Calcium and Carbon Stable Isotope Ratios as Paleodietary Indicators

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ABSTRACT Calcium stable isotope ratios are hypothesized to vary as a function of trophic level. This premise raises the possibility of using calcium stable isotope ratios to study the dietary behaviors of fossil taxa and to test competing hypotheses on the adaptive origins of euprimates. To explore this concept, we measured the stable isotope composition of contemporary mammals in northern Borneo and northwestern Costa Rica, two communities with functional or phylogenetic relevance to primate origins. We found that bone collagen $\delta^{13}C$ and $\delta^{15}N$ values could differentiate trophic levels in each assemblage, a result that justifies the use of these systems to test the predicted inverse relationship between bioapatite $\delta^{13}C$ and $\delta^{44}Ca$ values. As expected, taxonomic carnivores (felids) showed a combination of high $\delta^{13}C$ and low $\delta^{44}Ca$ values; however, the $\delta^{44}Ca$ values of other faunivores were indistinguishable from those of primary consumers. We suggest that the trophic insensitivity of most bioapatite $\delta^{44}Ca$ values is attributable to the negligible calcium content of arthropod prey. Although the present results are inconclusive, the tandem analysis of $\delta^{44}Ca$ and $\delta^{13}C$ values in fossils continues to hold promise for informing paleodietary studies and we highlight this potential by drawing attention to the stable isotope composition of the Early Eocene primate *Cantius*. Am J Phys Anthropol 000:000–000, 2014.

The detection, acquisition, and assimilation of food are central topics in the debate on euprimate origins (recent discussions: Cartmill, 2012; Gómez and Verdú, 2012; Rosenberger, 2013; Silcox, 2013; Sussman et al., 2013). In general, functional interpretations of euprimate apomorphies emphasize a diet of invertebrate prey, angiosperm reproductive tissues, or some combination of both (e.g., Rasmussen, 2002). These hypotheses are informed by the comparative method and the profound differences between these resources—with respect to sessility, temporal availability, and nutritional and physical attributes. Crucially, these foods also exist at different trophic levels, a distinction that predicts consumer tissues with discrete stable isotope compositions (West et al., 2006). Stable isotope analysis is therefore a plausible tool for testing competing hypotheses on the functional anatomy and ecology of early euprimates.

Such a test faces two challenges. First, any deep-time application of stable isotope analysis is limited to diagenesis-resistant tissues such as tooth enamel because it best preserves biogenic isotope compositions (Fig. 1). Second, the stable isotope composition of enamel bioapatite is by itself a relatively crude indicator of dietary behavior. Variation in carbon stable isotope ratios

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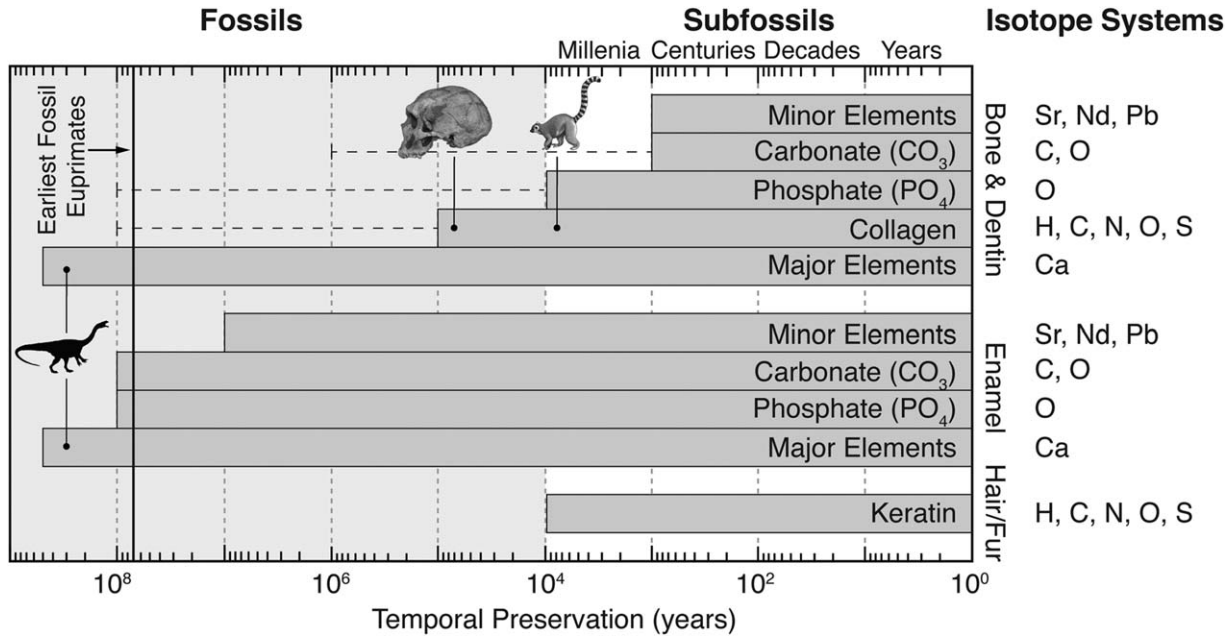

Fig. 1. Temporal ranges over which organic and mineralized tissues are likely to be preserved, together with different isotope systems. Bars depict the expected range of preservation, whereas the horizontal dashed lines represent the maximum theoretical age of preservation under exceptional conditions. The figure is based on Koch (2007) and modified from Clementz (2012) to reflect recent findings from Late Triassic (e.g., *Plateosaurus engelhardti*; depicted) and Late Cretaceous non-avian dinosaurs (Heuser et al., 2011). The antiquity of $\delta^{15}N$-based trophic discriminations is constrained by the preservation of bone collagen, which is unlikely to persist in the fossil record beyond 100 kya. Two of the oldest $\delta^{15}N$ values in the paleoanthropological literature are represented with depictions of *Homo neanderthalensis* and *Lemur catta* (sources: Richards and Trinkhaus, 2009; Crowley et al., 2012). The shaded field distinguishes fossil material ($>10^4$ years) from subfossil material ($<10^4$ years). The vertical black line marks the approximate appearance of euprimates in the fossil record.

can reflect trophic position due to the discrimination of ^{12}C at each trophic level (resulting in systematically higher $\delta^{13}C$ values), but primates often confound this pattern by consuming ^{13}C-enriched plant tissues in forest canopies, open-canopy woodlands, or C_4 grasslands. A diet based on these plant tissues can result in leopard-like $\delta^{13}C$ values (Lee-Thorp et al., 2000), thus obscuring trophic differences (Yeakel et al., 2007). This coarse level of resolution is an enduring problem for interpreting hominin diets (Klein, 2013). Oxygen stable isotope ratios can also inform paleoecological studies. However, they too can vary widely due to fluctuations in relative humidity, the consumption of plant tissues with different sensitivities to evaporative fractionation, and species-level variation in drinking behavior and physiology (discussions vis–à–vis primates: Levin et al., 2006; Moritz et al., 2012, Ecker et al., 2013; Krigbaum et al., 2013). Thus, the coupled analysis of $\delta^{13}C$ and $\delta^{18}O$ values is practical for identifying the isotopic niches of fossil taxa, but it is relatively insensitive to trophic differences.

Stable isotope applications that predate the Miocene have more promise as this time interval mitigates some of the confounding factors above. The available evidence suggests that habitats in North America were relatively open during the Eocene (Secord et al., 2008), which should result in a narrower range of $\delta^{13}C$ values due to negligible "canopy effects" (the vertical gradient of decreasing $\delta^{13}C$ values from a canopy to the understory; Kohn, 2010). In addition, because C_4 grasslands emerged 7–8 million years ago, C_4 plants were an improbable food source for Eocene primates. Thus, variation in $\delta^{13}C$ values should correspond more clearly with trophic level. Indeed, Secord et al. (2008) interpreted $\delta^{13}C$ and $\delta^{18}O$ values from Early Eocene specimens of *Cantius*, an adapiform, as evidence of frugivory in an open-canopy habitat. However, this study was devoid of unequivocal faunivores, or "control taxa" (Kohn and Cerling, 2002), that help define the isotopic end members of a dietary spectrum. It is therefore uncertain if the tandem of $\delta^{13}C$ and $\delta^{18}O$ values can truly differentiate the trophic positions of early euprimates.

Calcium stable isotope ratios offer a complementary approach for inferring dietary behavior in the primate fossil record. Calcium is a major component of global geochemical cycles and is crucial to life on Earth; it is also a stoichiometric component in bioapatite. The biological processing of calcium (specifically, the mineralization and demineralization of bone) fractionates Ca isotopes and causes the $\delta^{44/40}Ca$ (hereafter $\delta^{44}Ca$) values of some organisms to vary by as much as 4–5‰ (Skulan and DePaolo, 1999; DePaolo, 2004). Crucially, bone apatite $\delta^{44}Ca$ values are 1.3–1.5‰ lower than those of dietary calcium due to the preferential incorporation of ^{40}Ca. Chu et al. (2006) reported a virtually identical result for red deer (*Cervus elaphus*), suggesting a consistent difference between the $\delta^{44}Ca$ values of diet and bone ($\Delta_{diet\text{-}bone}$). The authors attributed such uniformity to rates of bone formation and resorption that equal the intake of dietary calcium, which is essentially constant during adulthood. A predictable $\Delta_{diet\text{-}bone}$, when combined with systematic decreases in $\delta^{44}Ca$ values (~1.0‰) at increasing trophic levels (Skulan et al., 1997), raises the possibility that $\delta^{44}Ca$ values can be used to discriminate trophic positions among fossil organisms (Clementz et al., 2003; Heuser et al., 2011; but cf. Reynard et al., 2010).

A caveat to using $\delta^{44}Ca$ values as a dietary indicator concerns certain life history stages. The rate of bone

formation and resorption can vary during early development and late senescence, which affects calcium flux (Skulan et al., 2007; Heuser and Eisenhauer, 2010; Morgan et al., 2012). For example, active bone formation during growth is expected to yield δ^{44}Ca values closer to those of the diet (Skulan and DePaolo, 1999). Another factor is the consumption of milk, a major source of calcium during infant growth and development (Chu et al., 2006). Accordingly, several studies have examined whether δ^{44}Ca values can isotopically identify lactation, weaning, or human dairy consumption during the Neolithic (Reynard et al., 2010, 2011, 2013). These studies suggest that any application of calcium isotope analysis to trophic questions should be limited to adults until these life history patterns are better understood.

STUDY DESIGN

We focused on two assemblages of contemporary mammals to explore the premise of using coupled carbon and calcium stable isotope ratios as a paleodietary indicator. We selected these communities because they include species with functional or phylogenetic relevance to primates. They also contain "control taxa" that capture the isotopic end members of the dietary spectrum, i.e., pure primary consumers and obligate carnivores (Table 1). We focused on Sabah, northern Borneo because it contains an extraordinary diversity of euarchontan species in the orders Scandentia ($n = 7$), Dermoptera ($n = 1$), and Primates ($n = 13$) (Emmons, 2000; Hazebroek et al., 2012). The Área de Conservación Guanacaste, northwestern Costa Rica also contains a diverse assemblage of tropical mammals, including three primate species and *Caluromys derbianus*, the archetypal marsupial analogue of stem euprimate foraging behaviors (Rasmussen, 1990; Rasmussen and Sussman, 2007). The habitat is further suited because it is a warm deciduous forest that could yield stable isotope ratios that resemble those from the Early Eocene (Secord et al., 2008).

To verify that our sample is sensitive to isotopic differentiation, we first measured bone collagen δ^{13}C and δ^{15}N values. This isotopic tandem is impractical for fossil species (Fig. 1), but it affirmed the systematic retention of ^{13}C and ^{15}N at each trophic level (Ambrose and DeNiro, 1986; Gannes et al., 1997). Thus, the isotopic niches in our two systems correspond with known or inferred ecological niches (Newsome et al., 2007). This result justifies the use of our communities as model systems for testing the predicted inverse relationship between bioapatite δ^{13}C and δ^{44}Ca values, a tandem that is expected to preserve in the fossil record. Support for this prediction would validate the use of calcium stable isotope analysis as a tool for studying the paleodietary ecology of primates.

MATERIALS AND METHODS

Study sites and sample acquisition

We surveyed species in (i) Sabah, northern Borneo, Malaysia; and, (ii) the Área de Conservación Guanacaste (ÁCG), northwestern Costa Rica. The lowland and lower montane rainforests of Sabah have tall (50–60 m), dense canopies with a mean annual rainfall of ~2700 mm and little seasonal variation (Walsh and Newberry, 1999; de Gouvenain and Silander, 2003). In contrast, the lowland deciduous forests of ÁCG have shorter (15–23 m), sparser canopies and mean annual rainfall is ~1,500 mm with profound seasonal variation. The six-month dry season corresponds with higher temperatures and widespread defoliation in the canopy (Kalacska et al., 2007; Holmes et al., 2011; Melin et al., 2013).

To estimate the isotopic baseline of vegetation in each habitat, and hence the trophic offset between primary producers and consumers, we assembled data on canopy and understory leaves (Borneo) and sun-exposed or shaded leaves (Costa Rica). For Borneo, we used the foliar δ^{13}C and δ^{15}N values of 11 canopy and 15 understory species in Lambir Hills National Park, Sarawak (Hyodo et al., 2010; Hyodo, pers. comm.). The δ^{15}N values agree well with those reported by Woodcock et al. (2012), who focused solely on foliar δ^{15}N values in Danum Valley, Sabah. For Costa Rica, we used foliar δ^{13}C and δ^{15}N values from ÁCG ($n = 87$ species; Powers and Tiffin, 2010). We categorized these leaves as sun-exposed or shaded based on our own research in ÁCG and the classifications of a local botanist. The category "sun-exposed" includes canopy species as well as smaller species found in edge habitats or areas of early succession (Supporting Information Table S1). Few studies have measured the δ^{44}Ca values of leaves, but the range of variation (reported here relative to BSE) is broadly consistent across North America (Page et al., 2008: −0.94 to −0.31‰; Holmden and Bélanger, 2010: −0.44 to 0.46‰), Europe (Cenki-Tok et al., 2009: −0.76 to −0.36‰), and Hawaii (Weigand et al., 2005: −0.43 to 0.74‰). Thus, we used the lower- and uppermost δ^{44}Ca values (−0.94 and 0.74‰) to capture the likely range of foliar values in our study systems.

Small fragments of bone (<200 mg) were sampled from adult specimens housed in the osteological collections of the Kinabalu National Park Museum (KNPM), Ranau, Malaysia ($n = 13$). Similarly, bone and tooth fragments were sampled from the Sector Santa Rosa Park Museum (SSRPM), Costa Rica ($n = 31$). Local restrictions account for such limited sample sizes; and, in some cases, quantity, which precluded measurement of δ^{13}C values in the bioapatite of the KNPM samples. The KNPM specimens were collected in Sabah between 1999 and 2009 as part of a larger biological survey. The SSRPM specimens were collected opportunistically by park staff and researchers between 2003 and 2012. In the final analysis, we augmented this data set with published values from additional primate specimens ($n = 7$) sourced from the SSRPM (Crowley et al., 2010). Finally, we classified the diet of each species into the following *a priori* categories: folivore, frugivore, omnivore, faunivore (of invertebrates), or faunivore (of vertebrates).

Sample preparation and analysis

A note on terminology: isotope ratios are presented as δ values, where $\delta = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$ and R = the ratio of the heavier isotope to the lighter isotope (i.e., $R = {}^{13}C/{}^{12}C$, ${}^{15}N/{}^{14}N$, or ${}^{44}Ca/{}^{40}Ca$) in parts per thousand (‰) relative to a reference standard: Pee Dee belemnite (PDB) for carbon; atmospheric N_2 (Air) for nitrogen; and bulk silicate earth for calcium (BSE; Simon and DePaolo, 2010).

Bone collagen δ^{13}C and δ^{15}N values. Methods follow Crowley et al. (2010). Bone samples were coarsely ground with an agate mortar and pestle and transferred to glass scintillation vials. The samples were

TABLE 1. Dietary classification, trophic level, and stable isotope composition of tissues in our study

Sources and species nomina[a]	Dietary classification[b]	Trophic level	Collagen $\delta^{13}C$	Collagen $\delta^{15}N$	Collagen C:N ratio (Atom %)	Bioapatite (enamel) $\delta^{13}C$	Bioapatite (bone) $\delta^{13}C$	Bioapatite (bone) $\delta^{44}Ca$	Data source
KNPM (Borneo)									
Sunda colugo (*Galeopterus variegatus*)	Folivore	Primary	−23.5	6.3	3.2				Current study
Red leaf monkey (*Presbytis rubicanda)*	Folivore	Primary	−21.9	1.1	3.2			−2.33 (0.09)	Current study
Prevost's squirrel (*Callosciurus prevosti)*	Frugivore	Primary	−22.7	4.6	3.2			−1.56 (0.18)	Current study
Short-nosed fruit bat (*Cynopterus bracyotis*)	Frugivore	Primary	−23.5	3.1	3.2			−1.46 (0.10)	Current study
Bornean gibbon (*Hylobates muelleri*)	Frugivore	Primary	−23.2	3.7	3.3			−3.12 (0.21)	Current study
Long-tailed macaque (*Macaca fasicularis*)	Omnivore	Intermediate	−22.6	3.9	3.3			−2.40 (0.25)	Current study
Pig-tailed macaque (*Macaca nemestrina)*	Omnivore	Intermediate	−22.2	6.2	3.3			−3.49 (0.14)	Current study
Lesser gymnure (*Hylomys suillus*)	Faunivore (I)	Secondary	−19.9	6.5	3.3			−2.46 (0.31)	Current study
Slow loris (*Nyctocebus coucang*)	Faunivore (I)	Secondary	−21.1	6.0	3.2			−1.63 (0.28)	Current study
Bornean tarsier (*Tarsius bancanus*)	Faunivore (I)	Secondary	−23.1	5.7	3.3			−1.64	Current study
Slender treeshrew (*Tupaia gracilis*)	Faunivore (I)	Secondary	−22.5	6.2	3.5			−1.36 (0.42)	Current study
Plain treeshrew (*Tupaia longipes*)	Faunivore (I)	Secondary	−24.2	8.6	3.6			−1.93 (0.21)	Current study
Montain treeshrew (*Tupaia montana*)	Faunivore (I)	Secondary	−22.0	5.7	3.2			−2.08 (0.30)	Current study
Large treeshrew (*Tupaia tana*)	Faunivore (I)	Secondary	−22.1	7.2	3.2			−1.82	Current study
Leopard cat (*Felis bengalensis*)	Faunivore (V)	Secondary	−20.7	8.6	3.3			−3.23	Current study
SSRPM (Costa Rica)									
Mantled howling monkey (*Alouatta palliata*)	Folivore	Primary	−24.0	4.4	3.4		−18.0		Crowley et al. (2010)
Mantled howling monkey (*Alouatta palliata*)	Folivore	Primary	−23.5	6.5	3.4		−19.0		Crowley et al. (2010)
Mantled howling monkey (*Alouatta palliata*)	Folivore	Primary	−24.9	4.4	3.3		−18.5		Crowley et al. (2010)
Mantled howling monkey (*Alouatta palliata*)	Folivore	Primary					−3.81[c]	−2.06	Current study
Two-toed sloth (*Choloepus hoffmanni*)	Folivore	Primary	−23.4	8.2	3.2		−17.5		Current study
White-tailed deer (*Odocoileus virginianus*)	Folivore	Primary	−23.0	6.9	3.2	−14.4	−16.0		Current study
Baird's tapir (*Tapirus bairdi*)	Folivore	Primary	−24.3	4.9	3.2	−15.2	−15.3		Current study
Paca (*Agouti paca*)	Frugivore	Primary				−16.5	−14.5		Current study
Black-handed spider monkey (*Ateles geoffroyi*)	Frugivore	Primary				−16.2		−2.30	Current study
Black-handed spider monkey (*Ateles geoffroyi*)	Frugivore	Primary	−22.7	3.9	3.3		−17.6		Crowley et al. (2010)
Black-handed spider monkey (*Ateles geoffroyi*)	Frugivore	Primary	−23.3	4.4	3.3		−18.0		Crowley et al. (2010)
Agouti (*Dasyprocta punctata*)	Frugivore	Primary				−12.7	−15.7		Current study
Agouti (*Dasyprocta punctata)*	Frugivore	Primary	−23.0	4.7	3.2	−17.7	−15.8		Current study
Variegated squirrel (*Sciurus variegatoides*)	Frugivore	Primary	−22.7	3.9	3.2	−18.6	−15.8		Current study
White-faced capuchin (*Cebus capucinus*)	Omnivore	Intermediate	−24.0	9.5	4.1		−18.3		Crowley et al. (2010)
White-faced capuchin (*Cebus capucinus*)	Omnivore	Intermediate	−23.2	9.8	3.4				Crowley et al. (2010)
White-faced capuchin (*Cebus capucinus*)	Omnivore	Intermediate				−16.8		−1.34	Current study
Striped hog-nosed skunk (*Conepatus semistriatus*)	Omnivore	Intermediate	−15.8	8.7	3.3	−15.7	−12.4		Current study
Common opossum (*Didelphis marsupialis*)	Omnivore	Intermediate	−22.1	7.6	3.3	−15.0	−15.3		Current study
Common opossum (*Didelphis marsupialis*)	Omnivore	Intermediate					−15.8		Current study
Coatimundi (*Nasua narica*)	Omnivore	Intermediate	−21.1	7.1	3.3	−15.7	−15.8		Current study
Coatimundi (*Nasua narica*)	Omnivore	Intermediate					−15.1		Current study
Big-eared climbing rat (*Ototylomys phyllotis*)	Omnivore	Intermediate	−19.0	8.5	3.6	−14.4	−13.9		Current study

TABLE 1. Continued

Sources and species nomina[a]	Dietary classification[b]	Trophic level	Collagen $\delta^{13}C$	Collagen $\delta^{15}N$	Collagen C:N ratio (Atom %)	Bioapatite (enamel) $\delta^{13}C$	Bioapatite (bone) $\delta^{13}C$	Bioapatite (bone) $\delta^{44}Ca$	Data source
Northern raccoon (*Procyon lotor*)	Omnivore	Intermediate	−21.6	9.7	3.2		−16.9		Current study
Collared peccary (*Tayassu tajacu*)	Omnivore	Intermediate	−23.0	5.1	3.3	−14.0	−16.3	−1.87	Current study
Nine-banded armadillo (*Dasypus novemcinctus*)	Faunivore (I)	Secondary	−19.1	9.9	3.3		−14.0	−2.34	Current study
Tamandua (*Tamandua mexicana*)	Faunivore (I)	Secondary	−22.8	7.9	4.1				Current study
Tamandua (*Tamandua mexicana*)	Faunivore (I)	Secondary	−21.3	7.5	3.4		−16.6	−1.88	Current study
Coyote (*Canis latrans*)	Faunivore (V)	Secondary	−16.1	12.7	3.4	−13.3	−11.4		Current study
Tayra (*Eira barbara*)	Faunivore (V)	Secondary	−21.1	7.9	3.3	−15.2	−13.7		Current study
Jaguarundi (*Herpailurus yaguarondi*)	Faunivore (V)	Secondary	−18.9	9.3	3.3	−11.7	−9.6		Current study
Ocelot (*Leopardus pardalis*)	Faunivore (V)	Secondary				−15.5	−15.1		Current study
Jaguar (*Panthera onca*)	Faunivore (V)	Secondary				−12.6			Current study
Jaguar (*Panthera onca*)	Faunivore (V)	Secondary	−20.1	8.9	3.3	−14.7	−16.0	−2.70	Current study
Puma (*Puma concolor*)	Faunivore (V)	Secondary				−15.1	−15.3		Current study
Puma (*Puma concolor*)	Faunivore (V)	Secondary	−22.0	8.7	3.3	−16.2	−16.6		Current study
Grey fox (*Urocyon cinereoargenteus*)	Faunivore (V)	Secondary	−20.6	8.3	3.4				Current study
Grey fox (*Urocyon cinereoargenteus*)	Faunivore (V)	Secondary	−18.5	6.7	3.5				Current study

[a] We report mean (±2 SEM) $\delta^{44}Ca$ values for samples run in duplicate; the analytical precision for all other isotopic ratios are described in the text.
KNPM = Kinabalu National Park Museum; SSRPM = Sector Santa Rosa Park Museum.
[b] Here we distinguish faunivores of (I) invertebrate prey from faunivores of (V) vertebrate prey
[c] This value is an outlier and presumed to be erroneous. It is therefore excluded from our analyses

subsequently demineralized via sonication in 0.5*N* hydrochloric acid for 10 min and refrigerated until the product was malleable (ca. 2–3 days). The hydrochloric acid was then removed via pipette and the samples were rinsed 5 times with ultrapure water and dried. Lipid extraction was performed via repeated 15-min cycles of sonication in petroleum ether (5 mL per sample), which was replaced between cycles. Finally, the samples were rinsed five times with distilled water, dried, and weighed (0.80 ± 0.20 mg) into 5 × 9-mm tin boats (part no. 041077, Costech Analytical, Valencia, USA). Combustion and analysis was performed using a Delta Plus XP isotope ratio mass spectrometer (Thermo-Finnigan, Bremen, Germany) interfaced with a NC2500 Elemental Analyzer (Carlo Erba, Milan, Italy) located in the Stable Isotope Laboratory at the University of California, Santa Cruz. Analytical precision (2 SEM) based on seven IAEA Acetanilide replicates was 0.04‰ and 0.03‰ for $\delta^{13}C$ and $\delta^{15}N$, respectively. The mean difference of 20 samples run in duplicate was 0.2‰ for carbon and 0.2‰ for nitrogen.

Bioapatite $\delta^{13}C$ and $\delta^{44}Ca$ values. Carbonate samples were prepared by powdering bone and enamel with a handheld rotating Dremel equipped with a dental drill bit. Approximately 20 mg of bone or 10 mg of enamel powder were immersed in 1 mL of 30% laboratory-grade hydrogen peroxide to remove organic material. The bone samples were reacted for 72 h and the hydrogen peroxide was refreshed after 48 h. The enamel samples were reacted for 24 h. Following these reactions, all samples were rinsed five times with ultrapure water. To remove non-lattice bound carbonate, samples were reacted with 0.5 mL of 1*M* acetic acid (buffered to pH 5.0 with calcium acetate) for 24 h at 4°C. The samples were rinsed with ultrapure water and lyophilized. Analyses were conducted on a Kiel IV carbonate device interfaced to a MAT 253 dual-inlet isotope ratio mass spectrometer (Thermo-Finnigan, Bremen, Germany) located in the Stable Isotope Laboratory, University of California, Santa Cruz. Samples were dissolved in 100% orthophosphoric acid and reacted for 10 min. The mean analytical precision (±2 SEM) was 1.95‰ (±0.05‰) based on four replicates of NBS 19, which compares well with the established value of 2.0‰. The mean difference of 6 samples run in duplicate was 0.24‰.

The analytical cost of calcium isotope analysis is presently two orders of magnitude greater than conventional isotopic analyses. Thus, for our Costa Rican dataset, we focused our analysis on a subset of "control taxa" (Kohn and Cerling, 2002) with well-known and divergent diets ($n = 7$, Table 1). All Bornean samples were analyzed using the ^{48}Ca–^{42}Ca double-spike method (Skulan et al., 2007), whereas the Costa Rican samples were analyzed using the ^{43}Ca–^{42}Ca double-spike method (Lehn et al., 2013). Both methods are well established, however the latter data are ~3–6 times more precise compared to the former, as determined by the external reproducibility (2SD) of standard measurements. All $\delta^{44}Ca$ values are reported relative to bulk silicate Earth (BSE) following Nielson et al. (2011).

The Bornean samples were cleaned and dissolved in 3M ultrapure nitric acid on a ~100°C hot plate overnight to facilitate dissolution. A mixed ^{42}Ca–^{48}Ca tracer (i.e., "double-spike"; $^{42}Ca/^{48}Ca$) was added to an aliquot of each solution to correct for isotopic fractionation

within the mass spectrometer during analysis. Chemical purification of the sample-tracer solution was achieved via column chromatography using element-specific DGA resin (Eichrom Technologies, normal variety) and nitric acid and DI water as the elutents (Nielsen et al., 2011). Next, ~3 μg of the dissolved, spiked samples were loaded onto degassed, zone-refined Re filaments and capped with ~1 μL of 20% phosphoric acid in preparation for analysis in Triton thermal ionization multi-collector mass spectrometer (Thermo-Finnigan, Bremen, Germany) located in the Center for Isotope Geochemistry at UC Berkeley. The intensities of the ^{39}K, ^{40}Ca, ^{42}Ca, ^{43}Ca, ^{44}Ca, ^{48}Ca, and ^{49}Ti ion beams were measured with two static cup configurations in order to calculate calcium isotope ratios as well as the contributions of potential isobaric interferences to those ratios. The contribution of mass interferences from ^{40}K and ^{48}Ti were found to be insignificant; accordingly, no corrections were applied. The Ca isotope standard SRM 915a (NIST synthetic carbonate) was analyzed at the beginning, middle, and end of each sample turret. Six replicates of 915a and a secondary standard (modern cow bone) were also measured during each session. The mean analytical precisions (±2 SEM) were −1.0‰ (±0.04‰) and −2.085‰ (±0.06‰), respectively. The experimental samples were measured in duplicate and uncertainties are reported as the mean (2SEM, Table 1).

The Costa Rican samples were transferred to Teflon vials and dissolved overnight in 6N HCl at 95°C on a hot plate. The solutions were evaporated to dryness and re-dissolved in 10 mL of 5% HNO_3. Calcium isotope ratios were measured according to the procedure described in Lehn et al. (2013). A ^{43}Ca–^{42}Ca double-spike having a molar ^{43}Ca/^{42}Ca ratio of 1 was mixed with 50 μg of sample Ca to achieve a molar spike/sample ratio of 0.33. To ensure isotopic equilibration, the mixtures were gently heated on a hotplate in capped Teflon vials. The mixtures were eluted through Teflon columns packed with Bio-Rad AG MP-50 cation exchange resin to isolate Ca from matrix elements. After drying the purified fractions, two drops of 35% H_2O_2 were added to oxidize organic compounds, and two drops of concentrated HNO_3 were added to convert Ca to nitrate form. Approximately 10–16 μg of Ca was loaded onto single filament assemblies containing degassed Ta ribbon, and 0.5 μg of 10% H_3PO_4 was added before drying at 3.5 amps. Ultrapure reagents were used for all steps, and blanks were negligible. Sample to blank ratios were ~500:1 or better. Calcium isotope ratios were measured using a Triton thermal ionization multi-collector mass spectrometer (ThermoFisher, Bremen, Germany) located in the Radiogenic Isotope Geochemistry Laboratory at Northwestern University. In the mass spectrometer, a 20V ^{40}Ca ion beam was attained after a 30 min warm-up, and ^{40}Ca/^{42}Ca, ^{43}Ca/^{42}Ca, and ^{43}Ca/^{44}Ca ratios were measured with a three-hop collector cup configuration for a total of 90 duty cycles requiring an additional 2.5 h. The ^{41}K beam was monitored during the first hop to ensure that ^{40}K did not isobarically interfere with ^{40}Ca. No corrections were required. The internal precision of the measurements was ±0.02‰ (2SEM). At Northwestern University, OSIL Atlantic Seawater is employed as the normalizing standard for the delta equation. Long-term accuracy for the method is continuously monitored by repeated analyses of the following standards: OSIL Atlantic Seawater [0.000 ± 0.003‰ (2SEM), $n = 159$], 915a [−1.862 ± 0.006‰ (2SEM), $n = 55$], and 915b [−1.132 ± 0.004‰ (2SEM), $n = 104$]. These data correspond to a global long-term, external reproducibility of ±0.04‰ (2SD), which is the error adopted for the Costa Rica samples analyzed in the present study. To compare with the UC Berkeley data, the δ^{44}Ca values measured at Northwestern University were converted to the bulk silicate earth (BSE) scale using the equations: $\delta^{44}Ca_{915a} = \delta^{44}Ca_{sw} + 1.86‰$ and $\delta^{44}Ca_{BSE} = \delta^{44}Ca_{915a} - 1.00‰$ (Nielson et al., 2011).

Statistical analyses

We used univariate and multivariate analysis of variance (ANOVA, MANOVA) to test whether vertical stratum (canopy, understory) or sunlight exposure (shaded, sun-exposed) can explain a significant amount of variation in the isotopic "baseline" (vegetation) of both systems, and whether trophic position could explain a significant amount of variation in the isotopic composition of the two mammalian communities. The Bornean and Costa Rican data sets were analyzed separately. For each isotope ratio, or pair of ratios, we compared primary consumers with faunivores using a one-tailed, planned contrast due to the predicted enrichment in ^{13}C and ^{15}N and depletion in ^{44}Ca at each trophic level. The univariate analyses of bioapatite δ^{13}C values were conducted separately for bone and enamel. The multivariate analyses of bioapatite δ^{13}C values were based on the calculated mean of both bone and enamel, as these tissues were undifferentiated across species (paired t-test: $t = 0.95$, $df = 17$, $P = 0.36$; Table 1). All statistical analyses were conducted using PROC GLM in SAS version 9.0 and the significance for all tests was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Isotopic baselines in Borneo and Costa Rica

Figure 2A illustrates the "canopy effect" in Borneo, where the mean (±1 SD) δ^{13}C value of canopy leaves (−29.7 ± 1.7‰) is substantially higher than that of understory leaves (−33.7 ± 1.6‰) as a result of decreasing irradiance and the recycling of CO_2 ($F_{1,24} = 39.82$, $P < 0.001$); in contrast, the δ^{15}N values do not differ ($F_{1,24} = 2.04$, $P = 0.17$; Fig. 2A). The magnitude of this effect is evident in the δ^{13}C values of two similar-sized primates. Although Bornean tarsiers (*Tarsius bancanus*) are predicted to have ^{13}C-enriched tissues relative to slow lorises (*Nycticebus coucang*)—because *T. bancanus* is an exclusive faunivore (Niemitz, 1984), whereas *N. coucang* consumes quantities of sap, floral nectar, and fruit (Wiens et al., 2006)—the reverse pattern is reported in Table 1. This result is likely due to the canopy effect: tarsiers capture their prey on or near the forest floor, whereas slow lorises forage in the mid and upper canopy. Thus, a strong canopy effect can overwhelm variation in δ^{13}C values due to diet-induced isotopic differences.

In Costa Rica, the distribution of foliar δ^{13}C values is less variable than that in Borneo (Levene's test: $F_{1,111} = 30.88$, $P < 0.001$), although a canopy effect is still detectable (Fig. 2B). The mean (±1 SD) δ^{13}C value of sun-exposed leaves (−29.1 ± 1.1‰) is narrowly higher than that of shaded leaves (−29.9 ± 1.4‰; $F_{1,85} = 6.44$, $P = 0.01$), suggesting little biological significance (but see Leffler and Enquist, 2002). The δ^{15}N values are marginally indifferent ($F_{1,85} = 3.59$, $P = 0.06$).

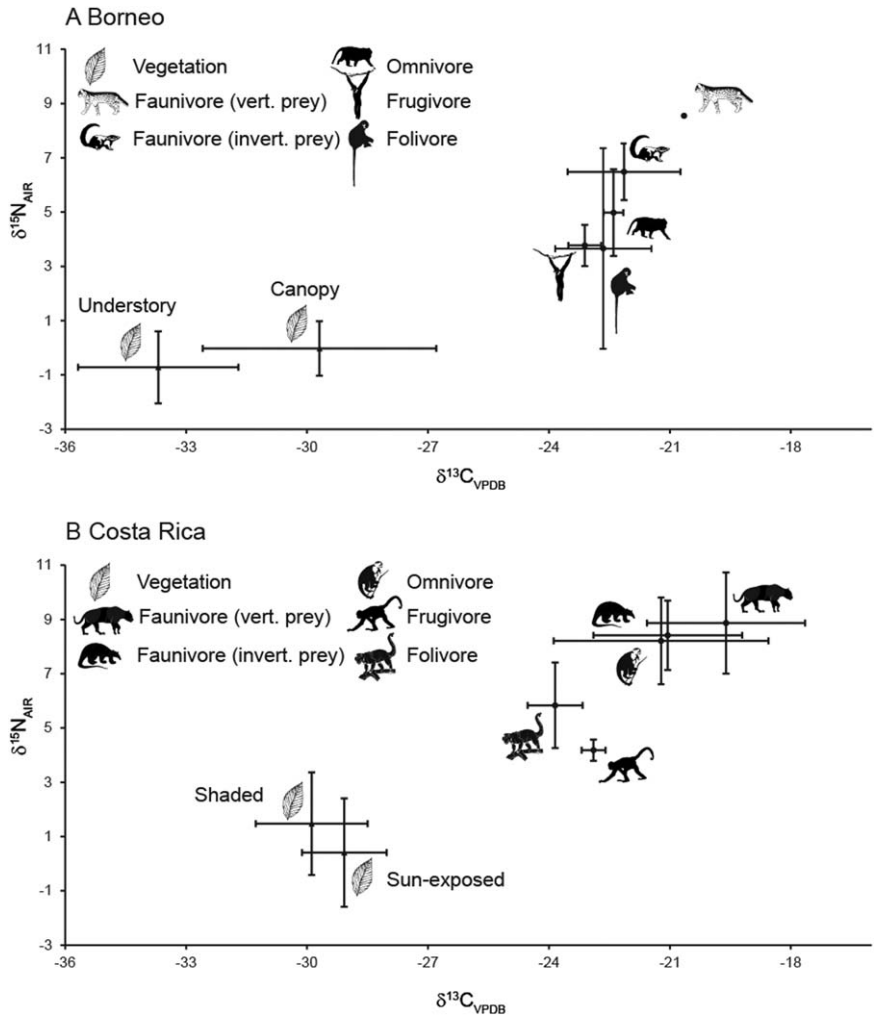

Fig. 2. Mean (±1 SD) bone collagen $\delta^{13}C$ and $\delta^{15}N$ values of mammals, grouped by dietary category, in two communities: **A**) Sabah, northern Borneo, Malaysia; and, **B**) Área de Conservación Guanacaste, northwestern Costa Rica. The isotopic baselines for these two communities are represented with mean (±1 SD) foliar $\delta^{13}C$ and $\delta^{15}N$ values from Hyodo et al. (2010) [Borneo] and Powers and Tiffin (2010) [Costa Rica].

Bone collagen $\delta^{13}C$ and $\delta^{15}N$ values

Although enamel is clearly the material of choice for stable isotope analysis of fossil materials (review: Clementz et al., 2009), we measured the isotopic composition of bone collagen to verify that trophic differences are detectable in our study systems. As predicted, we found ^{13}C- and ^{15}N-enrichment with increasing trophic level (Ambrose and DeNiro, 1986; Gannes et al., 1997), resulting in successive trophic "steps" that correspond with systematic increases in $\delta^{13}C$ and $\delta^{15}N$ values (Fig. 2A,B). The combined variation in the $\delta^{15}N$ and $\delta^{13}C$ values of herbivores and faunivores is significant (Borneo: $F_{2,9} = 8.29$, $P = 0.01$; Costa Rica: $F_{2,24} = 15.86$, $P < 0.01$), with the omnivores occupying an intermediate position, or half step. These values agree well with other studies, including those from dipterocarp forests in Sundaland (Nakagawa et al., 2007; Hyodo et al., 2010; Kawanishi et al., 2012).

For present purposes, a crucial result is that the mean (±1 SD) $\delta^{15}N$ values of faunivores in Borneo ($6.8 \pm 1.2‰$) and Costa Rica ($8.8 \pm 1.7‰$) are significantly higher than those of the primary consumers, i.e., frugivores and folivores, in Borneo ($3.8 \pm 1.5‰$, $F_{1,11} = 12.28$, $P < 0.01$) and Costa Rica ($5.2 \pm 1.5‰$, $F_{1,26} = 25.61$, $P < 0.01$), resulting in trophic steps of 3.0‰ and 3.6‰, respectively. The size of the former step is consistent with other studies (e.g., Hyodo et al., 2010), whereas the larger step in ÁCG can be attributed to the combination of higher temperatures and greater aridity (resulting in ^{15}N-enriched tissues; Ambrose, 1991) and the consumption of leguminous plants by some primates in ÁCG (resulting in ^{15}N-depleted tissues; Schoeninger et al., 1997). In sum, these findings justify the use of these communities to test predictions concerning calcium isotope ratios.

Bioapatite $\delta^{13}C$ and $\delta^{44}Ca$ values

The carbon in bone collagen is strongly labeled by the protein-fraction of diet, whereas the carbon in bioapatite is derived from blood bicarbonate, which is produced through respiration and closely tracks bulk dietary carbon, which includes carbohydrates, lipids, and proteins

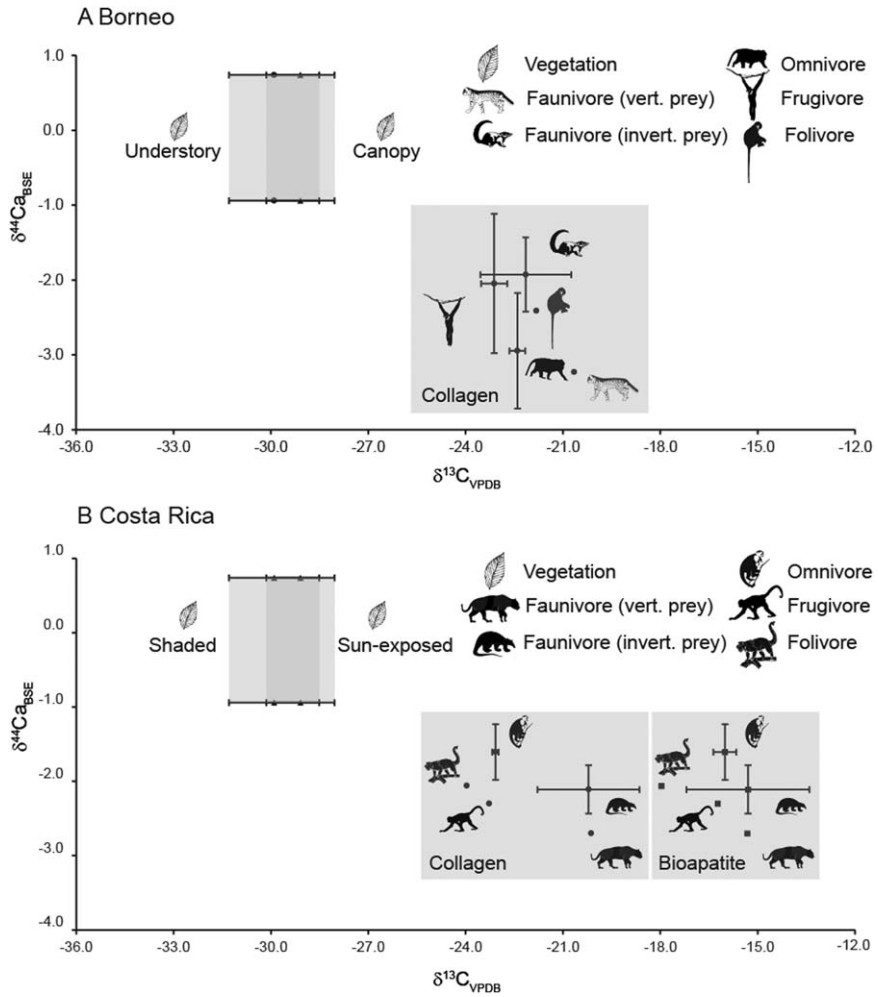

Fig. 3. Mean (±1 SD) bioapatite $\delta^{13}C$ and $\delta^{44}Ca$ values or bone collagen $\delta^{13}C$ values of mammals, grouped by dietary category, in two communities: **A**) Sabah, northern Borneo, Malaysia; and, **B**) Área de Conservación Guanacaste, northwestern Costa Rica. The isotopic baselines for these two communities are represented with mean (±1 SD) foliar $\delta^{13}C$ and $\delta^{44}Ca$ values ($\delta^{13}C$ values: Hyodo et al., 2010 [Borneo], Powers and Tiffin, 2010 [Costa Rica]; $\delta^{44}Ca$ values: Weigand et al., 2005; Page et al., 2008; Cenki-Tok et al., 2009; Holmden and Bélanger, 2010).

(Jim et al., 2004). As a result, the $\delta^{13}C$ values of bone collagen differ from those of bioapatite, although the difference, or spacing, varies as a function of diet (Table 1; review: Clementz et al., 2009). Concurrent analysis of bone collagen and bone apatite can provide a more complete picture of animal diet and physiology. We therefore calculated the difference (Δ) between the $\delta^{13}C$ values of bone apatite and collagen ($\Delta^{13}C_{bioapatite\text{-}collagen}$). The mean (± 1SD) $\Delta^{13}C_{bioapatite\text{-}collagen}$ value of the faunivores ($5.8 \pm 1.8‰$) is slightly less than that of the primary consumers ($6.3 \pm 1.3‰$). A consequence of this spacing, which agrees well with other studies (Clementz et al., 2009; Crowley et al., 2010), is that $\delta^{13}C_{collagen}$ values reveal trophic differences more clearly than do $\delta^{13}C_{bioapatite}$ values.

Figure 3A plots the $\delta^{13}C_{collagen}$ and $\delta^{44}Ca$ values of Bornean species (recall that sampling restrictions precluded $\delta^{13}C_{bioapatite}$ values). Thus, the main value of this figure is that it shows downward trophic steps from leaves to herbivores to omnivores to a single felid, the leopard cat (*Felis bengalensis*). The species that consume invertebrates (mostly treeshrews) have higher $\delta^{44}Ca$ values than expected; indeed, they resemble those of frugivores, a point that is revisited below. Figure 3B plots $\delta^{13}C_{collagen}$, $\delta^{13}C_{bioapatite}$, and $\delta^{44}Ca$ values from Costa Rica. This figure illustrates two practical points. The first is that variation in $\delta^{13}C_{bioapatite}$ values is relatively compressed compared to the $\delta^{13}C_{collagen}$ values. Thus, trophic differences based on coupled $\delta^{44}Ca$ and $\delta^{13}C_{bioapatite}$ values are less pronounced in a seasonally open habitat composed of C_3 plants ($F_{2,2} = 0.23$, $P = 0.81$), although the mean (±1 SD) $\delta^{13}C_{bioapatite}$ value of the primary consumers ($-16.8 \pm 1.4‰$) is less than that of the secondary consumers ($-14.3 \pm 2.4‰$; $F_{1,28} = 9.84$, $P < 0.01$). The second point is that we found modest evidence of downward trophic steps from leaves to primary consumers to a single felid, the jaguar (*Panthera onca*); however, the species that consume invertebrates again show higher $\delta^{44}Ca$ values than predicted (but similar to those from Borneo). We discuss these findings below.

Calcium isotope analysis: Still promising?

As predicted, the carnivorans at both sites revealed low $\delta^{44}Ca$ values; however, the mean (±1 SD) $\delta^{44}Ca$ values of faunivores overall (Borneo: $-2.0 \pm 0.6‰$; Costa Rica: $-2.3 \pm 0.4‰$) resembled those of primary

consumers (Borneo: $-2.1 \pm 0.8‰$, $F_{1,13} = 0.06$, $P = 0.81$; Costa Rica: $-2.2 \pm 0.2‰$; $F_{1,7} = 0.15$, $P = 0.72$). Several factors could account for this isotopic insensitivity to trophic level. First, the bulk calcium content of some fruits can be high, particularly figs (O'Brien et al., 1998), whereas the calcium content of insect prey can be negligible (Barker et al., 1998; Rothman et al., 2014). These factors are predicted to result in the depletion and enrichment of ^{44}Ca, respectively (Skulan et al., 2007; Heuser and Eisenhauer, 2010). Another plausible factor is the incidental ingestion of bioavailable calcium in soil particles. Soils are ^{44}Ca-enriched relative to plant tissues (DePaolo, 2004), and tarsiers, treeshrews, and armadillos are united in their tendency to forage in leaf litter; some collateral ingestion of soil seems inevitable. This conjecture can also extend to the tamandua given that arboreal termitaria are made of ground-sourced soils. Lastly, the exceptionally high $\delta^{44}Ca$ values of some omnivores is puzzling, particularly in the white-faced capuchin (*Cebus capucinus*), which consumes vertebrate prey. We speculate that this result reflects the dietary value of bird eggs in ÁCG (Rose, 1994). Egg white, or albumin, is extraordinarily enriched in ^{44}Ca relative to other biological tissues (Skulan and DePaolo, 1999).

Overall, the tandem of bioapatite $\delta^{13}C$ and $\delta^{44}Ca$ values failed to discriminate the primary and secondary consumers in our study. This result is disappointing, admittedly, as it weakens the premise of using coupled $\delta^{13}C$ and $\delta^{44}Ca$ values to test competing hypotheses on euprimate origins. Heuser et al. (2011) expressed a similar view: "regrettably, it is not possible to reconstruct [trophic level] effects in dinosaur ecosystems unambiguously from the [$\delta^{44}Ca$ values] of fossil bones", but they did detect a small enamel difference ($0.2‰$) between herbivores and *Tyrannosaurus rex*. Such a result is inconclusive but it is consistent with the present difference between taxonomic carnivores (felids) and other species, which raises the possibility of applying calcium isotope analyses to the study of fossil hominins. Hominins were nominal omnivores, but early *Homo* appeared to compete directly with felids for access to resources (Hoberg et al., 2001; Eppinger et al., 2006); and, by 4.4 Ma, hominins occupied habitats with a larger distribution of $\delta^{13}C$ values than those reported here (White et al., 2009). A wider range of $\delta^{13}C$ values holds greater potential for discriminating discrete foraging behaviors (e.g., Yeakel et al., 2009). And yet, a restricted range of $\delta^{13}C$ values does have at least one practical advantage, which we highlight below.

Guanacaste, Costa Rica as a model system

We focused on the Área de Conservación Guanacaste in part because it is a C_3 habitat with a rainy season (rainfall ~1,500 mm) and a warm dry season during which the canopy defoliates and the mean maximum temperature increases from 30–32°C to 32–36°C. Such conditions resemble those of two Early Eocene localities in the Willwood Formation, Bighorn Basin. Paleobotanical (leaf-margin) analyses indicate a comparable level of annual rainfall (1,200–1,400 mm), but lower mean annual temperatures of 11.5°C and 17.1°C (Secord et al., 2008), although the summer months were probably 18°C warmer than the annual mean (Snell et al., 2013). This inferred paleoclimate, together with a narrow range of $\delta^{13}C$ values in the mammalian assemblages, suggests a relatively open habitat based on similar spreads of $\delta^{13}C$ values in a savanna riparian habitat and an exposed understory in a subtropical monsoon forest (Ehleringer et al., 1987; Codron et al., 2005). More recently, a similar range was reported from sites in southwest Madagascar (Crowley et al., 2011). The present study adds the ÁCG of Costa Rica as a site with comparable values.

A practical advantage of the ÁCG concerns interpretations of Early Eocene adapiforms, such as *Cantius trigonodus* and *Cantius* sp., species with mean (±1 SD) $\delta^{13}C_{bioapatite}$ values of $-13.03 \pm 0.59‰$ and $-13.43 \pm 0.26‰$, respectively (Secord et al., 2008). These taxa are also relatively ^{18}O-enriched compared to other members of the community, with mean (±1 SD) $\delta^{18}O_{bioapatite\ SMOW}$ values of $21.10 \pm 0.61‰$ and $22.23 \pm 1.16‰$, respectively (Secord et al., 2008). This tandem of $\delta^{13}C$ and $\delta^{18}O$ values indicates a diet based on plant tissues that were sensitive to evaporative fractionation, and fruit is a strong candidate on the basis of functional anatomical interpretations (Covert, 2002). To corroborate this inference, we can correct for an atmospheric $\delta^{13}C$ value that was ~$2.1‰$ higher than today (Secord et al., 2008) and examine species in the ÁCG with analogous foraging ecologies, such as the black-handed spider monkey (*Ateles geoffroyi*) and the white-faced capuchin (*Cebus capucinus*). Indeed, comparable but lower ranges of $\delta^{13}C_{bioapatite\text{-}corrected}$ values (*A. geoffroyi*: −15.9 to −14.1‰; *C. capucinus*: −16.2 to −14.7‰; Table 1) attest to a diet premised on plant tissues, but it is the relatively high $\delta^{18}O_{bioapatite\ SMOW}$ values (*A. geoffroyi*: 30.6–31.2‰; *C. capucinus*: 30.0‰; B.E. Crowley, unpublished data) that speak to foraging activities in the upper canopy. Thus the stable isotope composition of *Cantius* mirrors that of frugivorous monkeys in the ÁCG, suggesting comparable foraging ecologies; however, this result cannot distinguish supplemental faunivory at capuchin-like levels. The present findings suggest that $\delta^{44}Ca$ values could be used in tandem with carbon and oxygen isotope ratios to determine whether Eocene primates such as *Cantius* were ecologically more similar to *Ateles* or *Cebus*.

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