Do Oxygen Isotope Values in Collagen Reflect the Ecology and Physiology of Neotropical Mammals?

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Stable isotope data provide insight into the foraging ecology of animals. Traditionally, carbon and nitrogen isotope values have been used to infer dietary and habitat preferences. Oxygen isotopes are used less frequently but may complement the ecological information provided by carbon and nitrogen, particularly in densely forested or arid environments. Additionally, because oxygen is preserved in both bioapatite and collagen, it is useful for paleoecological studies. To investigate the suitability of oxygen isotopes for complementing and building on ecological applications of carbon and nitrogen isotopes, we analyze all three isotopes in bone collagen for nearly identical assemblages of Costa Rican mammals in two ecologically distinct habitats - an evergreen rainforest and a seasonal dry forest. We assess the degree to which differences in habitat, activity pattern, diet, arboreality, and thermoregulation are revealed by each of the isotope systems. Our results highlight the potential of oxygen isotopes in modern and paleoecological contexts. In addition to reflecting habitat type, oxygen isotope values in collagen distinguish species on the basis of vertical habitat stratification and drinking behavior. Within a locality, individuals with low oxygen isotope values likely track meteoric water, whereas those with elevated values most likely consume evaporatively-enriched plant tissues, such as canopy leaves. These patterns will be useful in reconstructing paleoenvironments and interpreting ecological differences among taxa both extant and extinct.

Keywords: Costa Rica, collagen, niche overlap, carbon, nitrogen, oxygen

INTRODUCTION

Stable isotope data provide insight into the foraging ecology of species—including cryptic and extinct taxa—by preserving a record of ingested food and water. Traditionally, carbon ($\delta^{13}C$) and nitrogen ($\delta^{15}N$) isotope values have been used to infer differences in diet and habitat among species. However, the practical value of these isotope systems is limited in certain ecological contexts, such as densely forested or arid localities. Additionally, nitrogen isotope values can be measured only in proteinaceous tissues (i.e., collagen and keratin), which seldom preserve beyond

several thousand years (Tuross et al., 1988). Oxygen isotope ($\delta^{18}O$) values also have the potential to identify ecological differences among organisms (e.g., Bryant and Froelich, 1995; Lee-Thorp and Sponheimer, 2005; Nelson, 2013). Additionally, because oxygen is present in collagen and bioapatite, which is well preserved in the fossil record, it is well suited for investigating the foraging ecology of extant and extinct organisms. Yet few studies have investigated how variation in diet or habitat might affect oxygen isotope variation in modern or fossil communities (Bocherens et al., 1995, 1996; Kohn, 1996; Cerling et al., 2004; Lee-Thorp and Sponheimer, 2005; Levin et al., 2006; Secord et al., 2008; White et al., 2009; Pietsch et al., 2011; Moritz et al., 2012; Krigbaum et al., 2013; Nelson, 2013; Crowley, 2014; Carter and Bradbury, 2015).

The carbon, nitrogen and oxygen isotope values of animals are reflections of foraging behavior. Oxygen isotope values track those for carbon and nitrogen insofar as they are higher for plants and animals inhabiting dry, hot habitats compared to moist, cool ones (e.g., Cormie et al., 1994; Nelson, 2013). In addition, the $\delta^{18}O$ values of plants are positively related to height in stratified canopies (Sternberg et al., 1989; Ometto et al., 2005). These patterns are primarily driven by varying temperature, humidity, light, vapor pressure deficit, and the $\delta^{18}O$ of atmospheric water vapor beneath the forest canopy (Farquhar and Sharkey, 1982; Ometto et al., 2005; Barbour et al., 2007). Given that isotopic differences in canopy and understory vegetation are transmitted to the animals that forage in these strata, it is plausible that oxygen isotopes can distinguish arboreal species from those that feed on or near the forest floor (Krigbaum et al., 2013; Nelson, 2013; Carter and Bradbury, 2015).

In contrast to the prevailing pattern for carbon and nitrogen, faunivores tend to have lower $\delta^{18}O$ values than sympatric herbivores, particularly in arid habitats (Ambrose, 1992; Bocherens et al., 1995; Lee-Thorp and Sponheimer, 2005). Lower $\delta^{18}O$ values for faunivores may result from the consumption of ^{18}O-depleted animal fats and proteins or from frequent drinking. These same patterns may be able to distinguish faunivores that predominantly consume invertebrates from those that consume vertebrates. Additionally, oxygen isotope values may better discriminate folivores and frugivores than carbon or nitrogen isotopes values (Cerling et al., 2004; Krigbaum et al., 2013; Nelson, 2013). For example, fruits can exhibit higher $\delta^{13}C$ and lower $\delta^{15}N$ values than leaves, but these differences are relatively small and inconsistent (Cerling et al., 2004; Codron et al., 2006; Cernusak et al., 2009; Crowley et al., 2011). Although most plant tissues are not enriched in ^{18}O relative to source water, evapotranspiration increases $\delta^{18}O$ values of leaf tissues, particularly in arid localities or in the upper canopy (Sternberg et al., 1989; Yakir, 1997; Farquhar et al., 2007). In consequence, folivores, especially those foraging in the canopy, can have substantially higher $\delta^{18}O$ values than other animals (Cerling et al., 2004; Levin et al., 2006; Nelson, 2013; Carter and Bradbury, 2015).

Importantly, some animals may better reflect local temperature and humidity than others. For example, as nocturnal animals (particularly folivores) eat ^{18}O-depleted foods, they should be less affected by thermal stress and evaporative water loss than diurnal animals (Bocherens et al., 1996). Cooler nocturnal temperatures and the incorporation of oxygen from atmospheric CO_2, water vapor, and dewfall results in lower $\delta^{18}O$ values for foliage at night (Ometto et al., 2005; Seibt et al., 2007). Such diel differences may differentiate nocturnal and diurnal consumers. In addition, physiological factors, such as body size, drinking frequency, and water loss mechanisms could attenuate or obscure ecological signals. Body size affects metabolic rates and dictates the relative contributions of drinking water, ingested food, and inspired air to the $\delta^{18}O$ values of tissues (reviewed in Bryant and Froelich, 1995). Drinking water is the primary source of oxygen for all animals, even small-bodied species (Bryant and Froelich, 1995; Podlesak et al., 2008). Nevertheless, obligate drinkers are likely to have $\delta^{18}O$ values that follow environmental conditions more closely than species that drink infrequently (Levin et al., 2006; Pietsch et al., 2011; Moritz et al., 2012; Nelson, 2013). Finally, the degree to which a species pants or sweats can impact its $\delta^{18}O$ values. Whereas, sweat is isotopically indistinct from an animal's body water pool, oxygen expelled via panting is substantially depleted in ^{18}O compared to body water (reviewed in Bryant and Froelich, 1995). Panting also increases the pH of blood (Richards, 1970). Both of these factors can result in higher tissue $\delta^{18}O$ values for panters than sweaters. Such behavioral and physiological differences can influence paleoclimate and paleoenvironmental reconstructions if not accounted for properly.

Here we use a multi-isotope approach to investigate the degree to which oxygen isotope analysis can complement and build on ecological applications of carbon and nitrogen isotope analyses. Our focus is on mammalian assemblages in two Costa Rican tropical forests with profoundly different rainfall patterns—an evergreen rainforest and a seasonal dry forest. These forests have similar species compositions with taxa representing a range of dietary categories including carnivory, insectivory, omnivory, frugivory, and folivory. Such a system is ideal for testing the degree to which oxygen isotopes can discriminate diets, activity patterns, and foraging strata. We test the following hypotheses:

1. Oxygen isotope values are higher for animals inhabiting the seasonal dry forest than those in the evergreen rainforest.
2. Oxygen isotope values distinguish animals that consume different foods, forage at different heights within the forest canopy, and are active at different times of the day. We predict that: (i) faunivores have lower $\delta^{18}O$ values than herbivores, and among the faunivores, those that consume vertebrates have higher $\delta^{18}O$ values than those that primarily consume invertebrates; (ii) frugivores have lower $\delta^{18}O$ values than folivores; (iii) animals that feed on the forest floor have lower $\delta^{18}O$ values than those that feed in the canopy, and (iv) nocturnal animals have lower $\delta^{18}O$ values than diurnal animals.
3. Physiological adaptations impact $\delta^{18}O$ values. Animals that pant or drink infrequently have higher $\delta^{18}O$ values than those that sweat or drink frequently.

MATERIALS AND METHODS

Study Sites and Species

We sampled osteological collections from two protected ecosystems in Costa Rica (**Figure 1**). Sector Santa Rosa (SSR), Area de Conservación Guanacaste, encompasses over 108 km^2 (10,800 ha) of semi-deciduous forest in northwestern Costa Rica (10.84N, 85.62W). The region receives 800–2600 mm of annual rainfall (average 1473 mm) during a 6-month rainy season (Holmes et al., 2011; Melin et al., 2014a). Average daily temperatures range from 28°C in the rainy season to 30.3°C in the dry season (Campos and Fedigan, 2009). However, differences in maximum daily temperatures are more dramatic: 28°C in the rainy season vs. 34.5°C in the dry season. The forest at SSR is primarily secondary regrowth at various successional stages. Canopy height is variable (12–30 m), and the canopy is relatively discontinuous, particularly during the dry season when many large trees are defoliated (Kalacska et al., 2007; Holmes et al., 2011).

El Zota (EZ) Biological Field Station is a 10 km^2 (1000 hectare) private reserve located in northeastern Costa Rica (10.56N, 83.74W). Seasonality at EZ is less pronounced: most of the annual rainfall (ca. 4000 mm) occurs from April through June and November through December (Lattanzio and Laduke, 2012).

FIGURE 1 | Overview of the study area including maps of (A) Central America, and (B) Costa Rica with study areas highlighted, and (C) photographs of the forests at Sector Santa Rosa (SSR) and El Zota (EZ). Photographs by BC.

A brief and unpronounced dry season occurs between August and October (Senf, 2009). Average daily temperatures range from 18.5°C in the rainy season to 32.8°C in the dry season (Lindshield, 2006). The majority (700 ha) of EZ is primary tropical lowland rainforest and swamp forest (Lindshield, 2006; Senf, 2009). Tree plantations (270 ha) and pasture (30 ha) account for the remainder of the reserve (Lindshield, 2006; Senf, 2009). The canopy is relatively continuous throughout the forested portions of EZ, although gaps exist in plantation areas (Lindshield, 2006). Canopy heights range from 15 to 25 m with emergent trees reaching 40–60 m (Israel Mesen, personal communication).

Samples were obtained from osteological collections maintained by the field stations at EZ and SSR. Accession notes (when available) suggest that all specimens were less than 10 years old at the time of sampling. At each site we sampled all accessible osteological material from a diverse assortment of mammal taxa that span a variety of dietary, activity, and canopy height preferences (**Table 1**). There is substantial species overlap between the two sites. In order to minimize destruction of the osteological specimens, we sampled fragmented material, vertebrae, or ribs whenever possible. Therefore, minimal (<1‰; Tuross et al., 2008) isotopic differences may exist among bones due to variable composition and turnover rates. The species in this study include sweaters and panters, as well as species that avoid thermal stresses through behavioral mechanisms (**Table 1**). Species vary dramatically in their body masses, but the vast majority are >1 kg (**Table 1**). We verified that no statistical correlation exists between isotope values and log body mass ($R^2 < 0.03$ and $p > 0.05$ for all three isotope systems).

Sample Preparation and Analysis

We coarsely crushed samples using an agate mortar and pestle. We decalcified bone using 0.5 M hydrochloric acid and removed lipids using petroleum ether (following Crowley et al., 2010). Carbon and nitrogen isotope data are previously published for El Zota primates and all Santa Rosa individuals (Crowley et al., 2010; Melin et al., 2014b), and oxygen isotope data are previously published for primates from both sites (Crowley, 2014). New collagen data were measured at the UC Davis Stable Isotope Facility. Carbon and nitrogen were analyzed using a PDZ Europa ANCA-GSL elemental analyzer interfaced to a PDZ Europa 20–20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Data were normalized using ^{15}N-enriched glutamic acid (USGS-41), bovine liver, and nylon. The average differences in δ^{13}C and δ^{15}N between samples run in duplicate ($n = 6$) were 0.5 and 0.1‰, respectively. Collagen oxygen isotope values were analyzed using an Elementar PyroCube interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer. Data were normalized using IAEA 600 (Caffeine), IAEA 601 and 602 (benzoic acid), alanine, and glutamic acid. The average difference in δ^{18}O between samples run in duplicate ($N = 5$) was 0.4‰. We report δ^{13}C relative to V-PDB, δ^{15}N relative to AIR, and δ^{18}O values relative to V-SMOW.
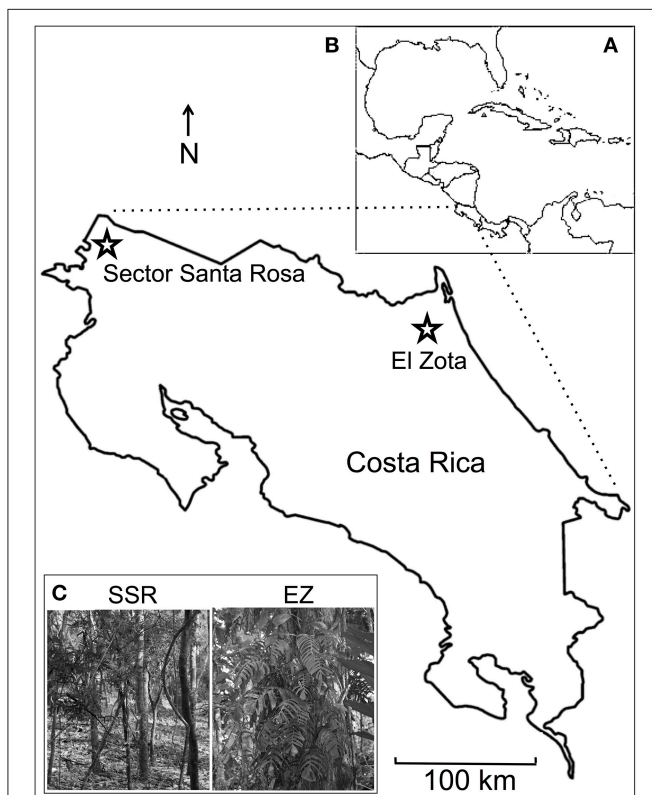
TABLE 1 | Sample sizes, body masses, primary thermoregulatory mechanism, foraging height, dietary, and diel activity classifications for mammalian taxa included in this study.

Family	Genus and species	Common name	SSR (N)	EZ (N)	Body mass (kg)	Thermo-regulation	Foraging height	Diet	Diel pattern
Atelidae	*Alouatta palliata*	Mantled howler monkey	3	4	7.3	Pant	Arboreal	Folivore	Diurnal
Atelidae	*Ateles geoffroyi*	Black-handed spider monkey	2	2	5.3	Pant	Arboreal	Frugivore	Diurnal
Bradypodidae	*Bradypus variegatus*	Three-toed sloth		2	4.3	Sweat[a]	Arboreal	Folivore	Cathemeral
Canidae	*Canis latrans*	Coyote	1		13.4	Pant	Terrestrial	Faunivore (V)	Nocturnal
Canidae	*Urocyon cinereoargenteus*	Gray fox	2	1	3.8	Pant	Terrestrial	Faunivore (V)	Nocturnal
Cebidae	*Cebus capucinus*	Capuchin	2	1	2.7	Pant	All	Omnivore	Diurnal
Cervidae	*Mazama americana*	Brocket deer	1	1	22.8	Sweat	Terrestrial	Folivore	Nocturnal
Cervidae	*Odocoileus virginiensis*	White-tailed deer	3	1	55.5	Sweat	Terrestrial	Folivore	Nocturnal
Cricetidae	*Ototylomys phyllotis*	Big eared climbing rat	1		0.1	Sweat	All	Omnivore	Nocturnal
Dasypodidae	*Dasypus novemcinctus*	Nine-banded armadillo	1	3	4.2	Sweat	Terrestrial	Faunivore (I)	Nocturnal
Dasyproctidae	*Dasyprocta punctata*	Agouti	1		2.7	Sweat	Terrestrial	Frugivore	Diurnal
Didelphidae	*Didelphis virginiana*	Common opossum	1		2.2	Sweat	All	Omnivore	Nocturnal
Felidae	*Panthera onca*	Jaguar	1		100.0	Pant	All	Faunivore (V)	Cathemeral
Felidae	*Puma concolor*	Puma	1		51.6	Pant	All	Faunivore (V)	Cathemeral
Felidae	*Puma yaguarondi*	Jaguarundi	1		9.0	Pant	All	Faunivore (V)	Cathemeral
Leporidae	*Sylvilagus brasiliensis*	Forest rabbit		2	20.9	Sweat	Terrestrial	Folivore	Nocturnal
Megalonychidae	*Choloepus hoffmanni*	Hoffman's two-toed sloth	1	2	6.0	Sweat[a]	Arboreal	Folivore	Nocturnal
Mephitidae	*Conepatus semistriatus*	Striped hog-nosed skunk	1		1.2	Pant	Terrestrial	Omnivore	Nocturnal
Mustelidae	*Eira barbara*	Tayra	1		3.9	Pant	All	Faunivore (V)	Diurnal
Myrmecophagidae	*Tamandua mexicana*	Tamandua	2		4.2	Pant	All	Faunivore (I)	Cathemeral
Procyonidae	*Nasua narica*	Coatimundi	1	1	4.0	Pant	All	Omnivore	Diurnal
Procyonidae	*Potos flavus*	Kinkajou		1	3.0	Pant	Arboreal	Frugivore	Nocturnal
Procyonidae	*Procyon lotor*	Northern raccoon	1	2	5.5	Pant	All	Omnivore	Nocturnal
Sciuridae	*Sciurus variegatoides*	Variegated squirrel	1	2	0.5	Sweat	Arboreal	Frugivore	Diurnal
Tapiridae	*Tapirus bairdi*	Baird's Tapir	1		300.0	Submerge	Terrestrial	Folivore	Nocturnal
Tayassuidae	*Tayassu tajacu*	Collared peccary	1		21.3	Submerge	Terrestrial	Omnivore	Nocturnal

Body masses calculated using MOM Version 4.0 (Smith et al., 2003).

[a]Sloths sweat, but they also frequently change their body temperatures to thermoregulate, which distinguishes them from the other species (Britton and Atkinson, 1938).

Statistical Analysis

We examined the effects of forest type, water loss mechanism, and diet, feeding height, and activity pattern on collagen isotope values using general Linear Mixed Models (LMMs). We first investigated isotopic differences among individuals according to habitat (moist vs. dry forest). Because of slight differences in species compositions at each locality, we included species as a random variable in these comparisons. We also examined the effects of water loss mechanism (panting vs. sweating) along with forest type on collagen $\delta^{18}O$ values.

To investigate the effects of diet, feeding height, and activity pattern, we ran general linear models (GLMs) and categorized species by their primary diet (folivore, frugivore, omnivore, vertebrate faunivore, invertebrate faunivore), canopy use (arboreal, terrestrial, both), and diel pattern (nocturnal, diurnal, cathemeral; see **Table 1**). We were not able to include interaction terms among diet, canopy use and diel pattern in our analyses due to insufficient degrees of freedom. The $\delta^{13}C$ value for a raccoon from EZ was identified as an outlier (Supplementary Table 1, Supplementary Figure 1), and was therefore excluded from all analyses. We speculate that this individual foraged on arthropods or C_4 grasses from the pasture portion of the reserve. We used planned contrasts to compare isotope values between (i) primary consumers (combining frugivores and folivores) and secondary consumers (combining vertebrate and invertebrate consumers), (ii) arboreal and terrestrial species, and (iii) diurnal and nocturnal species. Species that are omnivorous, cathemeral or forage at more than one height within the forest canopy were excluded from these planned comparisons. We chose to use planned contrasts rather than *post-hoc* comparisons because they are designed to test our *a priori* hypotheses, and are not susceptible to multiple comparison penalties. Because we tested directional predictions, we report one-tailed *p*-values for forest type comparisons and all planned contrasts. LMMs and GLMs with planned contrasts were conducted using PROC MIXED (forest comparison) or PROC GLM (diet, activity, feeding height) in SAS 9.4. Significance for all tests set at $\alpha = 0.05$.

Lastly, we compared the efficacy of different pairs of isotope systems (C:N, C:O, N:O) against the full 3-dimensional isotope system (C:N:O) at distinguishing groups by calculating the isotopic overlap between all pairs of groups within the dietary, trophic, activity, and feeding height categories. We also calculated overlap values between SSR and EZ limiting our analysis to the subset of taxa that were sampled at both sites. We calculated the overlap of both two dimensional ellipses (using

each isotope pair C:O, C:N, and N:O) and three dimensional ellipsoids (C:N:O) among groups in each category with the R-package nicheROVER v. 1.0 (Swanson et al., 2015). For a given pair of isotopic distributions (e.g., group A and B), the nicheROVER package employs a Bayesian algorithm to produce posterior estimates of the probability that a member of A falls within the 95% confidence ellipsoid of B (pr{A|B}), and the probability that a member of B falls within the 95% confidence ellipsoid of A (pr{B|A}). Because we were interested in the proportional amount of area/volume shared by the confidence ellipsoids of each pair of groups within a given category, we calculated the intersection between A and B, such that:

$$A \cap B / A \cup B = pr\{A|B\}pr\{B|A\}[(1 - pr\{pr\{A|B\}\}) + (1 - pr\{B|A\}) + pr\{A|B\}pr\{B|A\}]^{-1}.$$

Because nicheROVER is designed to analyze n-dimensional systems, these methods were the same for both 2-dimensional and 3-dimensional isotope systems.

RESULTS

Isotope data for each individual and summary data for each species are presented in Supplementary Table 1 and Supplementary Figure 1.

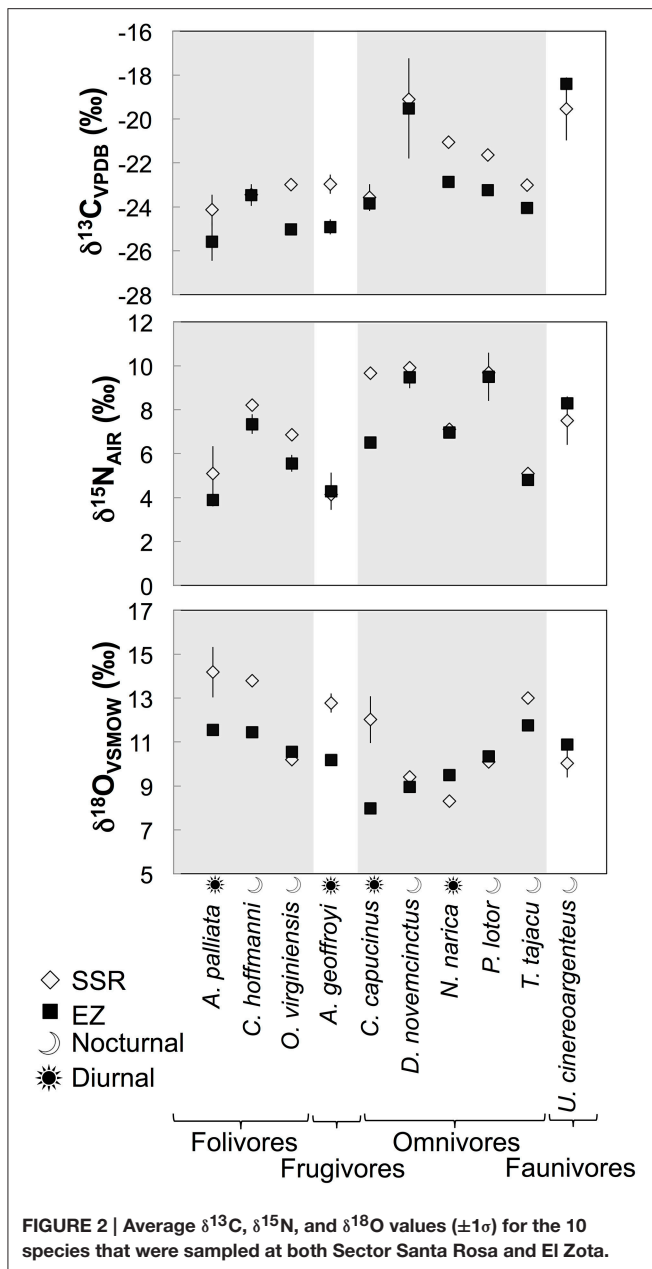### Effects of Habitat, Diet, Activity, and Height

Individuals from EZ have significantly lower $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$ values than those from SSR (**Table 2**). This pattern is robust for the majority of species sampled at both sites (**Figure 2**). We also detected significant differences in $\delta^{13}C$ values as a function of diet and canopy height, but not activity pattern (**Table 3**). The $\delta^{13}C$ values for frugivores and folivores are lower than those for species that consume animal matter (**Figure 3**; **Table 5**), leading to a significant difference in $\delta^{13}C$ values between herbivores and faunivores (**Table 4**). We detected only a marginally significant difference between terrestrial and arboreal species (**Table 5**). Surprisingly, semi-arboreal species that utilize multiple heights have lower $\delta^{13}C$ values than those that are either strictly arboreal or terrestrial (**Figure 3**; **Table 5**), but this appears to be confounded by diet (**Table 1**).

Nitrogen isotope values vary as a function of diet and activity pattern (**Table 3**). Frugivores have the lowest $\delta^{15}N$ values, followed by folivores. Vertebrate and invertebrate faunivores have the highest $\delta^{15}N$ values, whereas omnivores are intermediate (**Figure 3**; **Table 5**). The difference between

TABLE 2 | General linear mixed model results for forest comparisons.

Isotope	SSR Least squares mean (±1SE)	EZ Least squares mean (±1SE)	*F*-value	df	*P* (one-tailed)
Carbon	−21.7 ± 0.2	−22.8 ± 0.3	4.66	1.17	**0.0059**
Nitrogen	7.3 ± 0.2	6.6 ± 0.2	7.23	1.18	**0.0061**
Oxygen	11.1 ± 0.2	9.7 ± 0.3	11.75	1.18	**0.001**

Reported least square mean values account for taxon. Significant results are presented in bold.

FIGURE 2 | Average $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values ($\pm 1\sigma$) for the 10 species that were sampled at both Sector Santa Rosa and El Zota.

difference between arboreal and terrestrial species is significant (**Table 4**).

Niche Overlap

Multi-isotope overlap comparisons generally reinforce results seen in single system analyses. There is clear differentiation (i.e., little overlap) between some dietary, foraging height, and diel groups for multi-isotopic 2-dimensional ellipses and 3-dimensional ellipsoids (e.g., faunivores vs. frugivores, primary vs. secondary consumers, arboreal and semi-arboreal species, cathemeral vs. diurnal species). Other groups exhibit low differentiation (high overlap) in both 2-D and 3-D isotope systems (e.g., semi-arboreal vs. terrestrial fauna, and faunivores vs. omnivores), highlighting ecological similarities between these latter groups (**Figure 4**; Supplementary Table 2, Supplementary Figure 2).

Overlap values between 3-dimensional standard ellipsoids tend to be lower than those between 2-dimensional ellipses. This is due to the former measuring volume (‰^3) and the latter measuring area (‰^2; **Figure 4**; Supplementary Table 2, Supplementary Figure 2). The degree to which the addition of a third isotope improves our ability to distinguish groups varies considerably among isotope systems and categories. For example, when three isotopes are used, it may be possible to securely discriminate cathemeral vs. nocturnal and cathemeral vs. diurnal groups (ca. 21 and 11% overlap, respectively; **Figure 4**). However, overlap between diurnal and nocturnal taxa in C:N:O space is likely too large to be useful for separating these groups (39%).

In general, overlap among 3-D ellipsoids is only slightly lower than overlap calculated for 2-D C:N ellipses, but substantially lower than that calculated for most C:O or N:O ellipses (**Figure 4**; Supplementary Table 2, Supplementary Figure 2). For diet and trophic categories in particular, C:N ellipses and C:N:O ellipsoids produce very similar overlap estimates (**Figures 4**, **5**), which suggests that oxygen isotopes add little in the way of unique dietary information. There are, however, cases in which overlap estimates for C:O ellipses are more similar to C:N:O ellipsoids than other 2-dimensional isotope systems. For example, C:O is more effective than C:N or N:O at distinguishing localities, arboreal vs. terrestrial taxa, and nocturnal vs. diurnal taxa (**Figure 4**; Supplementary Table 2, Supplementary Figure 2).

Effects of Physiology

Using published data for water samples from various locations in Costa Rica (Lachniet and Patterson, 2002), we estimate that $\delta^{18}O$ values for surface waters at SSR and EZ are ca. -2 and -5‰, respectively. Additionally, based on the isotopic offset between bone collagen and drinking water for lab rats (Kirsanow and Tuross, 2011), we expect $\delta^{18}O$ values for bone collagen to be 12.5–15‰ higher than those in drinking water (i.e., expected $\delta^{18}O = 10$–13‰ at SSR and 8–11‰ at EZ). Average $\delta^{18}O$ values for most species at each site are within these expected ranges (11.3 $\pm$ 1.8‰ at SSR and 10.4 $\pm$ 1.3‰ at EZ; Supplementary Table 1, Supplementary Figure 1). The species with higher than expected $\delta^{18}O$ values include howler monkeys (*Alouatta palliata*), sloths (*Choelopus hoffmani, Bradypus variegatus*), coyotes (*Canis latrans*), and peccaries (*Tayassu tajacu*).

herbivores and faunivores is significant (**Table 4**). Nocturnal species have higher $\delta^{15}N$ values than diurnal or cathemeral species (**Figure 3**; **Table 5**). The difference between diurnal and nocturnal species is significant (**Table 4**).

Finally, we detected differences in $\delta^{18}O$ values as a function of diet and foraging height, but not activity pattern (**Table 3**). Frugivores have lower $\delta^{18}O$ values than folivores, and faunivores have lower $\delta^{18}O$ values than herbivores (**Figure 3**; **Table 5**). However, there is little overall variability in $\delta^{18}O$ values among groups and our comparison of herbivores vs. faunivores does not reach significance (**Table 4**). Arboreal species have higher $\delta^{18}O$ values than terrestrial species and semi-arboreal species that utilize multiple canopy heights (**Figure 3**; **Table 5**). The

TABLE 3 | General linear model results for diet, foraging height, and diel activity.

Isotope system	Source	df	SS	Mean square	F-value	P
Carbon ($R^2_{Adjusted}$ = 0.068)	Model	8	244.03	30.5	11.75	**<0.0001**
	Error	44	114.23	2.6		
	Corrected total	52	358.26			
	Diet	4	214.83	53.71	20.69	**<0.0001**
	Height	2	22.38	11.19	4.31	**0.02**
	Activity	2	6.82	3.41	1.31	0.28
Nitrogen ($R^2_{Adjusted}$ = 0.068)	Model	8	179.33	22.42	12.02	**<0.0001**
	Error	45	83.89	1.86		
	Corrected total	53	263.21			
	Diet	4	163.36	40.84	21.91	**<0.0001**
	Height	2	0.83	0.42	0.22	0.8
	Activity	2	15.14	7.57	4.06	**0.024**
Oxygen ($R^2_{Adjusted}$ = 0.033)	Corrected model	8	43.74	5.47	2.8	**0.013**
	Error	45	87.79	1.95		
	Corrected total	53	131.53			
	Diet	4	17.21	4.3	2.21	0.083
	Height	2	24.25	12.13	6.22	**0.0041**
	Activity	2	2.28	1.39	0.58	0.56

Significant results are presented in bold.

We found no differences in $\delta^{18}O$ values among species with different water loss mechanisms overall, or at either site ($p >$ 0.05, **Figure 6**). However, our data set is perhaps too limited to conclusively rule out this effect. Relative humidity at SSR is low, particularly during the day. Apparent differences in $\delta^{18}O$ values between panters and sweaters are more pronounced among the majority of diurnal animals at this locality (**Figure 6**), and a larger sample size may reveal a significant effect of water loss mechanism on $\delta^{18}O$ values.
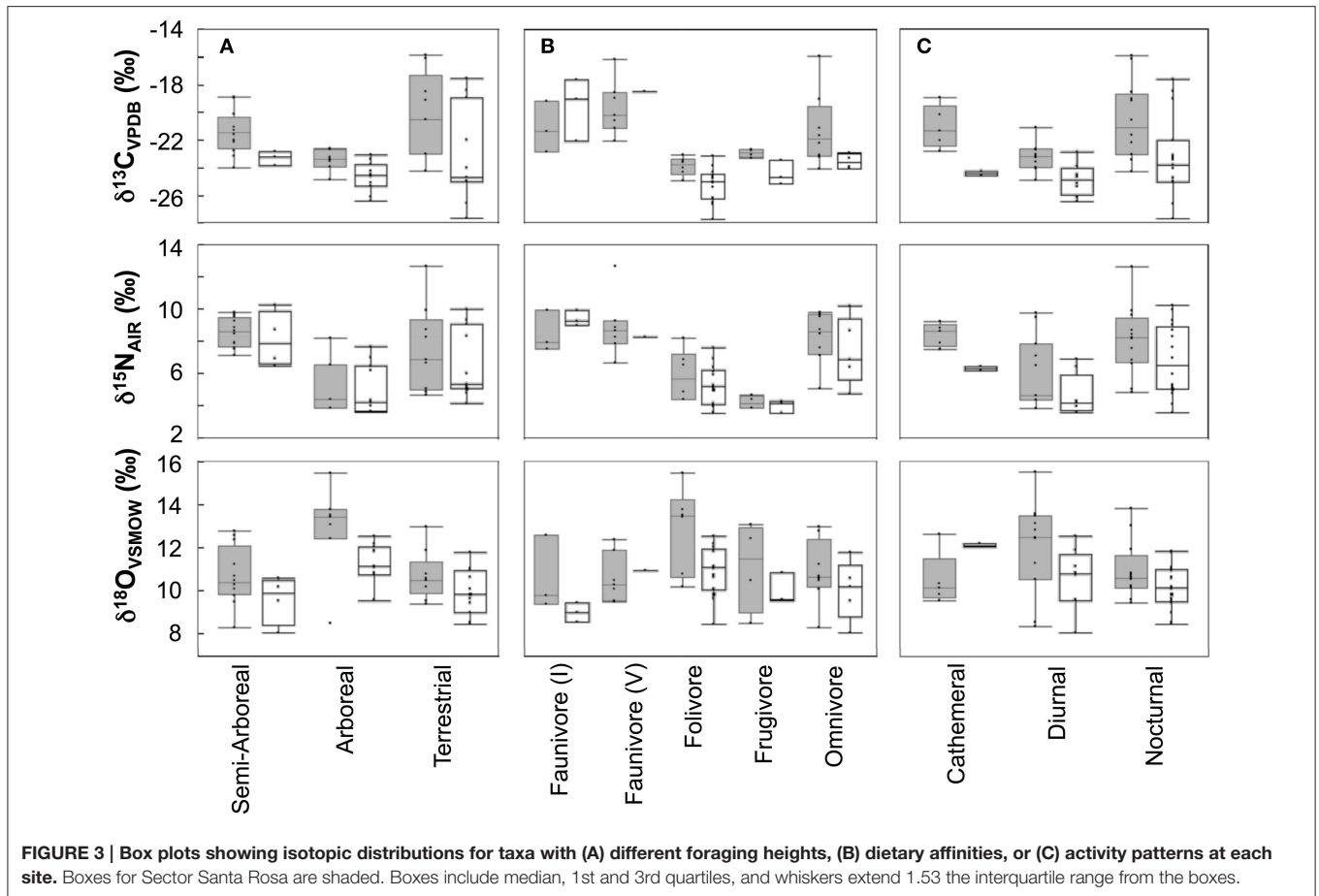
DISCUSSION

Do Oxygen Isotope Values Distinguish Individuals from Dry and Moist Forests?

As predicted, collagen $\delta^{18}O$, $\delta^{13}C$, and $\delta^{15}N$ values are higher for individuals from the deciduous dry forest at SSR (**Table 2**). These results suggest baseline isotopic differences between the two sites (most likely related to soil moisture), and evaporative enrichment that is more pronounced at SSR than at EZ. Patterns are broadly consistent across taxa that were sampled at both localities (**Figure 2**). Variability among taxa may be an artifact of small sample sizes; it could also result from meaningful ecological or physiological differences between individuals from the two sites. Among the four species with greater $\delta^{18}O$ spacing between habitats, three are diurnal (*A. palliata, Ateles geoffroyi,* and *Cebus capucinus*), suggesting that environmental disparities between habitats (e.g., evaporative enrichment) may be exacerbated for diurnal species. Differences in relative humidity and temperature between the two sites are likely less pronounced after the sun sets (Sternberg et al., 1989). Additionally, offset in $\delta^{18}O$ and $\delta^{15}N$ values between sites is generally higher for herbivores than for omnivores or faunivores (**Figure 2**), which affirms previous suggestions that the influence of relative humidity may decrease in importance with increasing trophic level (e.g., Kohn, 1996; Pietsch et al., 2011). The exceptions are herbivorous deer (*Odocoileus virginiensis*), which are nocturnal, and omnivorous capuchins (*C. capucinus*), which are diurnal.

Can Oxygen Isotope Values Distinguish Species that Consume Different Foods?

Contrary to our prediction, we did not detect significant differences in $\delta^{18}O$ values among dietary groups. Carbon and nitrogen are considerably more effective at differentiating dietary and trophic groups (**Figures 3, 4**; **Tables 3, 4**). Although the LMM results suggest that diet has a marginal influence on $\delta^{18}O$ values, planned contrasts indicate that herbivores and faunivores are indistinguishable. Each dietary group exhibits rather variable $\delta^{18}O$ values, resulting in considerable isotopic overlap among categories. Additionally, patterns among dietary categories are not consistent between sites. For example, faunivores that consume vertebrates have higher $\delta^{18}O$ values than those that consume invertebrates at EZ, but not at SSR. Omnivorous species exhibit a large range of $\delta^{13}C$ and $\delta^{15}N$ values, particularly at SSR, which likely reflects the variable amount of animal protein in their diets (Hyodo et al., 2010). Folivores have higher $\delta^{18}O$ values than other groups at SSR, which would be expected if they consume leaves that are affected by ^{18}O enrichment during evapotranspiration (Yakir, 1997). Lack of evidence for ^{18}O-enriched folivores at EZ may result from the elevated soil moisture and relative humidity at this site, which likely lead to less evaporative enrichment in leaves than at SSR (Ometto et al., 2005; Barbour et al., 2007).

FIGURE 3 | Box plots showing isotopic distributions for taxa with (A) different foraging heights, (B) dietary affinities, or (C) activity patterns at each site. Boxes for Sector Santa Rosa are shaded. Boxes include median, 1st and 3rd quartiles, and whiskers extend 1.53 the interquartile range from the boxes.

TABLE 4 | Planned contrast results for collagen isotopes.

Isotope	Contrast	df	Contrast SS	F-value	P (one tailed)
Carbon	Primary vs. Secondary Consumer	1	104.01	40.06	**<0.0001**
	Arboreal vs. Terrestrial	1	5.08	1.96	0.835
	Diurnal vs. Nocturnal	1	6.8	2.62	0.055
Nitrogen	Primary vs. Secondary Consumer	1	54.04	23.99	**<0.0001**
	Arboreal vs. Terrestrial	1	4.14	2.22	0.717
	Diurnal vs. Nocturnal	1	14.5	7.78	**0.00385**
Oxygen	Primary vs. Secondary Consumer	1	2.36	1.21	0.14
	Arboreal vs. Terrestrial	1	12.33	6.32	**0.008**
	Diurnal vs. Nocturnal	1	2.05	1.05	0.155

Significant results are presented in bold.

Are Oxygen Isotope Values Effective at Distinguishing Foraging Heights within the Forest Canopy?

Oxygen isotope values are effective at distinguishing arboreal and terrestrial species (**Figure 3**; **Table 4**). More pronounced differences in $\delta^{18}O$ values between foraging heights at SSR

than at EZ is a logical result; stratification in $\delta^{18}O$ values is predominantly driven by a gradient in relative humidity under the forest canopy (Sternberg et al., 1989). Whereas, we might expect substantial differences in relative humidity between the canopy and understory in the dry forest at SSR, relative humidity (and $\delta^{18}O$ values) should be more consistent in the moist forest at EZ.

Unexpectedly, foraging height has a negligible impact on $\delta^{13}C$ values. There is no discrete overstory at SSR (Kalacska et al., 2007). However, we had expected to find differences in $\delta^{13}C$ values among heights at EZ, which has a dense upper canopy (Lindshield, 2006). Although dietary factors could be obscuring any carbon isotope signal related to foraging at EZ (van der Merwe and Medina, 1991; Krigbaum et al., 2013), other researchers have also reported inconsistent $\delta^{13}C$ values among animals feeding at different canopy heights in African forests (Krigbaum et al., 2013; Nelson, 2013). This calls into question

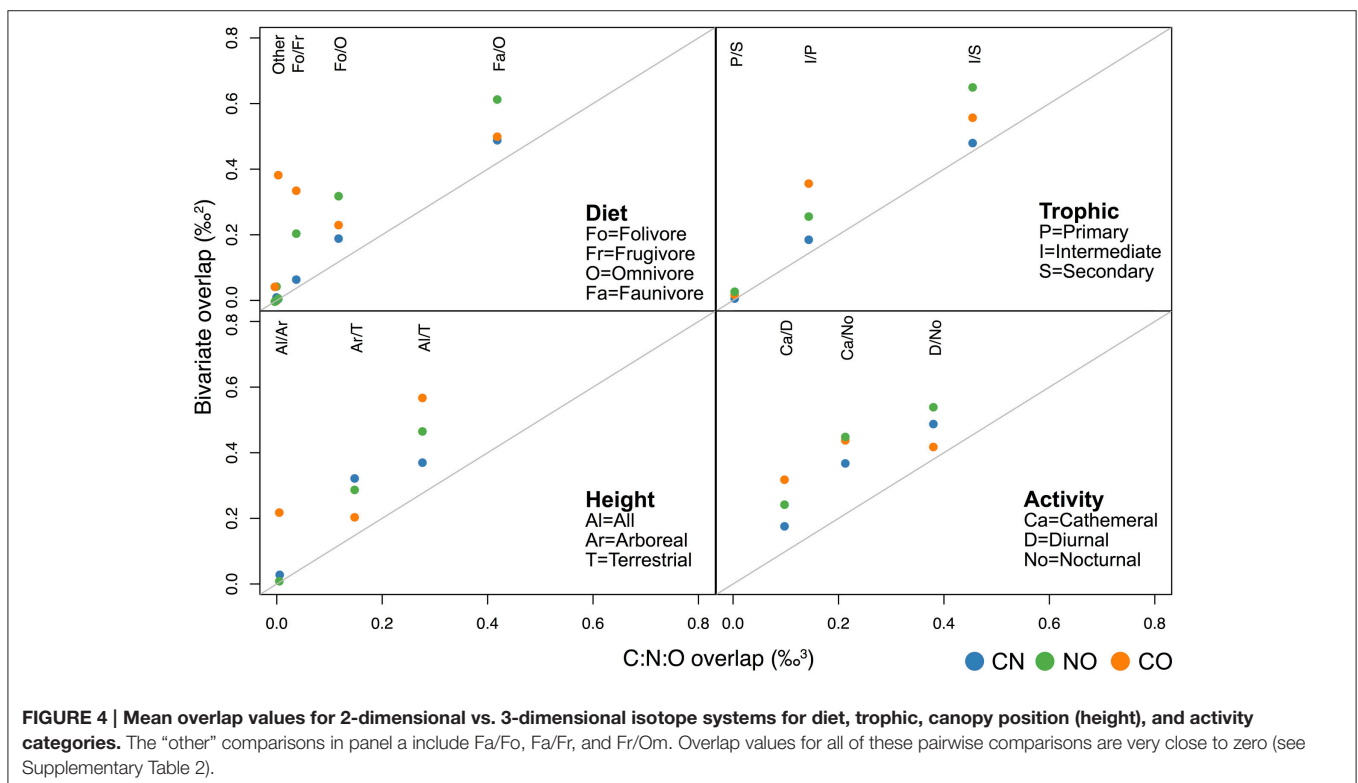the reliability of $\delta^{13}C$ for distinguishing terrestrial vs. arboreal taxa, or identifying strata within a dense canopy (Voigt, 2010). The ability for oxygen to discern foraging height appears to be more robust (Krigbaum et al., 2013; Nelson, 2013; Carter and Bradbury, 2015), suggesting that oxygen may have attractive utility for reconstructing paleoenvironmental conditions.
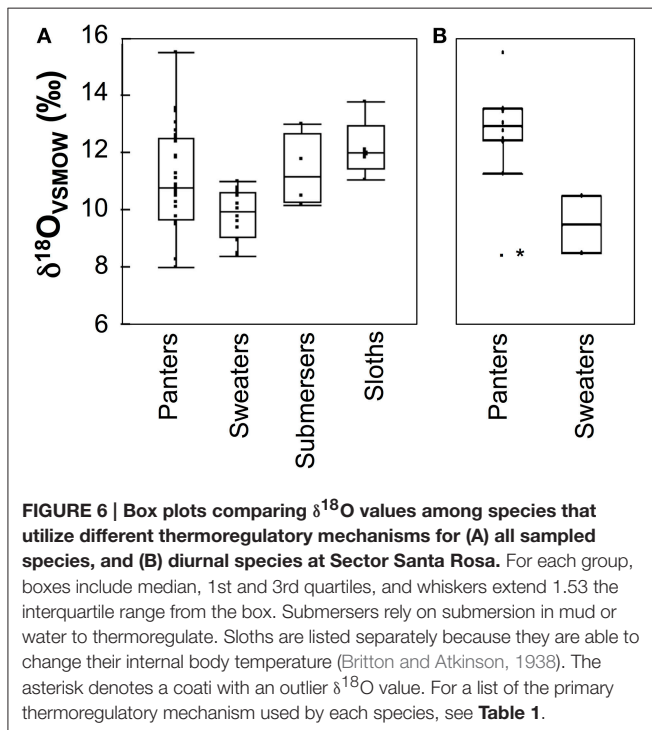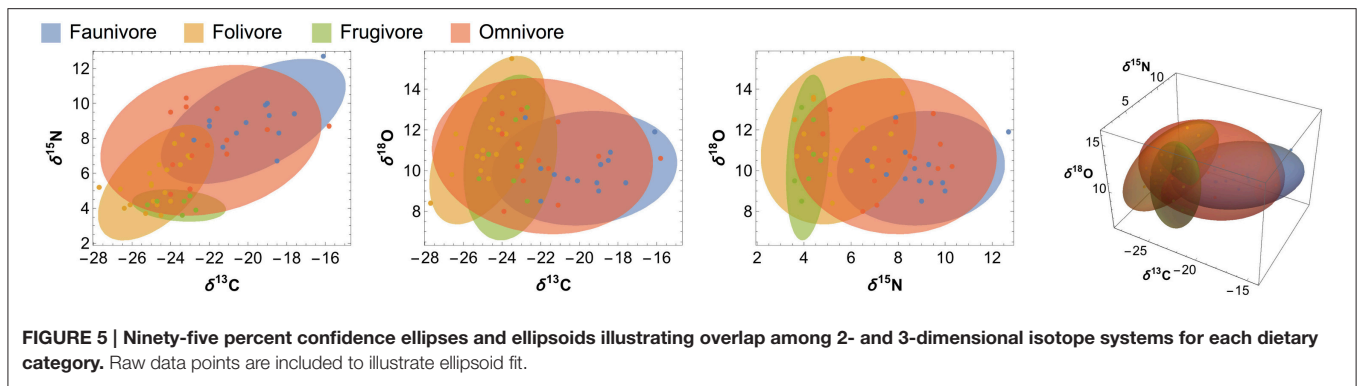
Can Oxygen Isotope Values Distinguish Species with Distinctive Diel Patterns?

Contrary to our expectations, oxygen isotope values in collagen are ineffective at distinguishing nocturnal and diurnal species. This suggests that either (i) there are insubstantial differences in relative humidity or the $\delta^{18}O$ value of respired CO_2 between night and day, or (ii) activity pattern is of minor importance compared to other factors, such as diet, foraging height, or physiology. Nitrogen isotope values differ significantly among diel groups. However, we suspect that this pattern reflects an interaction between activity, diet and possibly locality; most faunivores and omnivores are nocturnal or cathemeral and none of the diurnal species we sampled are dedicated faunivores (**Table 1**). Larger variability in $\delta^{15}N$ values for nocturnal animals compared to diurnal ones likely reflects a mix of higher $\delta^{15}N$ values for faunivores and omnivores, and lower $\delta^{15}N$ values for nocturnal herbivores, such as tapirs, deer, and agoutis. Considerable isotopic overlap between both 2-D and 3-D ellipsoids for nocturnal, diurnal and cathemeral taxa (**Figure 4**) suggests that carbon, nitrogen, and oxygen are not particularly well-suited for distinguishing diel groups, though the C:O isotope

TABLE 5 | Least square mean carbon, nitrogen, and oxygen isotope values for each dietary, foraging height, and diel activity category.

Category		$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)	$\delta^{18}O$ (‰)
Diet	Faunivore (I)	−20.3	8.8	10.5
	Faunivore (V)	−19.1	8.6	11.4
	Omnivore	−21.3	7.2	11.6
	Folivore	−25.6	5.4	11.0
	Frugivore	−24.2	4.5	9.4
Foraging height	Semi-arboreal	−23.3	7.6	11.0
	Arboreal	−21	7	11.0
	Terrestrial	−22.1	6.1	10.4
Diel activity pattern	Cathemeral	−22.3	6.5	9.8
	Diurnal	−22.6	6.3	12.1
	Nocturnal	−21.5	7.9	10.5

FIGURE 4 | Mean overlap values for 2-dimensional vs. 3-dimensional isotope systems for diet, trophic, canopy position (height), and activity categories. The "other" comparisons in panel a include Fa/Fo, Fa/Fr, and Fr/Om. Overlap values for all of these pairwise comparisons are very close to zero (see Supplementary Table 2).

FIGURE 5 | Ninety-five percent confidence ellipses and ellipsoids illustrating overlap among 2- and 3-dimensional isotope systems for each dietary category. Raw data points are included to illustrate ellipsoid fit.

FIGURE 6 | Box plots comparing $\delta^{18}O$ values among species that utilize different thermoregulatory mechanisms for (A) all sampled species, and (B) diurnal species at Sector Santa Rosa. For each group, boxes include median, 1st and 3rd quartiles, and whiskers extend 1.53 the interquartile range from the box. Submersers rely on submersion in mud or water to thermoregulate. Sloths are listed separately because they are able to change their internal body temperature (Britton and Atkinson, 1938). The asterisk denotes a coati with an outlier $\delta^{18}O$ value. For a list of the primary thermoregulatory mechanism used by each species, see **Table 1**.

system performs marginally better at distinguishing diurnal vs. nocturnal species than either C:N or N:O.

Physiological Adaptations Impact $\delta^{18}O$ Values

Differences in water loss mechanisms or drinking frequency may be responsible for some of the observed variability in $\delta^{18}O$ values at SSR and EZ (Levin et al., 2006; Moritz et al., 2012). Overall, mean $\delta^{18}O$ values do not differ among species with different water loss mechanisms, but this is likely due to the large range in $\delta^{18}O$ values exhibited by panters (**Figure 6**). We anticipate that some panters minimize water loss and thermal stress by traveling less, modifying their circadian activity, or submersing themselves in water or mud (e.g., Campos and Fedigan, 2009; Oliveira-Santoa et al., 2010). Although the influence of water loss mechanism on $\delta^{18}O$ values could complicate the interpretation of

ecological differences among species, we note that it is not large enough to obscure differences in $\delta^{18}O$ values among species that forage at different heights.

Higher $\delta^{18}O$ values for howler monkeys (*A. palliata*), sloths (*C. hoffmani; B. variegatus*), coyotes (*C. latrans*), and peccaries (*T. tajacu*) most likely reflect drinking water frequency. These species differ in their diet, foraging height, activity pattern, and water loss mechanisms, but they all share the ability to meet their water requirements through the food they consume (Zervanos and Day, 1977; Glander, 1978; Nagy and Montgomery, 1980; Golightly and Ohmart, 1984). Additionally, $\delta^{18}O$ values for these facultative drinkers are more elevated than other taxa at SSR than at EZ (Supplementary Table 1, Supplementary Figure 1). The lowest $\delta^{18}O$ values at both EZ and SSR are similar (ca. 8‰). However, the maximum $\delta^{18}O$ values at SSR are higher than those at EZ, which results in a larger overall spread in $\delta^{18}O$ values among taxa at the dry forest site (7.2 vs. 4.5‰). These data bolster the findings of Levin et al. (2006) that species that drink frequently have lower $\delta^{18}O$ values than those that drink infrequently, particularly in arid settings, and confirm that oxygen isotope values in bone collagen can distinguish species based on their sensitivity to evaporative enrichment in tropical forest settings.

Implications for Paleoenvironmental Reconstructions

Researchers are often limited to carbon and oxygen isotopes in enamel carbonate when working with the fossil record. However, collagen may be better suited for the investigation of environmental conditions and foraging ecologies for well-preserved historic, Holocene, or Late-Pleistocene specimens. It is straightforward to assess collagen integrity (e.g., Ambrose, 1990), and the ability to analyze three isotopes in one material reduces the need for multiple preparation techniques.

Our analysis shows that overlap values among collagen C:O ellipses are rather large compared to C:N ellipses or C:N:O ellipsoids (**Figure 4**; Supplementary Table 2, Supplementary Figure 2). Although oxygen and carbon may be just as effective as three isotope systems at distinguishing localities, nocturnal and diurnal species, terrestrial and arboreal species, or folivores and faunivores (**Figure 4**), we note that overlap estimates between some of these pairs range from 40 to 60%, which may be too

large to securely distinguish groups. The exceptions are arboreal vs. terrestrial taxa (ca. 20% overlap) and folivores vs. faunivores (ca. 5% overlap).

Combined, $\delta^{13}C$, $\delta^{15}N$, and $\delta^{18}O$ values in collagen are effective at distinguishing species with different diets, canopy positions, and physiological adaptations. Oxygen may also be able to distinguish species based on their drinking frequency. Fossil assemblages with more variable collagen $\delta^{18}O$ values among species likely indicate drier habitats than those with less variable $\delta^{18}O$ values. Additionally, within a particular assemblage, species with the lowest $\delta^{18}O$ values likely consumed water frequently. Their $\delta^{18}O$ values should track meteoric water most closely. Species with high $\delta^{18}O$ values are likely to be more enriched in ^{18}O than meteoric water, and are therefore suboptimal for interpreting paleoclimate. Although our results suggest some caution should be used when interpreting isotopic data, when used in combination, carbon, nitrogen, and oxygen isotope data from bone collagen may be able to provide comprehensive reconstructions of environments and extinct communities dating back to the Late Pleistocene.

AUTHOR CONTRIBUTIONS

BC, Conceived the project, collected and analyzed samples, wrote the manuscript. AM, Collected samples, designed and conducted data analyses, wrote the manuscript. JY, Conducted data analyses, wrote the manuscript. ND, Conceived the project, wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fevo.2015.00127

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