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Spatial and Seasonal Signals in Stable Isotopes of Incisor Enamel from Free-ranging, Thirteen-lined Ground Squirrels

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Abstract:

From early May through late September, 2012, we captured, tagged, and collected body masses from multiple, free-ranging thirteen-lined ground squirrels (*Ictidomys tridecemlineatus*) in southwestern Michigan, with 14 individuals recaptured 1 or more times through the season. Beginning in mid-June, we captured and euthanized 12 of these individuals (on average, about 1 per week) to allow study of their lower incisors. We serially micro-sampled enamel along squirrel incisors using laser ablation and determined stable isotope ratios ($\delta^{13}C$ and $\delta^{18}O$) with gas chromatography–isotope-ratio mass spectrometry (GC-IRMS). The resulting values fell into two spatial groups. Specimens collected within 25 m of a cornfield displayed more positive $\delta^{13}C$ values consistent with a C4 plant-rich diet (corn is a C4 plant) and appeared to show a seasonal peak in C4 plant use (higher $\delta^{13}C$ values) late in the season. In contrast, specimens collected in isolated fields separated by buildings and pavement from the cornfield exhibited more negative values of $\delta^{13}C$ consistent with a C3-plants diet. $\delta^{18}O$ values did not show distinct spatial grouping and were difficult to interpret. Although a cornfield is an artificial ecosystem, the difference in fractionation between C3 and C4 diet displayed in the stable carbon isotope profiles demonstrate the sensitivity of isotopic analyses of small teeth for elucidating small-scale geographic variation in food availability. Further use of this technique may provide additional evidence for fine-scale spatial and seasonal variation of modern diet and provide a model for further investigation of paleoseasonality using fossil rodent incisors.

Introduction:

Rodent incisors grow throughout the life of the animal, typically adding an increment of dentin and enamel each day at the incisor's base. Incremental dental tissues may record significant life-history events, such as the occurrence and timing of hibernation in ground squirrels and relatives (Goodwin et al. 2005; Goodwin and Ryckman 2006; Rinaldi 1999). However, this record is only retained for a few weeks to months because as an incisor grows, each increment added at the incisor's base is matched by erosion at its tip.

The chemistry of tooth enamel also may preserve ecological signals. Notably, stable isotope ratios of carbon ($^{13}C/^{12}C$) and oxygen ($^{18}O/^{16}O$) in enamel, expressed in parts per thousand in relation to a standard ($\delta^{13}C$ and $\delta^{18}O$ respectively), vary with diet ($\delta^{13}C$) and seasonal changes in the temperature of water sources ($\delta^{18}O$) (Hedges et al. 2005). For example, C3 plants (most trees, herbs, and some grasses) have substantially lower values of $\delta^{13}C$ than do C4 plants (warm-season grasses), and this difference persists in the tooth enamel of herbivores that feed on these plants (Cerling et al. 1993). Paleobiologists thus have much interest in the study of stable isotopes, and have used isotopic evidence to reconstruct temporal and geographic patterns in ancient environmental conditions (e.g., Cerling et al. 1993; Passey et al. 2002).

Studies done on mammals, such as cows, horses, alpacas, pigs, rabbits and voles, have shown that on controlled diets and oxygen sources, stable isotope profiles reflect dietary and oxygen sources (Passey et al. 2005; Sponheimer et al. 2003). Carbon is more relevant than oxygen to our project because $\delta^{13}C$ is known to vary in relation to diet, while oxygen isotope results are less reliable and more difficult to interpret.

Diet and climate conditions vary seasonally, and paleobiologists have interest in reconstructing such seasonality. One possible approach to doing so is to serially sample stable isotopes along the growth record of an ever-growing tooth. Until recently, this could only be done for large teeth, such as the ever-growing tusks of mammoths and mastodons (e.g., Koch et al. 1989), because required sample volumes were much larger than could be obtained from small teeth. However, recent advances in micro-sampling with laser ablation, and technical advances that facilitate analysis of small volumes of CO2 in gas chromatography–isotope-ratio mass spectrometry (GC-IRMS), now make possible serial sampling of very small teeth (Passey and Cerling 2006). Previous work demonstrated the ability of dental increments to serve as time recorders in thirteen lined ground squirrels (Kisser 2009). The laser-ablation method used by Passey has yielded a reproducibility better than 0.1% for all samples (Passey 2005). Thus, it now is possible to reconstruct diet, and to a lesser extent temperature-related environmental conditions, at ~weekly resolution during the life of even small mammals–a resolution generally unobtainable in paleoecological studies.

The overall goal of the project was to document the carbon ($\delta^{13}C$) and oxygen ($\delta^{18}O$) isotope make-up of the enamel within the incisors of modern, free-ranging thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) from May to September 2012 at single colony in Southwest Michigan. We documented a time-series of stable isotope values in one incisor each from 12 squirrels, using laser ablation coupled with GC-IRMS,. A parallel study tracked squirrel diet through collection and analysis of fecal pellets. Combined, the two studies purported to test the hypothesis that seasonal variation in food source (primarily C4/C3 plants), and temperature, would be recorded in stable

isotope profiles of carbon and oxygen in the squirrels' incisor enamel. Additionally, after preliminary analysis, we also explored small-scale spatial variation in food sources and the resulting differences in incisor stable isotope profiles.

Methods:

Field Methodology and Specimen Preparation

We studied a population of free ranging thirteen-lined ground squirrel (*Ictidomys tridecemlineatus*) at the Andrews University airpark, located ~ 1 mile northwest of Berrien Springs, Michigan. We began monitoring the population in early May and continued through October, 2012, visiting at least two times per week. Different individuals monitored the site from May through mid-July, and from mid-July to mid-October. Our fieldwork centered around 3 categories of events–initial captures, periodic recaptures, and final captures and euthanasia.

Initial capture– We initially attempted to use standard Sherman live traps, cleaned to remove contamination from prior use and baited with oats and peanut butter, to capture animals. However, no squirrels were trapped by this method. We then employed an alternative method of fencing, with stakes, a 3 foot circular radius around a squirrel burrow, waiting for an animal to emerge in order to feed, and then blocking the burrow hole with a plywood board using a twine pull cord before the squirrel attempted to reenter the burrow. We would then use heavy leather gloves to capture the squirrel by hand within the enclosure. This method was successful on first captures, but was very difficult to use for recaptures. Finally, we created wire traps out of 0.64-cm heavy wire mesh, with

a gravity driven trap door mechanism, tied together with wire and staked over the burrow. These were were much more successful for recaptures, and we used them the remainder of the field season.

At initial capture, we recorded capture coordinates with handheld GPS, weighed each squirrel to the nearest gram with a handheld balance, tagged it with a numbered and color-coded ear tag, and determined its sex and reproductive condition (mature/immature, and for females, pregnant or lactating). Thirty squirrels were captured and either tagged for further monitoring, or immediately euthanized (see below). We also collected fecal pellets deposited by the squirrel in the trap and stored them in clean, labeled plastic vials; we injected each squirrel intramuscularly with a solution of oxytetracycline (15 mg/kg body mass). Oxytetracycline binds to dental tissue formed while it is in the blood stream, and subsequently fluoresces in these tissues under ultraviolet light, thus marking specific growth increments in the teeth that correspond to known injection dates. Each squirrel was then released at the site of capture.

Recaptures– we attempted to recapture tagged squirrels every 1-2 weeks between initial capture and either final capture, or disappearance of the squirrel from our study area. At recapture, each squirrel was identified based on its tag, and its GPS coordinates, body mass and reproductive condition was recorded. We collected fecal pellets, re-injected with oxytetracycline if at least 10 days had elapsed since previous injection, and released the squirrel at site of recapture.

Although our focus was on the tagged squirrels during capture, it was often not possible to observe the tags from a distance. Thus, we also made 48 captures of untagged

squirrels. Field data was obtained from these additional captures, but we did not study them further.

Final captures and euthanasia–Beginning in June, we captured and euthanized 0 – 2 squirrels per week, for a total of 12 specimens. 8 of these squirrels were captured 2–12 weeks after initial captures, but 4 were taken at initial capture. If a captured animal was a lactating female, she was released. At final capture, we obtained the same data obtained during recapture. Then, within one hour of capture, the squirrel was transported to the lab and humanely euthanized by placing it in an enclosed chamber rapidly filled with compressed CO2. After euthanasia, the squirrel was immediately frozen to preserve body tissues for further study.

Specimen preparation–We prepared incisors for analysis by thawing frozen carcasses, preparing each as a study specimen (usually as a skeleton), and removing the incisor of one dentary. This was done by soaking the dentary in water for several weeks until the incisor could be extracted. Each incisor was then cleaned separately by soaking in a volume of 40 ml 10% H_2O_2 for approximately 5 hours, rinsed in DI water while being gently scrubbed with a small wire brush, and dried overnight in an incubator at 50º C.

Lab Methodology

We used the lab of Dr. Benjamin Passey (Johns Hopkins University) from Dec 17–20, 2012 to serially micro-sample incisor enamel with laser-ablation, and document $\delta^{13}C$ and $\delta^{18}O$ of the CO_2 released during micro-sampling through a coupled GC-IRMS system using helium as a transfer vehicle. We then adjusted and calibrated the $\delta^{13}C$ and

$\delta^{18}O$ values obtained from GS-IRMS following a published protocol (Passey and Cerling 2006).

Surface of the incisors were examined carefully under magnification to count enamel increments in the tooth. Because incisors typically deposit one increment per day, with the last increment before death deposited at the tooth's base, we were able to establish a basic chronology of tooth growth using incremental bands to assign each increment to day of growth, starting with the known date of euthanasia. This chronology allowed us to date each micro-sampled enamel segment on the tooth to within a few days of its initial deposition.

At the John Hopkins University isotope lab, incisors were mounted for analysis on a metal mounting platform, which was attached to a hollow rod, using a small amount of putty. The number and position of each incisor on the mounting platform was carefully recorded. This apparatus was subsequently inserted into a glass chamber, with the rod extending through a plate at each end that could be clamped onto the end of the chamber. The rod allowed inflow of helium into the chamber, and made it possible to rotate the mounting plate within the chamber to keep the surface of the enamel under the laser approximately perpendicular to the laser beam. We monitored seals between end plates and the chamber for leakage, and adjusted clamps as needed to stop leakage.

The mounting chamber was attached to 2 gas lines, for helium inflow and gas outflow, both with access sites for injecting gas of known volume and composition during standardization. Access sites displayed silicone septa, which were changed with each change of chambers. The analysis chamber was filled with helium, and the mounted specimens were allowed to degas for several hours before analysis began. During

analysis, helium flow was maintained at a rate of ~350ml/min. All connections were checked with a helium detector, and were adjusted if a leak was detected.

Each day, we prepared a CO2 standard by injecting 5 ml of CO_2 with a known isotopic composition into a flask first flushed then filled with N_2.

We followed a stereotypic sequence as we collected isotopic values for each incisor (or, in some cases, a single sequence was followed for 2 incisors investigated sequentially in one chamber):

a. Injection of wall gas of known composition
b. Reading of the CO_2 blank, by simply collecting CO_2 that arises from contaminants in the system, or from the specimens themselves, for 240 s
c. Injection of CO2 standard (25 μl) pre-chamber, by flushing the injection syringe 4 times with standard CO_2 then injecting 20 µl of the standard into the helium stream entering the chamber
d. Injection of CO2 standard (25 μl) post-chamber, using the same protocol noted above but into the gas leaving the chamber
e. Processed multiple laser-vaporized samples of tooth enamel, in series, with the pattern of ablation pits in each sample laid out by computer
f. Injection of an additional CO_2 pre-chamber standard after each 5-10 enamel samples
g. Close each analysis sequence with another blank, a final CO2 pre-chamber standard, and a final wall gas

For each analysis sequence, we recorded the following: specimen number; a sketch of specimen with location of each enamel sampling location; number, date, and time of commencement of each GC-IRMS analysis; and the following GC-IRMS output: sample ID, time analysis initiated, peak number (which tracks sample number unless a sample does not produce a peak), peak size.

We also maintained proper levels of dry ice, methanol, and liquid N_2 in a specially designed trap which consisted of 3 cooled chambers, the first with dry ice and methanol, the second and third containing liquid N_2. This trap concentrated CO_2 from each analysis prior to GC-IRMS. The trap times were set to have the sample exposed 240 seconds in the first trap, 90 seconds in the second trap and 60 seconds in the third trap.

The laser was calibrated to a spot size of 40 using trial and error between 22 and 50. We used 1 pass with a dwell time of 0.01, scan speed of 50, manual spot spacing, and created four to five lines of ablation pits (typically 16-20 pits total).

Results:

When $\delta^{13}C$ isotope profiles for the 12 specimens were plotted against the days before death, all showed a progressive decrease that commenced ~30 days prior to death (Fig. 1). This is likely an artifact of organics remaining in the enamel for some time after initial deposition due to incomplete mineralization, thus the 30 days prior to death were removed in subsequent analyses.

The resulting stable isotope profile values clustered into two groups: those with values around -15 $\delta^{13}C$, and a second clustering around -5 $\delta^{13}C$. One specimen (147) appears to have moved between groups during the study (Fig. 1).

The specimen also clustered spatially into two groups: those with relatively negative values occupied grassy habitat without easy access to an adjacent cornfield, and those with positive values were captured within 25 m of the cornfield (Fig. 2). Spatial clusters differed significantly in $\delta^{13}C$ values (equal variances not assumed; $t = -8.87$, $df = 5.65$, $p < 0.001$; Fig. 3).

$\delta^{13}C$ values trended more positively during the season among squirrels trapped , but this trend was not observed in the cluster isolated from the cornfield (Fig. 4). In contrast to the $\delta^{13}C$ values, the $\delta^{18}O$ values showed no clear spatial or seasonal patterns, although many specimens displayed a progressive decrease in these values over the course of the season (Fig. 5).

Fig. 1 - Grouped $\delta^{13}C$ Enamel Values Over Time Including 30 Days Prior to Death. Red symbols represent profiles of squirrels trapped within 25 m of a cornfield, blue symbols represent individuals isolated from the cornfield by buildings and pavement.

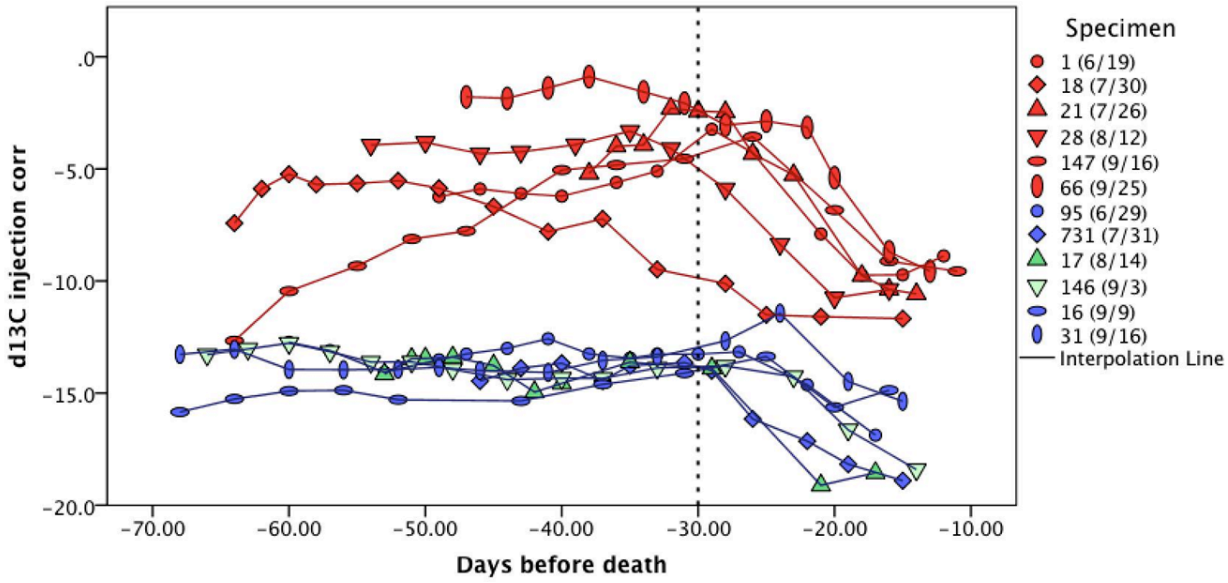


Fig. 2 - Capture GPS Coordinates by $\delta^{13}C$ Group at AU Airpark. Red symbols represent profiles of squirrels trapped within 25 m of a cornfield, blue symbols represent individuals isolated from the cornfield by buildings and pavement.

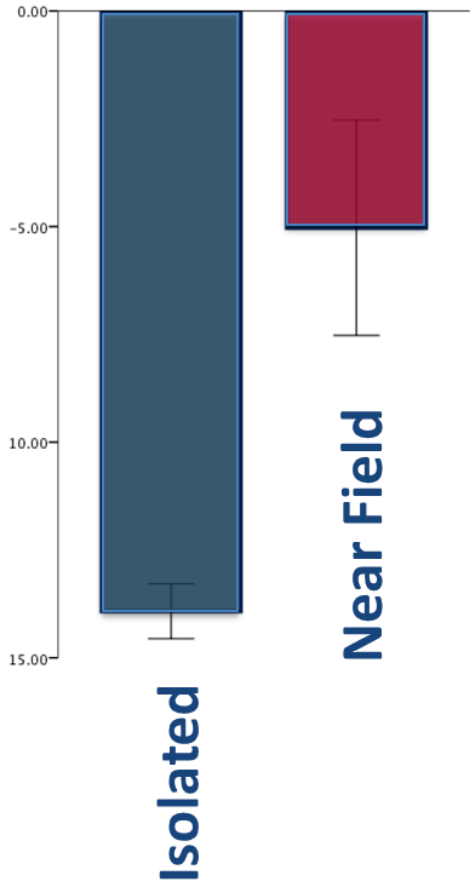


Fig. 3. - Statistical significance using T-test with equal variances not assumed; $t = -8.87$, $df = 5.65$, $p < 0.001$

Fig. 4- Grouped $\delta^{13}C$ Enamel Values Over Time (30 days prior to death removed). Red symbols represent profiles of squirrels trapped within 25 m of a cornfield, blue symbols represent individuals isolated from the cornfield by buildings and pavement.

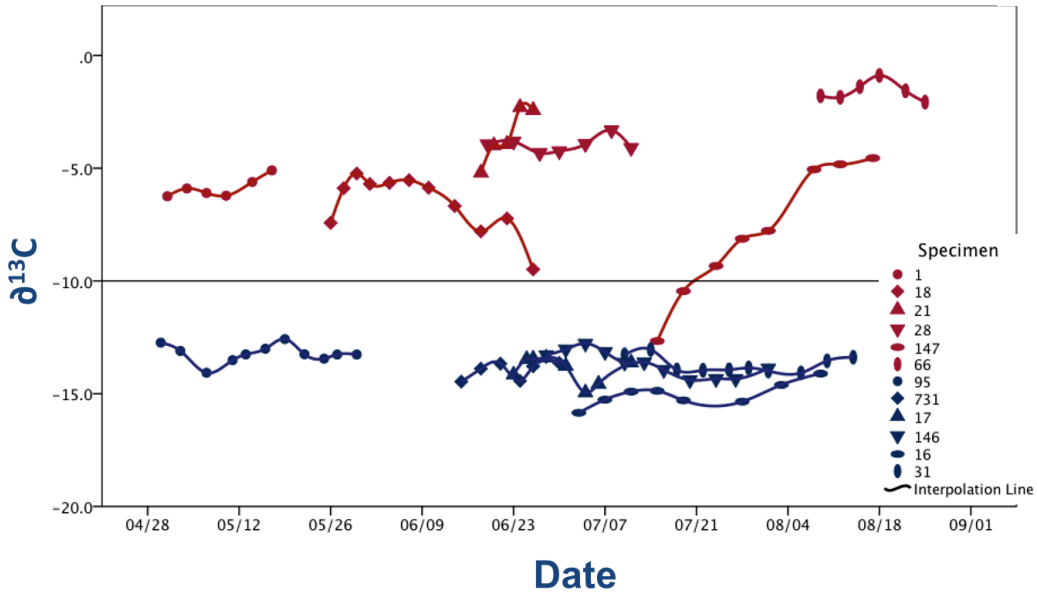
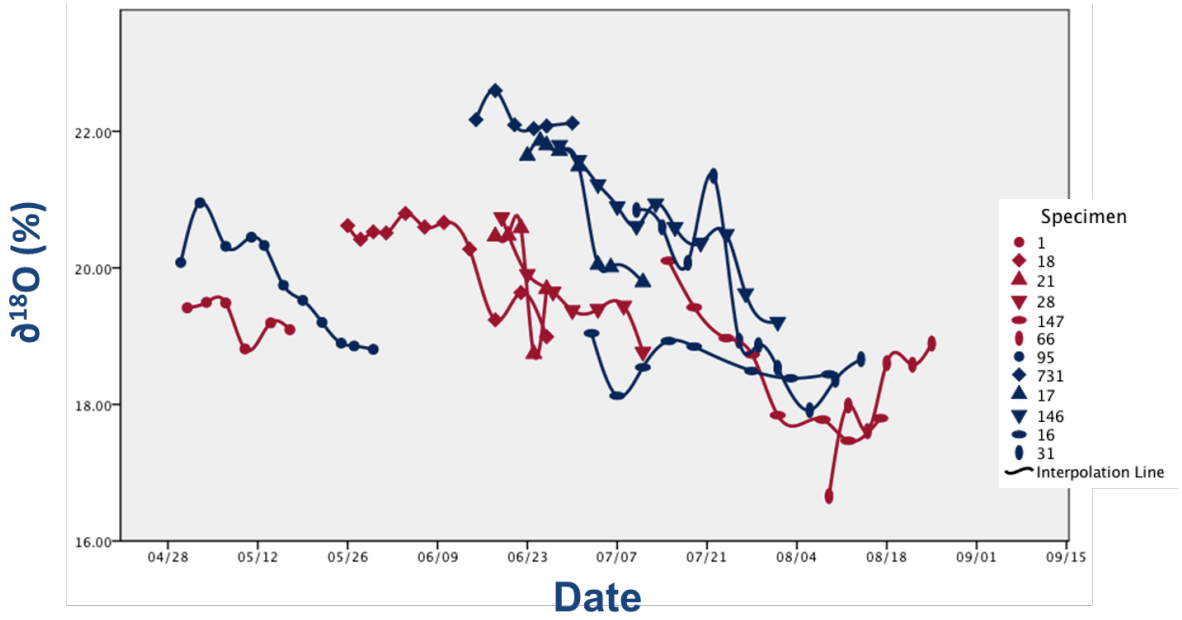


Fig. 5 - Grouped $\delta^{18}O$ Enamel Values Over Time (30 days prior to death removed). Red symbols represent profiles of squirrels trapped within 25 m of a cornfield, blue symbols represent individuals isolated from the cornfield by buildings and pavement.



Discussion:

Roughly 30 days preceding death, $\delta^{13}C$ values dropped in all specimens, no matter when in the active season they were collected. This drop appears to be an artifact of mineralization, perhaps due to organics present in the enamel matrix as it is mineralizing. Thus, we recommend that studies of isotope profiles in small incisors such as done here be cautious in interpreting the proximal part of the tooth record. Further study is needed to test the hypothesis that organics in the matrix are the cause of this decrease, and to determine how this artifact varies across species and higher taxa.

The oxytetracycline injections were only performed on 3 of the 12 specimens that we were subsequently able to collect, thus we did not use these as time markers in final analysis of the data. In the future, more regular oxytetracycline injections, starting at the time of initial capture would help calibrate more precisely the dates from daily enamel deposition. Even so, we suspect our estimated dates will be close given what is known about the circadian periodicity of dental growth.

We demonstrate that small-scale spatial variation in diet is reflected in the stable isotope values across teeth due to the differences in isotope profiles of C3/C4 vegetation. C3 plants, making up 90% of trees and grasses, have more negative values of $\delta^{13}C$ (on average, about -25.5 ppm) than C4 plants, largely warm-season grasses including corn and sugarcane, which have higher $\delta^{13}C$ values (on average, about -13.0 ppm). The gap between C3 and C4 isotopic values remains in tooth enamel of animals which depend on C3/C4 plants (Cerling et al. 1993), but are offset by approximately 13 parts per thousand because of fractionation during enamel growth (Fraser et al. 2008).

The artificial separation of squirrels by buildings and pavement overemphasized the difference in the isotope profiles in two distinct groups at our study site. Further research is needed in order to investigate whether reliable spatial grouping in $\delta^{13}C$ values can be found in more natural environments without artificial separation in the population.

The group with higher $\delta^{13}C$ values appears to show a seasonal pattern toward more positive values (higher proportion of C4 vegetation) late in the season. This trend is likely explained by variation of C3 and C4 vegetation. It is possible that a drought during the time of the study resulted in an increase in the amount of C4 vegetation the squirrels consumed later in the season, but we have no clear observational data to confirm this hypothesis.

It was difficult to discern any pattern in the oxygen isotope profiles data ($\delta^{18}O$). According to the literature, $\delta^{18}O$ values may provide a basis for inferring information about environmental water; "[F]actors that can affect the oxygen isotope composition… [are] …habitat, drinking behaviour, population dynamics, body size and thermophysiology," which should be considered before making inferences about environmental drinking water (Gehler et al. 2012). More study is necessary to more precisely measure and identify oxygen isotope profiles and any clues they might provide about dietary or seasonal signals available in rodent incisor data.

Our study was highly focused on recapturing squirrels. Recapture of squirrels proved to be problematic due to learned avoidance of wire squirrel traps. Further work needs to be done on more reliable methods of repeated recapture in order to provide better data over longer periods of the season on the same animals.

This study, along with the analysis of the squirrel diet through fecal pellet collection, may provide key information in the creation of a model which may assist in fine-scale (weekly) paleoecological interpretation of diet in fossil ground squirrels. For paleobiology, the application of the data collected and further insights into the limitations of the laser ablation micro-sampling method, necessitate further study of fossil collections to extrapolate about diet and seasonality from the past as evidenced by the fossil record.

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