supplementary material for this article is available at <http://advances.sciencemag.org/cgi/content/full/6/23/eaba3245/DC1>

[View/request a protocol for this paper from Bio-protocol.](#)

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