

RESEARCH ARTICLE

Stable carbon, oxygen, and nitrogen, isotope analysis of plants from a South Asian tropical forest: Implications for primatology

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Stable isotope analysis of primate tissues in tropical forest contexts is an increasingly popular means of obtaining information about niche distinctions among sympatric species, including preferences in feeding height, forest canopy density, plant parts, and trophism. However, issues of equifinality mean that feeding height, canopy density, as well as the plant parts and plant species consumed, may produce similar or confounding effects. With a few exceptions, researchers have so far relied largely on general principles and/or limited plant data from the study area as references for deducing the predominant drivers of primate isotope variation. Here, we explore variation in the stable carbon ($\delta^{13}\text{C}$), nitrogen ($\delta^{15}\text{N}$), and oxygen ($\delta^{18}\text{O}$) isotope ratios of 288 plant samples identified as important to the three primate species from the Polonnaruwa Nature Sanctuary, Sri Lanka, relative to plant part, season, and canopy height. Our results show that plant part and height have the greatest effect on the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ measurements of plants of immediate relevance to the primates, *Macaca sinica*, *Semnopithecus priam thersites*, and *Trachypithecus vetulus*, living in this monsoonal tropical forest. We find no influence of plant part, height or season on the $\delta^{15}\text{N}$ of measured plants. While the plant part effect is particularly pronounced in $\delta^{13}\text{C}$ between fruits and leaves, differential feeding height, and plant taxonomy influence plant $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ differences in addition to plant organ. Given that species composition in different regions and forest types will differ, the results urge caution in extrapolating general isotopic trends without substantial local baselines studies.

KEYWORDS

diet, plant ecology, primates, South Asia, stable isotope ecology

1 | INTRODUCTION

Stable isotope analysis is frequently applied to address problems in primate ecology. It is particularly useful for investigating long-term dietary behavior and ecology, especially of primates that are difficult to observe feeding, are shy or rare, or have been extirpated in their former habitats and are only represented by historic museum collections (e.g., Blumenthal, Chritz, Rothman, & Cerling, 2016; Crowley, Godfrey, & Irwin, 2011; Crowley, Rasoazanabary, & Godfrey, 2014; Crowley, Reitsema, Oelze, & Sponheimer, 2016;

Loudon, Sponheimer, Sauther, & Cuozzo, 2007; Macho & Lee-Thorp, 2014; Oelze, Head, Robbins, Richards, & Boesch, 2014; Sandberg, Loudon, & Sponheimer, 2012; Sponheimer et al., 2009). Stable isotope methods work best where there are well-known, straightforward environmental, or ecological correlates of variation in stable isotope ratios of carbon ($\delta^{13}\text{C}$), oxygen ($\delta^{18}\text{O}$), or nitrogen ($\delta^{15}\text{N}$). For example, Schoeninger, Iwaniec, and Glander (1997) found that hair $\delta^{13}\text{C}$ from four New World monkeys (*Alouatta palliata*, *Ateles geoffroyi*, *Cebus capucinus*, and *Brachyteles arachnoides*) reflected the density of canopy cover independently of a species' diet. Similar principles have also

been used to observe changes in primate habitats and diets through time. For example, Crowley, Godfrey, et al. (2012) used $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ values from bone collagen of four living species and eight extinct species of Madagascan lemurs to document contraction of lemur forest habitats on the island from 900 cal. years BP. In more open environments, stable isotopes in feces were used to determine the extent to which baboons in southern Africa rely on C_4 and surprisingly, CAM plant resources (Codron, Lee-Thorp, Sponheimer, de Ruiter, & Codron, 2006).

Researchers have also relied on subtle distinctions in stable isotopes within C_3 -dominated tropical forest ecosystems, where most primates reside, to draw out more detailed differences in primate feeding behavior. Oelze et al. (2014) compared stable carbon isotopes of *Pan* and *Gorilla* hair to local vegetation within the Loango National Park (Gabon) to argue that the former practiced arboreal feeding to a greater extent than the latter, which focused more on low canopy, herbaceous vegetation. Krigbaum, Berger, Daegling, and McGraw (2013) found no significant trends in $\delta^{13}\text{C}$ but argued that bone carbonate $\delta^{18}\text{O}$ values of seven sympatric primate species were correlated with feeding height in the Tai Forest, Côte d'Ivoire. Finally, $\delta^{15}\text{N}$ has been used to compare weaning periods among primate individuals (Fahy et al., 2014; Smith, Morgan, & Pilbeam, 2010), the prominence of hunting behaviors and animal consumption (Fahy, Richards, Riedel, Hublin, & Boesch, 2013), and health status (Loudon et al., 2007).

These studies are part of a growing body of research applying stable isotope analysis to primate ecology (Crowley, Reitsema, Oelze, & Sponheimer, 2016; Sandberg et al., 2012). Large-scale plant sample collection can be difficult and costly, or even practically impossible when dealing, for instance, with primate tissues collected from unhabituated living populations, historical museum collections, and fossil assemblages (e.g., Smith et al., 2010), and many applications of stable isotope analysis in primatology rely on general principles and/or small plant reference collections to underpin behavioral interpretations. The lack of baseline information places significant constraints on the confidence with which one can resolve sources of isotopic variability, which leads to the unavoidable reality that in cases where less prior ecological information is available, where an indirect indicator of diet or behavior is most needed, stable isotopes are less useful as an ecological indicator. For example, Krigbaum et al.'s [2013] study of feeding height lacks plant data to support the assertion of increasing $\delta^{18}\text{O}$ with height, as opposed to, for example, differing dietary proportions of fruit or foliage. Where large plant stable isotope datasets of foodstuffs have been collected in association with primate populations, more detailed understanding of isotopic variation related to their diets is possible. For example, Crowley et al. (2014) used a large $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ baseline dataset to demonstrate that subtle differences in lemur $\delta^{15}\text{N}$ and $\delta^{13}\text{C}$ are a result of lower consumption of arthropods and plant exudates in dry forests during the dry season.

These large plant datasets have also demonstrated significant complexity in interpretation of the most important environmental influences on plant $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$, with less research focusing on $\delta^{18}\text{O}$. In particular, equifinality of effects has been noted between different ecological drivers of plant isotopic variation and, in turn,

primate tissue isotope variation in C_3 forest ecosystems. For example, small $\delta^{13}\text{C}$ differences (c. 1–2‰) in primate tissues could plausibly reflect feeding on different plant parts, in different canopy layers, or under different canopy density, or isotope changes in vegetation itself rather than feeding behavior. Oelze et al. (2014) use plant data to suggest a subtle $\delta^{13}\text{C}$ difference (c. 1‰) in sympatric *P. t. troglodytes* and *G. g. gorilla* hair in Loango, Gabon, is driven by differential fruit consumption and feeding height. By contrast a similar (c. 1‰) distinction between these primates in Cameroon has been associated with different canopy density preferences, although without plant data for context (Macho & Lee-Thorp, 2014).

Similarly, although Krigbaum et al. (2013) argue that $\delta^{18}\text{O}$ values in bone apatite primarily reflect feeding height, oxygen isotope variation could, for instance, also reflect different drinking behaviors (Cerling, Hart, & Hart, 2004) or differences in preferences for foliage versus non-photosynthetic plant parts (Carter & Bradbury, 2016; Crowley et al., 2016). With some notable exceptions (e.g., Blumenthal, Chritz, Rothman, & Cerling, 2012; Blumenthal et al., 2016; Carlson & Kingston, 2014; Crowley, 2012; Crowley, McGoogan, et al., 2012), there have been few attempts in forest primate stable isotope ecology studies to disentangle these different sources of variation, and these have focused on $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. There have been no attempts to simultaneously address the carbon, nitrogen, and oxygen isotopic composition of primate foods. Finally, seasonal habitats have been understudied in isotopic studies of tropical forests, as most plant isotope datasets used to shed light on primate isotope ecology primarily come from the dense evergreen tropical forests in Africa and the Amazon (Blumenthal et al., 2012; Cerling et al., 2004; Oelze et al., 2014; Sternberg, Mulkey, & Wright, 1989; Van der Merwe and Medina, 1991).

To address these issues, we analyzed a large sample of primate plant foods in Polonnaruwa Nature Sanctuary, Sri Lanka for their carbon, nitrogen, and oxygen isotopic composition. Specifically, we aimed to investigate the impacts of plant part, height, and season to a) identify the primary environmental and ecological correlates of isotopic variation in primate foods in a monsoonal, seasonally dry, tropical forest and b) provide a floral baseline for isotopic applications to South Asian primate communities, which have a long history of primatological observation (Dittus, 1974, 2013; Hladik, 1977; Hladik & Hladik, 1972), but have been understudied with regards to their stable isotope ecology.

1.1 | Stable carbon, oxygen, and nitrogen isotope dynamics in tropical forests; general principles

C_3 plant taxa dominate tropical forest ecosystems (Ehleringer & Monson, 1993). $\delta^{13}\text{C}$ values of C_3 plants are affected by environmental variables such as temperature, relative humidity, soil moisture, and degree of solar insolation (Handley et al., 1999; Heaton, 1999; Kohn, 2010). In a tropical forest setting these parameters result in the "canopy effect," whereby vegetation growing under a closed, dense forest canopy is strongly depleted in ^{13}C , which has variously been explained by recycling of respired, ^{13}C -depleted CO_2 (Medina & Minchin, 1980; Van der Merwe and Medina, 1991; Vogel, 1978), low light (Ehleringer, Field, Lin, & Kuo, 1986; Farquhar, Ehleringer, &

Hubick, 1989), and high humidity (Graham et al., 2014). On the basis of this work it is hypothesized that plants growing in the understory of a closed canopy have lower $\delta^{13}\text{C}$ values compared to C_3 plants growing in the subcanopy and canopy (Buchmann, Guehl, Barigah, & Ehleringer, 1997; Jackson et al., 1993; Van der Merwe & Medina, 1989, 1991). However, the carbon isotopic range associated with the canopy effect is not constant, and has been observed to vary from approximately 3–10‰ across tropical forests (Blumenthal et al., 2016; Carlson & Kingston, 2014; Cerling et al., 2004; Martinelli et al., 1998; Van der Merwe & Medina, 1989).

High relative humidity throughout the year results in reduced evapotranspiration in tropical forest plants relative to arid regions. However, in forest regions with a dry season canopy density and composition also varies intra-annually. During drier parts of the year, lower precipitation results in more open forest ecosystems and increased insolation that, as a result of a reduction in the “canopy effect,” leads to seasonally higher plant $\delta^{13}\text{C}$ values relative to periods of the year with higher precipitation and denser forest formations (Buchmann et al., 1997; Ometto et al., 2006).

Stable isotope variation between parts of the same plant is well-documented. For example, non-photosynthetic (heterotrophic) tissues, including fruits, seeds, stems, and wood, have repeatedly been found to have higher $\delta^{13}\text{C}$ values (c. 1–2‰) than photosynthesising leaves (Blumenthal et al., 2012, 2016; Codron et al., 2005; Martinelli et al., 1998; Medina et al., 1991). While this has not yet been studied in a seasonal tropical forest, we would expect a similar pattern in the Polonnaruwa tropical forest. Young leaves may also be ^{13}C -enriched relative to mature leaves (Cernusak et al., 2009), although this pattern varies among species (Blumenthal et al., 2016).

The oxygen isotope composition of terrestrial plant tissues reflects plant water, absorbed by roots from soil water with minimal isotopic fractionation (Cernusak et al., 2016). Soil water reflects precipitation-derived (meteoric) water, which in the tropics relates to rainfall amount (Gonfiantini, Roche, Olivry, Fontes, & Zuppi, 2001). Thus, the isotopic composition of plant water in non-photosynthetic tissues relates to meteoric water. In leaves, evapotranspiration leads to preferential loss of H_2^{16}O and hence ^{18}O -enrichment at the sites of evaporation, which leads to bulk leaf water with higher $\delta^{18}\text{O}$ (Cernusak et al., 2016). The evaporative enrichment of leaf water is inversely related to relative humidity (Cernusak et al., 2016; Flanagan, Comstock, & Ehleringer, 1991; Yakir, Berry, Giles, & Osmond, 1994). Thus, plant matter growing in shaded, humid conditions during a wet season should have lower $\delta^{18}\text{O}$ than those growing in more open, arid areas with high irradiance in the dry season (Sternberg et al., 1989). Moreover, gradients in stomatal conductance suggest that we should expect a vertical stratification in plant $\delta^{18}\text{O}$ (Sternberg et al., 1989). Increased humidity on the forest floor results in low leaf $\delta^{18}\text{O}$ values for forest floor taxa, and ^{18}O -enrichment along a vertical gradient has been observed in a lowland Neotropical forest (Sternberg et al., 1989).

The oxygen isotopic composition of plant tissues varies due to the effects of evapotranspiration on leaf water and differences in biochemical composition (Barbour, 2007; Cernusak et al., 2016; Cernusak, Pate, & Farquhar, 2002). For example, evaporative enrichment of leaf water leads to higher $\delta^{18}\text{O}$ in leaf cellulose relative

to cellulose from roots and fruits (Epstein, Thompson, & Yapp, 1977; Sternberg, DeNiro, & Keeley, 1986; Sternberg et al., 1989; Yakir, 1992). Sucrose, present at a high proportion in fruits, consistently and directly reflects leaf water isotopic composition, with an offset of c. +27‰ (Barbour, Cernusak, & Farquhar, 2005), but cellulose, present at the highest proportion in leaves, may not be in full isotopic equilibrium with leaf water, depending on rates of cellulose synthesis and sucrose import (Barbour & Farquhar, 2000; Helliker & Ehleringer, 2002; Hill, Waterhouse, Field, Switsure, & Après, 1995). Regardless, the dominant impacts of transpiration through stomata mean that we would expect leaves to show higher $\delta^{18}\text{O}$ compared to other plant parts.

Nitrogen isotope variability in ecosystems reflects the complex balance between nitrogen fixation, recycling, and release within the biosphere (Evans, 2001; Robinson, 2001; Szpak, 2014). Factors such as precipitation and nutrient status have been argued to influence the balance between nitrogen uptake and release and, therefore, plant $\delta^{15}\text{N}$. Lower $\delta^{15}\text{N}$ values are expected in tropical ecosystems with higher mean annual precipitation according to global surveys (Amundson et al., 2003; Handley et al., 1999). On an intra-annual scale, one might expect plant $\delta^{15}\text{N}$ to be lower during the wet season relative to the dry season, due to seasonal changes in tree cover and soil N. However, local soil effects have been shown to have significant impacts on $\delta^{15}\text{N}$ (see Craine et al., 2009). Recent studies show similarly contradictory findings relating to the impact of these environmental variables on primate tissues (Loudon, Sandberg, Wrangham, Fahey, & Sponheimer, 2016; Oelze et al., 2016; Schoeninger, Most, Moore, & Somerville, 2016). Beyond the primary effects of nitrogen source and fixation pathways, intra-plant $\delta^{15}\text{N}$ variability has also been observed, possibly relating to differences in N relocation and biomolecular composition (Handley & Raven, 1992; Evans, 2001). Higher $\delta^{15}\text{N}$ values are observed in leaves and lower $\delta^{15}\text{N}$ values in woody tissues and roots (Blumenthal et al., 2016; Evans, 2001), likely related to biochemical composition as there is greater nitrogen content in the former. Additionally, vertical gradients in foliar $\delta^{15}\text{N}$ values have been observed in forests, with changes in ^{15}N discrimination possibly relating to gradients in nitrogen concentration and/or acquisition (Ometto et al., 2006; Wania, Heitz, & Wanek, 2002).

On the basis of general principles of carbon, nitrogen, and oxygen isotope variation in tropical forest plants, we can make the following predictions: (1) plants should have higher $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ values in the canopy relative to the understory, as well as during the dry season as a result of greater irradiation; (2) plant part should influence $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ significantly, with leaves having lower $\delta^{13}\text{C}$ and higher $\delta^{18}\text{O}$ than fruits, seeds, bark and pith; (3) plant $\delta^{15}\text{N}$ values should be higher in the canopy relative to the understory, as well as during the arid dry season within the Polonnaruwa forest; (4) photosynthetic parts should have higher $\delta^{15}\text{N}$ values than non-photosynthetic parts.

2 | METHODS

All methods comply with protocols approved by the appropriate Institutional Animal Care Committee, the American Society of Primatologists (ASP) Principles for the Ethical Treatment of Non

Human Primates, and the legal requirements of the country in which the research was conducted.

2.1 | Study area

We conducted this study in the Polonnaruwa Nature Sanctuary and Archaeological Reserve in the so-called north-central “Dry Zone” of Sri Lanka (07°56'N, 81°00'E) (Figure 1). The “Dry Zone” is a relative nomenclature for the island of Sri Lanka, receiving less annual rainfall (c. 1,000–1,700 mm) (Puvaneswaran & Smithson 1993a,b; Roberts, Boivin, & Petraglia, 2015) compared to evergreen tropical rainforests of Africa and South America where the majority of primate stable isotope ecology has been undertaken. Most precipitation at Polonnaruwa is brought by convectional rains that grade into the Northeast Monsoon, with an excess of rainfall (for plant growth) occurring from October to January (the so-called “wet season” at the reserve). Rainfall peaks at between 250 and 400 mm a month between November and January (Dittus 1977; pp. 269 Figure 2). Convectional rains bring some rain again in April. Drought, where rainfall is inadequate to support plant growth, occurs during the “dry season” at Polonnaruwa for 3–5 months (May–September) when the forest is also subject to the strong, desiccating warm winds of the Southwest Monsoon (Gausson, 1955; Mueller-Dombois, 1968). This is the only tropical forest in Sri Lanka where all three-monkey species live together today and where they have been observed for over four decades (e.g., Dittus, 1974, 1977, 2013).

The Polonnaruwa forest can be classified, following Dittus (1977), into three main strata: an understory that consists of a non-woody herb and woody shrub layer up to 5 m in height, a more or less continuous closed canopy tree layer between 5 and 15 m, and a discontinuous emergent layer between 15 and 30 m. The semi-evergreen canopy is dominated by *Drypetes sepiara*, followed by *Walsura piscidia*, *Vitex pinnata*, and *Premna tomentosa*. Emergent tree species are less common and include *Schleichera oleosa*, *Adina cordifolia*, and *Manilkara hexandra* (Dittus, 1977). Most trees of the canopy retain their foliage year round, although the taller crowns in

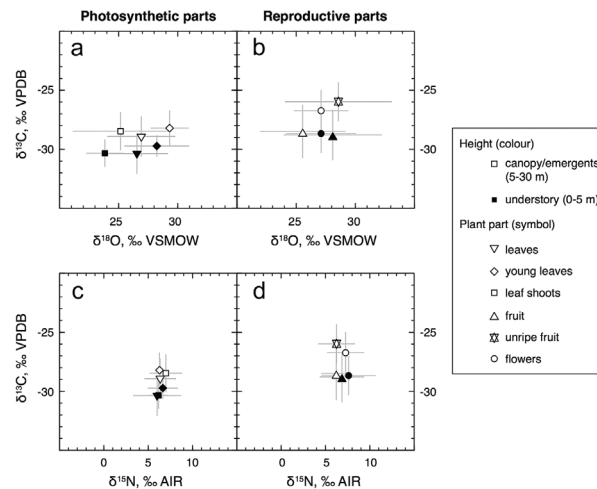


FIGURE 2 Stable isotope composition of plants grouped by part and height for photosynthetic (a, c) and reproductive (b, d) parts. Symbols correspond to different plant parts and color corresponds to height

this layer (e.g., *V. pinnata*), will defoliate their desiccated leaves during the peak of the dry season (Dittus, 1974, 1977). Two drought-adapted species, having thick waxy leathery leaves, retain their foliage in the dry season: the emergent *M. hexandra*, and the major constituent species of the canopy *D. sepiara*. The overall effect of differences in dry season-induced defoliation by species and tree height results in increased insolation of the canopy (Dittus, 1974, 1977). This feature, as well as measures of species diversity, has been argued to place the forest as an intermediate type between tropical evergreen rainforest and seasonally (partly) defoliated forest (Dittus, 1977; Walter, 1971).

2.2 | Sample collection

We use ongoing feeding observations of primates in the Polonnaruwa Nature Sanctuary made by WD to determine their principal vegetative dietary components during the dry and wet seasons (Supplemental Tables S1 and S2). This dataset is broadly complementary to older observation datasets for primates in the same reserve (Dittus, 1974; Hladik, 1977; Hladik & Hladik, 1972). Supplemental Tables S1 and S2 provide species information, height in the canopy, and growing conditions for all vegetation samples ($n = 288$). Two sampling seasons were undertaken, in August (2014) and February (2015), to test for differences in the isotopic variation in primate plant foods in dry versus wet seasons. Where available, at least three individual plants were sampled from different locations within the forest to capture variation in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ within a given species (Supplemental Tables S1 and S2). If important to primate diets, fruits, flowers, leaves, and leaf shoots were obtained from the same tree or shrub.

Height in the canopy was recorded by visual estimation according to the forest stratification described by Dittus (1977) (i.e., low: 0–5 m, middle: 5–15 m, upper: 15–30 m). While more detailed range-finding measurements could have been taken it was considered that these were the most important height categories from the perspective of primate ecology and based on the forest structure. Where possible,

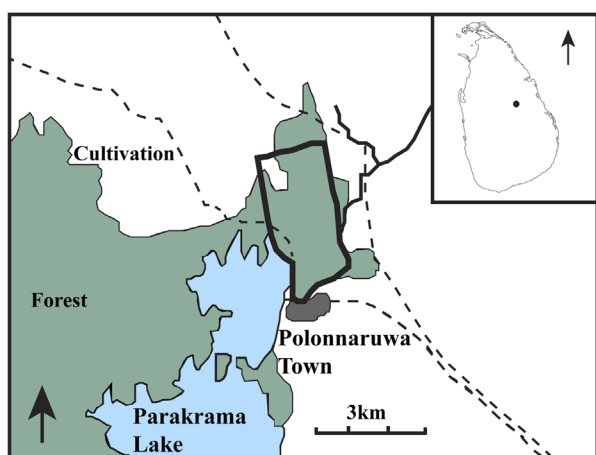


FIGURE 1 Map of the Polonnaruwa Nature Sanctuary, Sri Lanka and its environs

multiple samples of different plant parts were collected at different heights in the canopy within the same tree. Each of the 288 plant samples represent between eight and ten samples of each plant part, at each location within a plant/tree, that have been combined in order to take into account natural $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, and $\delta^{15}\text{N}$ variation. Plant parts were obtained using a combination of a secure ladder system and skilled local assistants climbing into the trees. In both cases a pair of leaf cutters was used to obtain the relevant plant part. Samples were placed in brown, acid-free paper bags, and dried thoroughly using an oven. They were then stored in a food dehydrator in order to avoid rotting.

2.3 | Stable isotope analyses

All dried vegetation samples were homogenized into dried bulk organic matter powder using a 6770 Freezer/Mill® (SPEX® SamplePrep) with liquid nitrogen immersion bath. 1 mg and c. 3 mg of sample was then combusted using a Sercon Europa EA-GSL and $^{13}\text{C}/^{12}\text{C}$ and $^{15}\text{N}/^{14}\text{N}$ ratios of the resulting gas were measured on a Sercon Geo 2022 IRMS in continuous flow mode with helium carrier gas (80 ml/min flow rate) at the University of Oxford. Stable isotope ratios are reported using the conventional δ -notation, where $\delta^{13}\text{C}$ or $\delta^{15}\text{N} = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1,000$ and R_{sample} and R_{standard} are the $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ ratios in the sample and standard for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. $\delta^{13}\text{C}$ values were compared against international sucrose (IAEA-CH-6 ($\delta^{13}\text{C} = -10.4\text{‰}$, SD = 0.0)—iaea.org) and polyethylene (IAEA-CH-7 ($\delta^{13}\text{C} = -32.2\text{‰}$, SD = 0.1)—iaea.org) standards using a two-point calibration. $\delta^{15}\text{N}$ values were similarly compared to nitrate (IAEA-N₂ ($\delta^{15}\text{N} = 20.3\text{‰}$, SD = 0.2)—iaea.org) and caffeine (IAEA-600 ($\delta^{15}\text{N} = 1.0\text{‰}$, SD = 0.2) – iaea.org) standards. Replicate analysis of an in-house alanine international standard ($\delta^{13}\text{C} = -26.9\text{‰}$, SD = 0.1; $\delta^{15}\text{N} = 1.6\text{‰}$, SD = 0.2) suggests that machine measurement error was c. $\pm 0.1\text{‰}$ for $\delta^{13}\text{C}$ ($n = 50$) and $\pm 0.2\text{‰}$ for $\delta^{15}\text{N}$ ($n = 50$).

Aliquots of powder from the same samples were weighed out separately for the measurement of oxygen isotopes using a Sercon Geo 2022 IRMS coupled within a Sercon HTEA at 1,400°C over glassy carbon at the University of Oxford. Bulk organic matter $\delta^{18}\text{O}$ from leaves, fruits, and flowers was considered to be an appropriate measure of primate food $\delta^{18}\text{O}$ in this study, although it is acknowledged that this approach misses possible oxygen input from drinking water. Oxygen isotope values were compared against the international standards EMA-P1 ($\delta^{18}\text{O} = 21.0\text{‰}$, SD = 0.7—Elemental Microanalysis Ltd) and EMA-P2 ($\delta^{18}\text{O} = 26.9\text{‰}$, SD = 2.2—Elemental Microanalysis Ltd), using a two-point calibration. Multiple replicate analysis of EMA-P1 suggests that instrumental precision was c. $\pm 0.2\text{‰}$ for $\delta^{18}\text{O}$ ($n = 50$). The resulting measurements were reported as “per mil” in the δ notation relative to the standards of VSMOW. Levels of isotopic variation within the plant samples were analyzed by taking ten separate $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{18}\text{O}$ subsample measurements (and their standard deviations) from the two single bulk samples PL183 and PL178 (Supplemental Tables S3 and S4). In all cases isotopic variation (standard deviation) within a sample is minimal (PL183: $\delta^{13}\text{C} = 0.1$, $\delta^{15}\text{N} = 0.2$, $\delta^{18}\text{O} = 0.2$; PL178: $\delta^{13}\text{C} = 0.1$, $\delta^{15}\text{N} = 0.2$, $\delta^{18}\text{O} = 0.2$).

2.4 | Statistical analysis

Statistical analyses were performed in R (R Core Team, 2013). We use the “lme4” package to generate linear mixed-effects models to explore the effects of multiple explanatory variables and interactions between these variables (Bennington & Thayne, 1994; Sokal & Rohlf, 2012) on the carbon, nitrogen, and oxygen isotopic composition of plants. Mixed models assume normality, homoscedasticity (homogeneity of variance), and independence (minimal collinearity) between predictor variables. For each isotope, we generate a model that describes isotopic variation attributable to part + height (e.g., understory fruit, canopy fruit, understory leaves, canopy leaves, etc.) and season (wet and dry) as fixed effects variables and controlling for tree number and plant species as random effects. A fixed effect is removed if it is associated with unacceptably high collinearity, which is estimating using variance inflation factors (extreme collinearity: $\text{VIF} > 10$) and kappa values (extreme collinearity: $K < 10$) (Yu, Jiang, & Land, 2015). Normality and homoscedasticity are assessed by visual examination of qq-plots and residual-fitted value plots. Isotopic differences among part-height categories were further examined using Kruskal–Wallis one-way analysis of variance and postdoc multiple comparisons.

3 | RESULTS

Supplemental Tables S1 and S2 show the $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, and $\delta^{15}\text{N}$ measurements of each plant sample collected during the dry season and wet season, respectively. Table 1 summarizes mixed models for $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, and $\delta^{15}\text{N}$ measurements.

Mixed model results, presented in Table 1, demonstrate that carbon and oxygen isotopic variation is attributable to plant part and canopy height, controlling for species and tree number, with acceptable levels of collinearity, normality, and homoscedasticity. Season was removed as a fixed effect variable from carbon and oxygen isotope models because it was associated with unacceptably high VIF and K values. The nitrogen isotope model was discarded because the assumption of homoscedasticity was violated.

The carbon, oxygen, and nitrogen isotopic composition of plants grouped by part and height is presented in Figure 2, and statistical results for pairwise comparisons among part and height groupings is presented in Table 2. We found statistically significant differences in $\delta^{13}\text{C}$ values (Kruskal–Wallis, chi-squared = 69.0479, $df = 10$, $P = 6.77 \times 10^{-11}$). Among photosynthetic parts, understory leaves have lower $\delta^{13}\text{C}$ values than canopy leaves ($P < 0.05$) and canopy young leaves ($P < 0.05$). $\delta^{13}\text{C}$ values of young leaves in the understory are similar to young leaves in the canopy ($P > 0.05$). Among reproductive parts, canopy flowers have higher $\delta^{13}\text{C}$ values than understory leaves ($P < 0.05$), young leaves ($P < 0.05$), and shoots ($P < 0.05$), as well as canopy leaves ($P < 0.05$). Fruit in the understory and canopy have higher $\delta^{13}\text{C}$ values than understory leaves ($P < 0.05$) but are similar to canopy leaves ($P > 0.05$). Unripe fruit have higher $\delta^{13}\text{C}$ values than understory leaves ($P < 0.05$) and understory shoots ($P < 0.05$).

We found statistically significant differences in $\delta^{18}\text{O}$ values (Kruskal–Wallis, chi-squared = 69.0479, $df = 10$, $P = 0.0002749$).

TABLE 1 Summary of mixed multiple regression models

Model	ANOVA					Likelihood ratio test			Collinearity	
	Effect	Df	Sum Sq	Mean Sq	F value	Df	χ^2	P	Max VIF	Kappa
Carbon	Part and canopy	10	266.80	26.68	13.55	11	153.96	<0.001	5.67	16.74
Oxygen	Part and canopy	10	176.13	17.61	3.35	11	125.41	<0.001	5.71	16.74

Young leaves in the canopy have higher $\delta^{18}\text{O}$ values than fruit in the canopy and shoots in the understory or canopy ($P < 0.05$). Young leaves and mature leaves have similar $\delta^{18}\text{O}$ values ($P > 0.05$). $\delta^{15}\text{N}$ values were similar ($P > 0.05$) among parts and height groupings.

4 | DISCUSSION

As predicted, we show that plant part and canopy height are major drivers of isotopic variation in primate foods, consistent with the importance of this variable in primate niche partitioning (Clutton-Brock & Harvey, 1977). However, we show that expected isotopic patterns associated with part and height are not uniform. For example, we detected a vertical canopy effect in $\delta^{13}\text{C}$ among mature leaves as predicted, but not among other photosynthetic parts such as young leaves and shoots or among reproductive parts. Similarly, we find some support for predicted differences between photosynthetic and reproductive parts, depending on height. For example, flowers in the canopy have higher $\delta^{13}\text{C}$ values than both understory and canopy leaves, but flowers in the canopy and understory are similar. Fruit in the understory and canopy both have higher $\delta^{13}\text{C}$ values than understory leaves, but $\delta^{13}\text{C}$ values of fruit are similar to canopy leaves.

Significantly, the linear model shows that part and height explain $\delta^{18}\text{O}$ variation in plants, although we only find significant pairwise differences among parts. In the canopy, young leaves have higher $\delta^{18}\text{O}$ values than fruit or shoots, but we detect no difference between mature leaves and fruit or between young and mature leaves. Thus, oxygen isotopes do not consistently differ between photosynthetic and reproductive plant parts, contrasting with expectations that oxygen isotopic enrichment associated with evapotranspiration should result in higher $\delta^{18}\text{O}$ values in leaves compared to fruit and other tissues (Barbour, 2007). Vertical stratification of $\delta^{18}\text{O}$ values has been observed in both humid (Sternberg et al., 1989) and seasonally dry (Ometto, Flanagan, Martinelli, & Ehleringer, 2005) neotropical forests, in leaf cellulose and leaf water, respectively. However, we find no pairwise differences in $\delta^{18}\text{O}$ between understory and canopy leaves, possibly relating to lower vertical environmental gradients (humidity, vapour pressure) in Polonnaruwa, or because intra-plant differences are partly obscured by variable exchange with leaf water by different biomolecular fractions, which are integrated in bulk leaf analysis. We find no significant pairwise differences in $\delta^{15}\text{N}$ between part and height groupings. We did not include woody tissues, and could not test for possible differences between woody and non-woody tissues, as observed elsewhere (Blumenthal et al., 2012, 2016).

Thus, isotopic differences associated with plant part and height are intertwined, and may vary in relative importance across study areas (Blumenthal et al., 2016; Oelze et al., 2014). This has important implications for interpretations of isotopic variation in primate tissues. For example, $\delta^{13}\text{C}$ variation in primate tissues has been variously interpreted as indicating diet (Carlson & Crowley, 2016; Oelze et al., 2014), feeding height (Carlson & Crowley, 2016), and forest density (Macho & Lee-Thorp, 2014). These should be viewed as context-specific inferences rather than generalizable expectations. For example, despite abundant evidence for vertical stratification in plant leaf $\delta^{13}\text{C}$ values, difficulties in distinguishing feeding height using carbon isotopes in primate tissues (Cerling et al., 2004; Krigbaum et al., 2013; Nelson, 2013) likely stem from feeding on different plant parts, each with varying degrees of $\delta^{13}\text{C}$ vertical stratification. Likewise, while $\delta^{13}\text{C}$ differences between leaves and fruit have been identified in some forests (Blumenthal et al., 2012), with suggestions that in some cases it is possible to distinguish folivory and frugivory, we show that

TABLE 2 Pairwise comparisons for plant part and canopy groupings

Isotope	Comparison	Significance ^a
Carbon	Canopy flowers and canopy leaves	Yes
	Canopy flowers and understory leaves	Yes
	Canopy flowers and understory shoots	Yes
	Canopy flowers and understory young leaves	Yes
	Canopy fruit and understory leaves	Yes
	Canopy leaves and understory leaves	Yes
	Canopy unripe fruit and understory leaves	Yes
	Canopy unripe fruit and understory shoots	Yes
	Canopy young leaves and understory leaves	Yes
	Understory fruit and understory leaves	Yes
Oxygen	All other pairwise comparisons	No
	Canopy young leaves and canopy fruit	Yes
	Canopy young leaves and canopy shoots	Yes
	Canopy young leaves and understory shoots	Yes
Nitrogen	All other pairwise comparisons	No
	All pairwise comparisons	No

^aSignificance level of 0.05. Exact *P* values not provided by R function for Kruskal–Wallis multiple comparisons (kruskalmc).

this distinction may be obscured by height differences in the availability and preference for particular fruits and leaves.

Similarly, Carlson and Crowley (2016) pointed out that primates consuming leaves may have higher $\delta^{18}\text{O}$ than those consuming fruits due to evapotranspiration in leaves. However, our findings indicate that inferences will be complicated by the effects of feeding height in the canopy, whereby a leaf low in the canopy may have equifinal $\delta^{18}\text{O}$ values relative to fruits higher in the canopy. Additionally, while variation in folivorous primate bone apatite $\delta^{18}\text{O}$ values in Kibale have been linked to vertical niche partitioning (Carter & Bradbury, 2016), this pattern among may not be generalizable to seasonally dry forests, due to a reduction in vertical gradients of humidity. Alternatively, vertical stratification of $\delta^{18}\text{O}$ values may relate to behavioral or physiological variation unrelated to food.

Thus, we caution that ecological inferences from primate tissue isotope data in isolation, without accompanying baseline values, are ambiguous as a result of difficulties in distinguishing between many possible explanations related to feeding and habitat preferences. Oelze et al. (2014) noted that the consumption of different plant taxa within and between ecosystems might result in inconsistent isotopic effects associated with plant part. It is only possible to account for these uncertainties in modern studies where feeding observation data are available (Crowley et al., 2014; Crowley, McGoogan, et al., 2012; Oelze et al., 2014). Identifying the effects of species is difficult or impossible in studies using tissues sampled from populations for which no direct data on species consumption is available, such as unhabituated primates or from tissues preserved in museum or fossil collections. Codron et al. (2005) showed that species composition can be the major driver of $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ variation of foods that may in turn be used to infer diet. We do not intend to dissuade users of stable isotope methods in primatology, but caution that isotopic differences commonly assumed to reflect canopy height or plant part, for example, can be mimicked or obscured by a combination of site- or population-specific behavioral, ecological, and taxonomic factors. Thus, it is important to always acknowledge such alternative explanations for observed isotopic variation of primate tissues.

These results are important for primate isotope ecology in the Polonnaruwa tropical forest given that the three Sri Lankan primates, *Macaca sinica*, *Semnopithecus entellus priam*, and *Trachypithecus vetulus*, can be observed consuming varying proportions of different plant parts and feeding at different canopy heights. Long-term observational data demonstrates that *T. vetulus* is a specialized folivore, obtaining 70% of its food from the mature leaves of three species. *S. priam thersites*, by contrast, obtains c. 70% of its food from ten tree species, including 48% leaves, 7% flowers, and 45% fruits by wet weight (Hladik, 1977; Hladik & Hladik, 1972). The generalist macaques exploited 89% of the 46 species of trees within the Reserve, with fruits constituting over 70% of their diet, alongside flowers, leaves, mushrooms, fungi, grasses, roots, tubers, resins, and invertebrates (Dittus, 1974). On the basis of these isotopic and feeding differences, tissues of the generalist *M. sinica* and *S. priam thersites* might be expected to have higher $\delta^{13}\text{C}$, lower $\delta^{18}\text{O}$ and more variable $\delta^{15}\text{N}$ values compared to the specialized leaf consumer *T. vetulus*. In particular, when averaged out over the year, 100% fruit versus 100% leaf diets could lead to a distinction of c. 2‰.

However, there are also significant differences in the observed heights at which these primates feed. *T. vetulus* feeds high in the canopy and rarely ventures to the ground, while *S. priam thersites* and *M. sinica* feed in the lower canopy and frequently on the ground (Dittus, 1974). Due to equifinal isotope effects observed here, while high leaf consumption may lead to lower $\delta^{13}\text{C}$ in *T. vetulus* tissues, the higher feeding height of this primate would drive $\delta^{13}\text{C}$ in the opposite direction. By contrast high leaf consumption and high feeding height might be expected to both drive higher $\delta^{18}\text{O}$, though the trends observed in the plant dataset analyzed here are more equivocal and not likely to be clear cut. More broadly, the variable feeding heights of *S. priam thersites* and *M. sinica* suggest that definite plant part differences in $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ will be difficult to constrain. This is especially the case given that *T. vetulus*, *S. priam thersites*, and *M. sinica* are known to feed on different plant taxa throughout the year, which have been argued to result in inconsistent plant part isotopic effects (Oelze et al., 2014).

4.1 | Implications for isotopic studies in primatology

Analysis of $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, and $\delta^{15}\text{N}$ of sympatric primate tissues, in modern and prehistoric settings, has provided ecologists with a means of obtaining quantifiable data of ecological differences from primates that are shy, hard to observe, or even extinct (Codron et al., 2006; Crowley, Godfrey, et al., 2012; Loudon et al., 2007; Macho & Lee-Thorp, 2014; Oelze et al., 2014; Sandberg et al., 2012; Sponheimer et al., 2009). However, while we can show how significant these differences between taxa and populations are, there is greater difficulty in interpreting what they mean. In most cases interpretations in this regard have been based on general isotopic principles or guided by observational data of primate diets and behaviors in a modern context. Such approaches often underestimate the complexity of $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, and $\delta^{15}\text{N}$ in tropical forest plants. Existing large datasets of tropical forest plants have shown considerable complexity in $\delta^{13}\text{C}$, $\delta^{18}\text{O}$, and $\delta^{15}\text{N}$ variation (Blumenthal et al., 2016; Carlson & Crowley, 2016; Carlson & Kingston, 2014; Crowley, McGoogan, et al., 2012), where different environmental factors can produce overlapping or conflicting effects.

This study highlights the potential complexity of isotopic variation related to canopy height, insolation, and growing season in tropical forest habitats. Most previous isotopic studies of tropical plants relevant to primate ecology are focused on humid tropical forests (Blumenthal et al., 2016; Carlson & Kingston, 2014; Crowley et al., 2014; Crowley, McGoogan, et al., 2012), but the possibility that major ecological correlates of isotopic variation may differ between and within environments suggests that additional large-scale studies are needed to understand baseline isotopic variation across a wider range of primate habitats. Where we seek to transplant understandings of ecology-linked primate tissue isotope variation into prehistoric primate communities, we must be cautious of over-interpretation. For example, while Nelson (2007) argued that high $\delta^{18}\text{O}$ and low $\delta^{13}\text{C}$ in the Miocene ape, *Sivapithecus*, indicate that it fed high in the canopy in dense forest habitats. However, in a more recent publication she acknowledges that the same effect could equally result from feeding higher in the canopy, feeding in a less continuous canopy, relying more on leaves, or the consumption of particular plant species (Nelson, 2013).

In agreement with previous guidelines for large-scale primate isotope studies (Blumenthal et al., 2016; Crowley, 2012), we note that it is critical to test, where possible, how isotopic parameters of interest react to canopy height, plant part, plant species and forest density in the local study area of relevance to the primates of interest, rather than relying only on general principles. It is also essential that these isotope baselines are expanded to dry tropical forests (Crowley, McGoogan, et al., 2012) and monsoonal forests in order to understand the main environmental factors influencing primate diet in these different formations. In the case of the Polonnaruwa Nature Sanctuary, Sri Lanka, plant part is the major driver of plant $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ variation. This would suggest that in subsequent studies of primate tissue $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ from this forest, this parameter should be given primacy in ecological interpretations. However, the factors of differential feeding height and differential consumption of different plant taxa between primate species may complicate simple observation of plant part effects.

5 | CONCLUSIONS

Primates exhibit some of the most “generalist” feeding strategies of any taxa on the planet. This makes the reconstruction of their dietary practices using stable isotope analysis of their tissues particularly complex. Some have argued, on the basis of general isotopic principles that information regarding feeding height in the canopy, plant part preferences, preferences for micro-habitats with different degrees of shade, fluctuations in the contribution of animal protein, and weaning effects, are all discernible isotopically within primate tissues (Carter & Bradbury, 2016; Sandberg et al., 2012). However, where attempts have been made to verify the impact of these different parameters on large datasets of plant $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{18}\text{O}$, relevant to primate diet, they have demonstrated considerable complexity of interpretation. This is particularly the case in monsoonal forests, such as that at the Polonnaruwa Nature Sanctuary, Sri Lanka, where season can have a significant, variable impact on the manifestation of the “canopy effect” through the year.

Data from this forest indicate that plant part plays the dominant role in structuring the $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ of plants of immediate relevance to primate diets. In particular, the specialized leaf consumer *T. vetulus* can be expected to have lower $\delta^{13}\text{C}$ than more generalist feeders, *M. sinica* and *S. prima thersites*, by as much as 2‰. The data from this forest suggests that feeding height has the potential to obscure or dampen plant part impacts on primate tissue $\delta^{13}\text{C}$. However, it is the more variable plant part differences seen in $\delta^{18}\text{O}$ that are likely to be particularly affected by varied feeding height and differential consumption of different plant taxa amongst primate taxa at the Polonnaruwa Nature Sanctuary. It appears that this seasonal, dry forest, and the different ecological behaviors of its incumbent primates, make equifinal isotope effects particularly problematic, with the exception of plant part differences in $\delta^{13}\text{C}$.

The relative impacts of canopy height, plant part, plant species, and forest density on $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$, and soil composition, plant species, and plant part on $\delta^{15}\text{N}$, clearly remain complex, and highly variable between different forest types and regions. Greater quantification of the importance of these parameters in different local ecosystems has the potential to enrich our understanding of the ecological factors that

influence the $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and $\delta^{18}\text{O}$ of different primate taxa and thus inform the use of stable isotopes in analysis of their feeding behaviors, social interactions and, ultimately, their conservation.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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