Received: 18 February 2020 | Revised: 19 January 2021 | Accepted: 24 January 2021

DOI: 10.1002/ajp.23239

RESEARCH ARTICLE

AMERICAN JOURNAL OF PRIMATOLOGY WILEY

Condensed tannins in the diet of folivorous diademed sifakas and the gap between crude and available protein

Emma G. Thurau[1,2,3] | Andry Narcisse Rahajanirina[4,5] | Mitchell T. Irwin[5,6]

[1]Department of Anthropology, The Graduate Center of the City University of New York, New York, USA

[2]New York City Consortium of Evolutionary Primatology (NYCEP), New York, USA

[3]Department of Anthropology, Hunter College of City University of New York, New York, USA

[4]Mention Anthropobiologie et Développement Durable, Faculté des Sciences, Université d'Antananarivo, Antananarivo, Madagascar

[5]Division of Research, ONG SADABE, Antananarivo, Madagascar

[6]Department of Anthropology, Northern Illinois University, DeKalb, Illinois, USA

Correspondence
Emma G. Thurau, Department of Anthropology, Hunter College, 695 Park Ave, HN 772, New York, NY 10065.
Email: ethurau@gradcenter.cuny.edu

Funding information
Global Wildlife Conservation, Grant/Award Number: 5095.006-0175; Northern Illinois University; Primate Conservation, Grant/Award Number: 1508

Abstract

Tannins, a type of plant secondary metabolite, are well-known for their ability to precipitate proteins and thereby reduce the protein available to consumers. Most primate studies have focused on condensed tannins (CTs) as they were thought to be the most effective type of tannin at preventing protein acquisition, but there is growing recognition that other types of tannins can bind to proteins, suggesting the division among tannin types is not as clear-cut as previously thought. Although previous studies have documented the presence of CTs in primate diets and primates' behavioral responses to them, our understanding of tannins remains limited because few researchers have used Sephadex column purification to accurately determine tannin concentrations, and few have used in vitro assays to determine available protein content and the tannins' effectiveness in binding protein. In this study, we documented diademed sifaka (*Propithecus diadema*) diet from June to August 2018 at Tsinjoarivo, Madagascar (in two forests with varying degrees of habitat disturbance) and quantified CT concentration and actual available protein in foods. Eleven of the fourteen top foods tested contained CTs (concentrations: 4.8%–39.3% dry matter). An in vitro assay showed available protein was strikingly low in six of the eleven top foods (e.g., little to no apparent available protein, despite high crude protein). Overall, our findings suggest sifakas acquire less protein than previously recognized and probably have adaptations to counteract tannins. Such studies of available protein are critical in understanding dietary constraints on sifaka populations and the evolution of their diet choice strategies; despite the conventional wisdom that leaves are protein-rich, folivorous primates may indeed be protein-limited. However, further studies are necessary to determine if sifakas have counter-adaptations to tannins, and if they absorb more protein than our analyses suggest, perhaps receiving protein that we were unable to detect with the current techniques (e.g., pollen).

KEYWORDS
nutritional ecology, plant secondary metabolites

1 | INTRODUCTION

Plant secondary metabolites (PSMs) are a highly diverse group of plant compounds (Coley, 1983; DeGabriel et al., 2009; Feeny, 1976; Freeland & Janzen, 1974); many are thought to have evolved specifically to deter herbivores, and are known to have substantial effects on their foraging choices and health. Generally, PSMs affect herbivores in three key ways. First, they can cause decreased foraging on specific plants (Moore & DeGabriel, 2012). For example, Pass and Foley (2000) found that common brushtail possums (*Trichosurus vulpecula*) exhibited decreased feeding when salicin (a type of phenolic glycoside) was added to their food items. Second, PSMs can impose metabolic costs for detoxification (Moore & DeGabriel, 2012). For example, Au et al. (2013) found *T. vulpecula* exhibited increased whole-body protein turnover when consuming diets with increasing benzoate (a type of aromatic carboxylic acid) suggesting a protein cost for detoxification. Third, PSMs can decrease the overall nutritional quality of the plant (Moore & DeGabriel, 2012). For example, Roy and Bergeron (1990) found that meadow voles (*Microtus pennsylvanicus*) formed piles of coniferous tree twigs before ingesting them, these twigs exhibit reduced total phenolic levels after several days, suggesting the twigs increased in nutritional quality after phenolic levels decreased (Iason & Villalba, 2006).

The best-known and most common PSMs are tannins. Traditionally tannins have been defined as a heterogenous group of water-soluble defensive compounds, well-known for their ability to bind and precipitate proteins and other macromolecules (Foley & McArthur, 1994; Moore & DeGabriel, 2012; Salminen & Karonen, 2011). Most often in the literature, tannins have been split into two classes: hydrolyzable and condensed tannins (CTs; Bryant et al., 1991; Freeland & Janzen, 1974; Milton, 1998; Mole & Waterman, 1987). However, Salminen and Karonen's (2011) seminal review of tannins suggest there are actually three major classes of tannins that can be defined structurally. First, phlorotannins exhibit a fairly simple structure with two or more phloroglucinol units that are attached to each other by either a C–C or C–O–C bond. Second, hydrolyzable tannins (HTs) exhibit the most complex structure relative to other classes of tannins and can be broken into three subclasses; (1) gallic acid derivatives, (2) galloyl tannins, and (3) ellagitannins. CTs are the most common group and can be broken up into two major groups; (1) procyanidins and (2) prodelphinidins. Procyanidins exhibit two or more monomeric (+)-catechin or (−)-epicatechin while prodelphinidins exhibit (+)-g gallocatechin or (−)- epigallocatechin units (Salminen & Karonen, 2011). Most often in the literature, primatologists tend to focus on CTs and their protein precipitation capacity, because although HTs are toxic to the gut microbes and cause gastrointestinal damage they tend to be easily broken down and less likely to function to deter herbivores. However, this a false dichotomy since ellagitannins can bind proteins (Salminen & Karonen, 2011).

Tannins can have various effects on herbivores including altered food consumption rates, altered growth rates, altered digestive efficiencies, weight loss, reduced fecundity, and pathological effects (Cork & Foley, 1991; DeGabriel et al., 2009; Rothman et al., 2006; Wallis et al., 2012). Several primate species exhibit CT avoidance (Ganzhorn et al., 1985; Ganzhorn, 1988, 1989; Kool, 1992; Leighton, 1993; Oates et al., 1980; Simmen et al., 1999; Wrangham & Waterman, 1983), while other primate species exhibit no influence of CTs on food selection (Arlet et al., 2015; Beaune et al., 2017; Carrai et al., 2003; Felton et al., 2009; Ganzhorn, 1989; Kool, 1992; Milton, 1998; Reynolds et al., 1998; Wrangham et al., 1998). This suggests an unclear or variable relationship in primate diets; tannins may be selected against by some species but not others, perhaps in part because some primates have adaptations (e.g., tannin-binding salivary proteins) that block the action of tannins (Beaune et al., 2017; Espinosa Gómez et al., 2015; Remis et al., 2001; Espinosa Gómez et al., 2018). However, although it is clear that CTs are present in the diet of some primates, we still know very little about the true prevalence and effectiveness of tannins (e.g., the degree of reduced protein digestibility), largely due to two major methodological issues.

First the methods used in prior studies to quantify tannin concentrations relied on external standards (e.g., quebracho), which has been shown by Rothman et al. (2009) to introduce massive errors, either overpredicting or underpredicting CT concentrations in food. This is likely why some studies quantifying feeding selection give misleading results. Second, tannins are a diverse group of molecules. Many different tannin subgroups exist, and these subgroups can interact with each other and impact their biological activity; therefore, if a study only measures the concentration of CTs in diet and assumes uniform biological activity of tannins, it may be a poor measure of the true impacts (Marsh et al., 2019; Salminen & Karonen, 2011). Further as pointed out by Salminen and Karonen (2011), herbivores consume the whole tannin mixture within the plant, not specific CTs or HTs (Salminen & Karonen, 2011).

Previous studies by Irwin (2008a, 2008b) found that diademed sifakas (*Propithecus diadema*) in both fragmented and continuous forest sites at Tsinjoarivo exhibited dietary shifts in the lean season, including high folivory, increased dietary diversity, decreased food consumption, and decreased protein and nutritional intakes. Generally, primates during the lean season are expected to ingest more foods to compensate for decreased nutritional density, yet the sifakas at Tsinjoarivo exhibited the opposite (Irwin et al., 2014). Based upon this, Irwin et al. (2014) suggested that intrinsic features of lean season foods (i.e., PSMs) may impact consumption levels - in other words, animals eat less to avoid the accumulation of detrimental chemicals, coupled with reduced activity to conserve energy. This hypothesis is supported by decreased feeding effort and increased dietary diversity observed during the lean season (Irwin et al., 2014; Marsh et al., 2006). However, despite these behavioral inferences no study has yet documented tannin consumption in Tsinjoarivo, and only one (Powzyk & Mowry, 2003) documented the presence of tannins in *Propithecus diadema* (diademed sifaka) foods; this study, though useful in confirming tannin presence, used quebracho, which may lead to over or underestimations of tannin concentrations (Rothman et al., 2009).

In this study, we: (1) quantified the tannin concentration in sifaka lean season foods at Tsinjoarivo, (2) quantified the crude protein based on nitrogen concentration in foods, and (3) used an in vitro assay to estimate the available protein in foods, and therefore the effectiveness of the tannins on decreasing protein intake. Our hypothesis builds on previous studies by Irwin et al. (2014) suggesting intrinsic features of lean season foods limit consumption levels and protein intake of diademed sifakas. We therefore hypothesize plant species consumed by the sifaka will contain tannins and these, in turn, will reduce the protein absorbed by the sifaka. Based on this, we expect to see that most, if not all, foods exhibit tannins, and these tannins in turn reduce the food's available protein.

2 | METHODS

2.1 | Ethics statement

All data collection procedures were approved by Northern Illinois University IACUC committee (IACUC LA12-0011). Field data collection protocols were also approved by Madagascar's Ministry of Environment, Ecology and Forests (Permit 106/18/MEEF/SG/DGF/DSAP/SCB.Re). This study adhered to the American Society of Primatologists Principles for the Ethical Treatment of Nonhuman Primates.

2.2 | Study site and subjects

Tsinjoarivo forest contains mid-altitude rainforest (1400–1650 m) located 80 kilometers SE of Antananarivo (Irwin, 2008a, 2008b). Tsinjoarivo is part of a semi-continuous corridor between Ranomafana (150 km SSW) and Mantadia (100 km NE) National Parks and is part of the new Tsinjoarivo-Ambalaomby protected area, which gained official protection in 2020.

The study subjects, *P. diadema*, have been studied since 2002 (Irwin, 2008a). Diademed sifakas are large-bodied lemurs endemic to eastern and northeastern rainforests of Madagascar (Mayor et al., 2004). At Tsinjoarivo, *P. diadema* live in groups of two to nine individuals; groups contain one adult male, one to two adult females, and up to seven immatures (Irwin, 2008a, 2008b).

Their diet is predominantly composed of leaves (53% of feeding time) along with fruits (24%), flowers (15%), and seeds (7%). The contribution of leaves to their diet varies seasonally and depends on the availability of fruits; in the abundant season (when fruit availability is highest), leaves only make up 20% of diet, whereas in the lean season, leaves comprise up to 90% of the diet (Irwin, 2008a).

2.3 | Data collection

Data were collected using all-day focal animals follows (Altmann, 1974), simultaneously targeting the adult male and adult female of four study groups: two groups in Ankadivory (CONT4, CONT5) and two groups in Mahatsinjo (FRAG4, FRAG5; Figure 1). Ankadivory exhibits a relatively intact continuous forest landscape while Mahatsinjo is a fragmented forest landscape (Irwin et al., 2010). Data collection took place during four two-week cycles from June to August 2018.

Feeding behavior was recorded using continuous sampling. We recorded (1) feeding times, start and stop, to the nearest second;

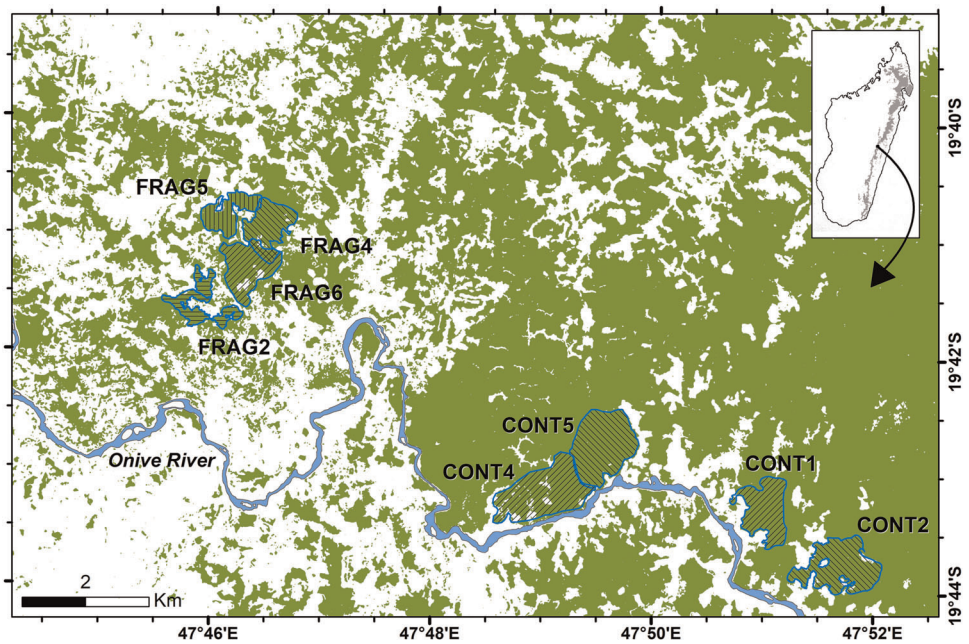

FIGURE 1 Map of Tsinjoarivo forests including *Propithecus diadema* study group home ranges at Mahatsinjo (FRAG2, 4, 5, 6), Vatateza (CONT1, 2), and Ankadivory (CONT4, 5)

(2) plant part, and (3) plant species. A feeding bout began when a food item entered an animal's mouth and ended when the animal (1) stopped consuming the item for more than 10 s, (2) began feeding on a new item, or (3) moved onto a new activity.

2.4 | Sample collection

Food samples were collected for the representative top five foods (determined by duration of time spent feeding) of all individuals studied. Foods were collected from both sites; a single sample was used for all individuals at a site, but if that food was represented in both sites' top foods, separate samples were collected from each site. After collection, the plants were processed to retain only parts consumed by the sifakas, weighed, dried using a Nesco electric dehydrator at 41°C (105°F), placed in a Ziploc bag with desiccant, and stored in the dark. Drying took approximately 12–48 H; we weighed a sample every 2–4 H and considered it to be dry when the sample's weight stabilized. In total we collected 15 samples, comprising 10 distinct species.

2.5 | Lab methods

Samples were analyzed at Northern Illinois University following established techniques (Rothman et al., 2012). Before analysis samples were milled to a uniform particle size using a Thomas Wiley® Mill with a 1 mm screen. Four main analyses were performed: acid butanol assay to screen for tannins ($n = 14$), purification through Sephadex ($n = 11$), quantification of tannins levels using the acid butanol assay with the internal standard ($n = 11$) and in vitro nitrogen assay ("Avail N"; $n = 15$, two plants were duplicates from the same forest: young leaves *Pittosporum verticillatum*).

The acid butanol assay is a spectrophotometric assay that estimates the soluble CT content within a plant extract (Porter et al., 1986; Rothman et al., 2009). This assay was used to screen all foods ($n = 14$), following Rothman et al. (2009). Briefly, samples were weighed to approximately 0.2 g, immersed in 10 ml 70% (vol/vol) aqueous acetone, sonicated in ice water for 20 min, centrifuged for 10 min, and then the supernatant was extracted and stored. This process was repeated three times with the supernatants combined to 30 ml of extract (representing 6.67 mg/ml crude plant material). Following this, 100 μl of sample extract was mixed with 600 μl of 5% HCL in n-butanol and 20 μl of 2% $FeNH_4(SO_4)$ in triplicate. Of the three tubes per sample, two were then heated for 50 min at 90°C, while one was left at room temperature to control for plant pigment unrelated to tannins. A spectrophotometer (Thermo Scientific Spectrophotometer Genesys 10 S UV-VIS) then measured the red anthocyanidin products of the oxidative polymerization of the proanthocyanidin (CTs) at 550 nm. The red color is produced when the interflavan bond is broken, therefore the intensity of color produced by the butanol-HCL reactions is due to the anthocyanidin products released. But as discussed above, the heterogeneity of the CT present in the plant extract challenges the use of this assay, hence the need for a standard curve of six different standards (Gould et al., 2009; Rothman et al., 2009). Absorbance was calculated as ([{ABS A + ABS B}/2] − ABS CONTROL). Two duplicates of each sample were run to help determine if any laboratory error occurred. If the sample absorbance was greater than 0.100, the sample was considered to contain CTs as described in Rothman et al. (2006).

Sephadex column purification was used to produce pure tannin extracts for the samples that screened positive ($n = 11$). This extract was then used as an internal standard to calibrate the relationship between observed color change and tannin concentration within the plant tissue. This was necessary because tannins are a diverse group of molecules that vary from species to species, and in the color change produced per quantity of tannin present (Rothman et al., 2009)

Each sample was weighed out to 2.0 g and suspended in 40 ml 70% (vol/vol) aqueous acetone, sonicated in ice water for 20 min, centrifuged (2500*g*) for 10 min, and then the supernatants were extracted and stored. This process was repeated three times and the supernatants were combined to reach 120 ml of extract (representing 16.67 mg/ml of crude plant material). The acetone was then evaporated leaving approximately 40 ml of the solution, this residue was then redissolved in 95% ethanol (aqueous) and applied to a slurry of 33 g of equilibrated Sephadex LH-20 (Sigma-Aldrich) in a fritted glass funnel. This column allowed the solvent to flow through, to be collected underneath in a flask; 95% ethanol was applied to the slurry until the absorbance of the eluant at 280 nm was close to zero (indicating non-tannin material was fully washed through the Sephadex).

Next, 70% (vol/vol) acetone was applied to remove the brown bands of tannins remaining in the Sephadex. This second eluant (tannin extract) was collected in a flask and left to evaporate. Following this, the extract was frozen at −50°C, lyophilized, weighed and stored in a desiccator. Each sample was then used to create a solution (0.5 mg of tannin in 2 ml of 70% acetone); this solution was used to create a 10-point standard curve (serial dilution from 0.025 to 0.25 mg/ml) for each food, which was measured using the acid butanol assay (see Rothman et al., 2009). A regression equation was calculated in the form of $y = mx + b$ (y = absorbance, m = slope, x = tannin concentration, $b = y$ intercept); since each plant has as different standard curve, the regression equation was unique to each plant extract. The CT concentration was determined using the absorbance concentration of tannins in the extract (solving for x), then dividing the tannin concentrations determined from this acid butanol assay ("x") by the total amount of plant material present in the plant extract.

Finally, an in vitro available nitrogen assay ("AvailN") was used to quantify the protein available to the sifaka with and without the effect of tannins (DeGabriel et al., 2008; Marsh et al., 2003; Marsh et al., 2006; Wallis et al., 2012). This assay simulates digestion of plant material and measures "available protein" using the loss in protein in the digested sample. The specific effect of tannins can be tested using polyethylene glycol (PEG), a nonabsorbable tannin-

blocking agent that binds to the tannin and prevents the tannin from binding to the protein allowing the protein to be digested.

Samples were first analyzed for protein by determining crude protein percent (CP; nitrogen content multiplied by 6.25 conversion factor) using a Leco FP- 828 P combustion analyzer. We simultaneously determined the quantity of dry matter (DM) in each sample, to account for the amount of moisture present in the sample.

Second, ground samples were weighed (0.8050 + 0.0050 g) into an Ankom fiber filter bags (F57; four bags per sample). We ran each sample in duplicate for the non-PEG and PEG treatment, making a total of four bags for each sample. Two of these bags were placed in 3000 ml jars with 33.33 g/L of PEG 4000 and 0.05 M Tris-BASE buffer, and the other two were placed with 0.05 M Tris-BASE buffer. Beakers were then incubated at 37°C for 24 h in a Daisy incubator (Ankom), and samples were subsequently rinsed thoroughly with hot tap water and distilled water (DeGabriel et al., 2008; Wallis et al., 2012)

Samples were then placed back into four jars, with 70 ml per sample of cellulase, sodium acetate and glacial acetic acid solution added to simulate fiber digestion, incubated at 37°C for 48 h, and then rinsed. This process was repeated but with 70 ml per sample of 2.00 g of 1:1000 pepsin in 1 L of 0.1 N hydrochloric acid (pH 1.0), to simulate protein digestion, samples were then incubated for 24 h and then rinsed. Samples were then oven-dried in an oven at 50°C for 1–2 h until samples were dry (DeGabriel et al., 2008; Wallis et al., 2012) and transferred to a desiccator.

Next, each sample's postdigestion weight was recorded (bag weight subtracted from the sample plus bag weight) and the nitrogen content for each sample was determined (Leco) and converted into protein concentration (6.25 conversion factor). The actual percent of residual protein was then determined by dividing the residual protein by the DM determination (RP%/DM).

Next, we determined percent protein digested and available protein (%CP digested). Degree of protein digestion was determined as:

$$\%\text{Protein digested} = 100\left(\frac{\text{Protein predigestion-Protein postdigestion}}{\text{Protein before digestion}}\right)$$

Available protein was calculated as:

$$\text{Available protein}(\%) = \left(\frac{\%\text{Protein digested} \times \text{DM adjusted CP}}{100}\right)$$

This available protein was calculated separately for the non-PEG treatment (biologically realistic assay) and the PEG treatment (with action of tannins blocked). For each treatment, the value was an average of the two sample bags. The PEG available protein was subtracted from the non-PEG available protein to determine how much protein was bound by tannins. "CT relative efficiency" was quantified by dividing the % protein effectively bound by tannins (i.e., percent reduction in non-PEG available protein relative to PEG available protein) by the concentration of CTs within the sample (in other words, the degree of action of the tannins weighted by their concentration). We recognize that other factors may affect protein absorption (such HTs that were not accounted for in the acid butanol analysis), but this variable should largely express the variability in the effectiveness of tannins in different plant species. Finally, the tannin content in the overall diet for each habitat (CONT and FRAG) was estimated using the weighted average of the CT concentrations in the foods sampled. This was calculated as: Average CT concentration = $(\Sigma(\text{CT concentration})_i \times (\text{Proportion of feeding time})_i)/\Sigma(\text{Proportion of feeding time})_i$, where "i" is the food ($n = 6$ for FRAG, $n = 8$ for CONT). This assumes that the rarely-used foods we did not sample were similar in CT concentration to the ones we did sample.

3 | RESULTS

3.1 | Top foods

The sifaka diet was characterized by young leaves (46.52% of feeding time) and flower buds (38.57%), fruits and seeds (13.24%), mature leaves (1.15%), petioles (0.37%), soil (0.13%), gall (0.01%), and distal growing stems (0.01%; Figure 2).

The top five foods of the eight focal individuals included 14 foods (plant part/species combination; Tables 1 and 2). All groups and sexes shared the top food, buds of *Bakerella clavata* var 1, but the other top foods varied between sites and sexes, with certain species only consumed at one site. For example, unripe fruits and seeds of *Abrahamia ditimena* were only fed on in CONT groups, as this species is virtually absent in FRAG home ranges, while other foods within one site's top foods made up a tiny proportion of the other site's diet.

3.2 | CT concentrations

Eleven of the fourteen top foods were positive for CTs (Table 3). Of the feeding time on analyzed foods, the vast majority was on CT-positive foods (95% in fragmented forests and 92% in continuous forests). The young leaves of *Symphonia microphylla* exhibited the highest CT concentration (39.3% DM). The most-eaten food, buds of *Bakerella clavata* var 1, exhibited 10.1% CTs. Only three top foods lacked CTs (cf. *Clerodendrum* sp., *Pittosporum verticillatum*, and *Solanum mauritianum*). All three made up small proportions of the diet (3.4%, 4.1%, and 4.7%).

When present, CT concentration in plant tissues varied from 4.8% to 39.3% (DM). The CT concentration in plant tissues of particular species also varied by site; for example, the young leaves of *Bakerella clavata* var 2 exhibited 19.0% tannin concentration in continuous forests and 7.7% tannin concentration in fragmented forests. The CT concentration in plant tissues of species also varied within sites; for example, the young leaves of *Embelia concinna* (two samples were collected to meet sample size for laboratory analysis) of one plant sample was 4.8% CT concentration while another plant sample was 19.8% CT concentration.

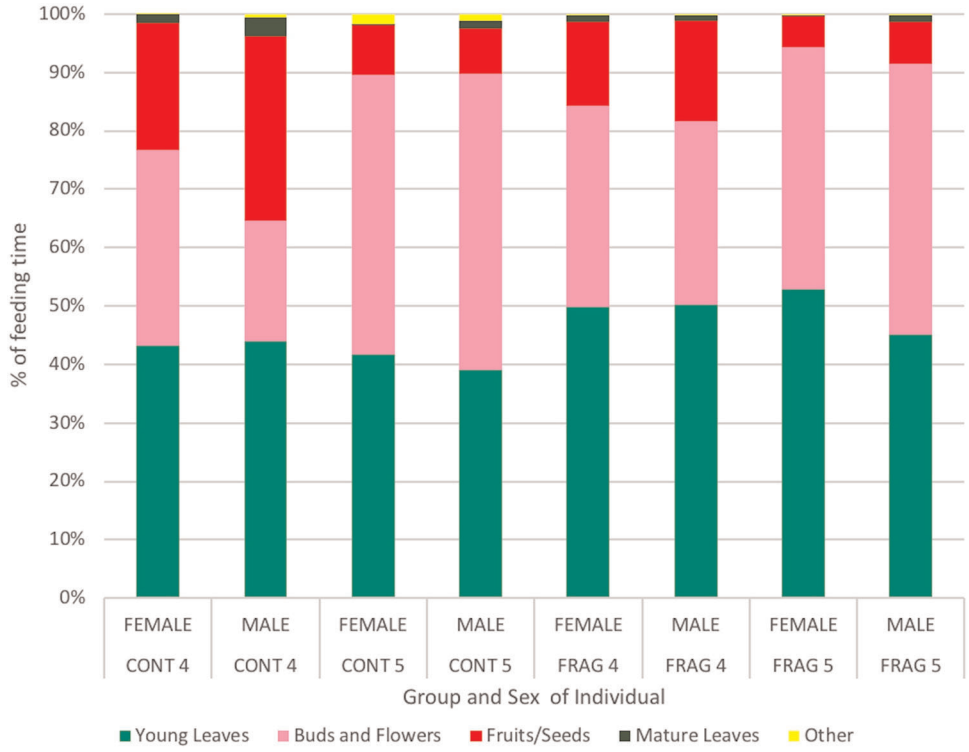

FIGURE 2 Plant parts consumed by *P. diadema* across groups and sex

Further, different parts of the same plant varied in CT concentrations: young leaves of *Pittosporum verticillatum* were negative for CTs while unripe fruits with seeds were positive for CTs (10.4%) as well as the buds and young leaves of *Bakerella clavata* var 1 (10.1% vs. 8.6%).

Though we did not compare these values statistically, both CONT and FRAG foods exhibited similar CT concentrations, although the highest values occurred in CONT groups (Figure 3). The weighted average per site of percent CTs in plant tissues for CONT groups was 11.3% and FRAG groups was 9.6%. This is largely due to the reliance of CONT groups on the young leaves of *Symphonia microphylla* (3.8% of feeding time, CT concentration = 39.3%). Further, CT concentration in young leaves of *Bakerella clavata* var 2 varied between continuous forest and fragmented forests (19.0% vs. 7.7%).

3.3 | Available protein and CT effectiveness

In terms of available protein, 6 of the 11 foods positive for CTs were 100% effective in binding to proteins (Table 4). In other words, the amount of protein digested from sample bags when the tannin-binding PEG was absent was 0%, indicating that sifakas derived no protein from that food. The protein bound up in the other five foods varied from 0.0% to 62.0%.

"CT relative efficiency" (protein reduction relative to CT concentrations) in the food, varied from 0.0483 to 0.9769 (Table 4). For the latter (*Embelia concinna* young leaves), the CT concentration was 4.8% and the concentration of protein bound was 4.7%; in other words, each gram of tannin bound almost its own weight in protein. There was considerable variation; some foods had low CT concentrations but were highly effective in binding to protein, while others had high CT concentrations but were relatively ineffective (e.g., *Symphonia microphylla*).

The buds of *Bakerella clavata* var 1 showed 8.3% crude protein; however, they exhibited 0.0% available protein for both PEG and non-PEG, suggesting something other than tannins is binding protein. There is also evidence of intraspecific variation: *Embelia concinna* was a top food for both FRAG groups; however, one sample showed 0.9769 CT efficiency and the other was 0.3200.

Overall, most top foods only exhibited low levels of available protein. The top food common to all study animals had 0% available protein, as did all second-ranked foods in CONT groups. CONT4's top foods exhibited extremely low available protein when compared to foods eaten by CONT5. Only one of the top five foods eaten in CONT4 exhibited available protein (*Pittosporum verticillatum* unripe fruit with seeds, 1.7%). Top foods from the CONT5 diet exhibited slightly more available protein; both the male and female consumed young leaves of cf. *Clerodendrum* sp. (4.1% available protein), and the female also ate the young leaves of *Gouania* cf. *mauritiana* (17.1% available protein) in her top foods, but the male did not. The top five foods in FRAG groups had only one food high in available protein: both FRAG4 animals consumed ripe fruits with seeds of *Solanum mauritianum* (14.1%). Additionally, three animals consumed *Embelia concinna* young leaves (2.8%), both FRAG5 animals consumed young leaves of *Pittosporum verticillatum* (2.1%) and the FRAG5 female consumed young leaves of *Schefflera vantsilana* (1.4%).

TABLE 1 Top five foods in diet of *P. diadema* males and females of Ankadivory

CONT4 female	% of diet	CONT4 male	% of diet	CONT5 female	% of diet	CONT5 male	% of diet
Bakerella clavata var 1 (BD)	32.14%	*Bakerella clavata* var 1 (BD)	19.56%	*Bakerella clavata* var 1 (BD)	34.86%	*Bakerella clavata* var 1 (BD)	42.54%
Bakerella clavata var 1 (YL)	6.65%	*Symphonia microphylla* (YL)	7.52%	*Bakerella clavata* var 1 (YL)	9.82%	*Bakerella clavata* var 1 (YL)	7.95%
Symphonia microphylla (YL)	6.12%	*Pittosporum verticillatum* (URFSD)	6.97%	*Bakerella clavata* var 1 (FL)	7.43%	cf. *Clerodendrum* sp. (YL)	6.22%
Abrahamia ditimena (URSD)	5.39%	*Abrahamia ditimena* (URSD)	6.67%	cf. *Clerodendrum* sp. (YL)	4.92%	*Bakerella clavata* var 2 (YL)	6.14%
Pittosporum verticillatum (URFSD)	4.68%	*Bakerella clavata* var 1 (YL)	4.77%	*Gouania* cf. *mauritiana* (YL)	4.75%	*Bakerella clavata* var 1 (FL)	5.38%
Total	54.98%	Total	45.49%	Total	61.78%	Total	68.23%

Abbreviations: BD, flower buds; FL, flowers; ML, mature leaves; URFSD, unripe fruits and seed; URSD, unripe seeds; YL, young leaves.

TABLE 2 Top five foods in diet of *P. diadema* males and females of Mahatsinjo

FRAG4 female	% of diet	FRAG4 male	% of diet	FRAG5 female	% of diet	FRAG5 male	% of diet
Bakerella clavata var 1 (BD)	31.74%	*Bakerella clavata* var 1 (BD)	22.61%	*Bakerella clavata* var 1 (BD)	34.84%	*Bakerella clavata* var 1 (BD)	37.14%
Embelia concinna (YL)	14.17%	*Embelia concinna* (YL)	14.03%	*Embelia concinna* (YL)	13.32%	*Bakerella clavata* var 2 (YL)	10.92%
Bakerella clavata var 2 (YL)	10.05%	*Bakerella clavata* var 2 (YL)	11.18%	*Bakerella clavata* var 2 (YL)	11.80%	*Embelia concinna* (YL)	8.96%
Allophylus pinnatus (YL)	4.97%	*Solanum mauritianum* (RFSD)	9.77%	*Pittosporum verticillatum* (YL)	6.58%	*Pittosporum verticillatum* (YL)	4.93%
Solanum mauritianum (RFSD)	4.17%	*Bakerella clavata* var 1 (FL)	5.09%	*Schefflera vantsilana* (YL)	6.47%	*Bakerella clavata* var 1 (FL)	4.02%
Total	65.10%	Total	62.68%	Total	73.01%	Total	65.97%

Abbreviations: BD, flower buds; FL, flowers; ML, mature leaves; URFSD, unripe fruits and seed; YL, young leaves.

TABLE 3 Condensed tannins (CT) in top foods consumed by *P. diadema* in both fragmented and continuous forests sites

Species	Part	Site	CT	% in diet (average of all groups)	Regression equation of standard curve	R^2	CT concentration of extract (mg/ml)	% CT in plant tissue (% DM)	Rank
Symphonia microphylla	YL	CONT	+	3.76%	y = 1.963x + 0.0734	0.969	0.3370	39.3%	1
Schefflera vantsilana	YL	FRAG	+	3.77%	y = 2.7908x + 0.0245	0.965	0.3456	21.9%	2
Embelia concinna	YL	FRAG (A)[a]	+	12.52%	y = 3.0145x + 0.0737	0.932	0.1544	19.8%	3
Bakerella clavata var 2	YL	CONT	+	3.58%	y = 0.5785x + 0.0135	0.963	1.2809	19.0%	4
Pittosporum verticillatum	URFSD	CONT	+	2.59%	y = 3.9785x + 0.1157	0.975	0.1887	10.4%	5
Bakerella clavata var 1[b]	BD	CONT	+	32.25%	y = 2.4756x + 0.0723	0.978	0.3198	10.1%	6
Gouania cf. *mauritiana*	YL	CONT	+	3.02%	y = 5.6308x + 0.0385	0.960	0.0638	8.7%	7
Bakerella clavata var 1	YL	CONT	+	7.55%	y = 1.4095x + 0.0628	0.971	0.5805	8.6%	8
Abrahamia ditimena	URSD	CONT	+	2.84%	y = 1.224x + 0.0198	0.953	0.5770	8.6%	9
Bakerella clavata var 2	YL	FRAG	+	11.03%	y = 1.2594x + 0.0735	0.949	0.5221	7.7%	10
Embelia concinna	YL	FRAG (B)[a]	+	12.52%	y = 9.7394x + 0.1938	0.924	0.0513	4.8%	11
cf. *Clerodendrum* sp.	YL	CONT	-	4.74%					
Pittosporum verticillatum	YL	FRAG	-	4.10%					
Solanum mauritianum[c]	RFSD	FRAG	-	3.37%					

Abbreviation: DM, dry matter.

[a]Two samples (A/B) were collected in fragmented forest to meet sample size necessary for laboratory analysis.

[b]Sample was collected from both fragmented and continuous sites; samples from FRAG sites were moldy, so CONT samples were used as a proxy.

[c]Sample was collected from CONT forests and used as proxy for FRAG forests (FRAG forest samples were moldy).

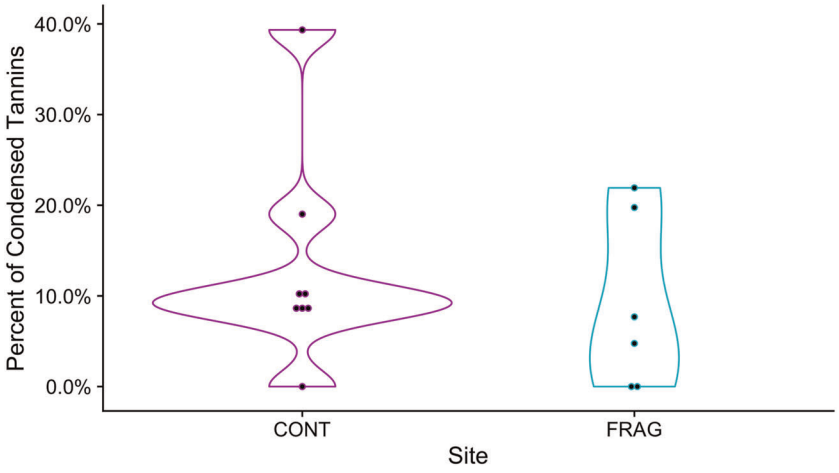

FIGURE 3 Violin plot showing the percent condensed tannins within top foods both negative and positive for condensed tannins within both continuous and fragmented forest sites

4 | DISCUSSION

4.1 | CTs and protein in the diademed sifaka diet

This study confirms that diademed sifakas consume tannin-rich foods, with foods varying from 0% to 39.3% CT (concentration by mass). Most of the top foods in both sites were positive for CTs, which mirrors other studies that have detected CTs in primate diets (Carrai et al., 2003; Davies et al., 1988; Ganzhorn et al., 1985; Ganzhorn, 1988; Glander, 1982; Gould et al., 2009; Leighton, 1993; Milton, 1979; Norscia et al., 2006; Oates et al., 1977; Remis & Kerr, 2002; Reynolds et al., 1998; Simmen et al., 1999; Wrangham & Waterman, 1983). However, further studies are necessary in terms of food selection (foods vs. non-foods) before determining if diademed sifakas simply tolerate high-tannin foods indiscriminately, or if foods they are selecting are lower in tannins relative to unselected foods. Further, it is important to point out that tannins are only one of several PSMs that may impact feeding behavior. Therefore, further studies are necessary to consider other PSMs, their interactions with tannins and their impact on feeding.

Species that were negative for CTs still exhibited some differences in available protein with PEG and available protein without PEG. This could be due to the actions of other tannins (e.g., gallotannins and ellagitannins) that are also able to bind to proteins that are not detected by the acid butanol method (non-CT tannins; Karonen et al., 2019; Salminen & Karonen, 2011). Overall our study supports the call by Marsh et al. (2020) for looking at total tannins rather than a singular class of tannins. Further, several species contained high crude protein content (18.5%, 20.4%, 25.7% of DM) but with far lower available protein (both with and without PEG), suggesting factors other than tannins result in decreased protein availability, such as the binding of protein to cell walls; further studies should consider these effects. This occurred with the sifakas' top food, *Bakerella clavata* var 1 buds: despite 8.3% crude protein, this food exhibited 0% available protein with and without tannins being able to act.

Overall, the two forest types (CONT and FRAG) were similar in terms of CT concentration, prevalence, and relative efficiency of the CTs within the diet; most variation among foods occurs within sites rather than between them. However, this is likely due to the convergence of diet that occurs during the lean season. Irwin et al. (2014) found that CONT and FRAG groups both shift to leaves and flowers in the lean season and increase reliance on mistletoe, but their abundant season diets diverge in terms of species consumed. Therefore, future studies should include the abundant season, when CONT groups eat high-quality foods (fruits) while FRAG groups shift to eating fruits but from different species, and maintain high levels of *Bakerella clavata* in their diet (Irwin, 2008a). Although we expect that tannins will be less important in the abundant season diet, because of the shift to rely on fruits, it is possible that CONT and FRAG abundant season foods vary meaningfully in tannin concentration and effectiveness.

It is also interesting to note that plant parts of the same species differed in concentration and tannin efficiency (e.g., the young leaves of *Embelia concinna*). This is corroborated by other studies that found differing CT concentrations within plants, across populations and temporally, further substantiating the suggestion that plant defenses can differ within and among populations of the same species (Forkner et al., 2004; Moore & DeGabriel, 2012). Therefore, the results presented here should be taken with caution since several foods were collected from plants not directly fed on by the sifakas, largely due to the need to collect necessary sample size for lab analyses, as well as the fact that sifakas typically use small patches, especially in the lean season. Therefore, further studies should consider sampling more widely within each food type.

4.2 | Sifaka diet choice and strategy in a comparative context

During the lean season, fruit availability is reduced and sifakas switch from consuming fruit to consuming young leaves, buds, and flowers, with a nearly 40% drop in overall mass ingested when compared to

TABLE 4 Tannin effectiveness of plant species found within both continuous and fragmented forests

Species	Plant part	Site	% CT	% Crude protein/DM	Avail protein with PEG	Avail protein (no PEG)	Amount of protein bound	% of protein concentration bound	CT relative efficiency
Embelia concinna	YL	FRAG	4.8%	11.0%	7.5%	2.8%	4.7%	62.3%	0.9769
Bakerella clavata var 2	YL	FRAG	7.7%	10.0%	6.3%	0.0%	6.3%	100.0%	0.8218
Bakerella clavata var 1	YL	CONT	8.6%	7.9%	3.1%	0.0%	3.1%	100.0%	0.3600
Embelia concinna	YL	FRAG	19.8%	9.8%	6.3%	0.0%	6.3%	100.0%	0.3200
Bakerella clavata var 2	YL	CONT	19.0%	10.2%	5.6%	0.0%	5.6%	100.0%	0.2923
Pittosporum verticillatum	URFSD	CONT	10.4%	7.2%	3.9%	1.7%	2.2%	55.7%	0.2075
Abrahamia ditimena	URSD	CONT	8.6%	4.6%	1.1%	0.0%	1.1%	100.0%	0.1241
Schefflera vantsilana	YL	FRAG	21.9%	7.6%	3.7%	1.4%	2.3%	61.5%	0.1026
Gouania cf. *mauritiana*	YL	CONT	8.7%	25.7%	17.7%	17.1%	0.6%	3.2%	0.0641
Symphonia microphylla	YL	CONT	39.3%	7.0%	1.9%	0.0%	1.9%	100.0%	0.0483
Bakerella clavata var 1	BD	CONT	10.1%	8.3%	0.0%	0.0%	0.0%	0.0%	0.0000
Solanum mauritianum	RFSD	FRAG	0%	18.5%	14.5%	14.1%	0.4%	2.8%	
cf. *Clerodendrum* sp.	YL	CONT	0%	20.4%	9.3%	4.1%	5.2%	55.9%	
[a]*Pittosporum verticillatum*	YL	FRAG	0%	9.6%	5.6%	2.1%	3.5%	63.0%	
	YL	FRAG	0%	8.3%	4.2%	2.8%	1.4%	32.4%	

Abbreviations: CT, condensed tannin; DM, dry matter; PEG, polyethylene glycol.

[a]Two samples were collected in fragmented forest to meet sample size necessary for laboratory analysis.

abundant-season intake (Irwin et al., 2015; Irwin, 2008a, 2008b). Irwin et al. (2015) suggested sifakas exhibit this strategy in response to intrinsic plant characteristics. Our study supports this hypothesis, as diets documented here are largely protein-deficient, perhaps reflecting a strategy of reducing activity and food intake to help in tolerating inferior foods in the short term. In other words, if foods that can be easily found in the lean season are not delivering protein, it's better not to expend energy searching for them.

Strikingly, sifakas appear to be consuming food items with little available protein, perhaps to obtain non-protein macronutrient energy (or because of an adaptation to tannins, such as tannin binding salivary proteins), while obtaining protein from a small number of foods negative for CTs or with very little tannin effectiveness. Only two foods sampled were protein-rich, the young leaves of *Gouania* cf. *mauritiana* (available protein: 17.1%) and the ripe fruit and seeds of *Solanum mauritianum* (available protein: 14.1%). The young leaves of *Gouania* cf. *mauritiana* exhibit CTs (8.7% CT concentration) but only effectively bind 0.6% of protein and the ripe fruit and seeds of *Solanum mauritianum* were negative for tannins, allowing the sifakas to obtain high levels of protein from these foods. In total, these foods made up only small amounts of their diet (4.8%–9.8%), being either the fourth or fifth top food; neither was abundant in the landscape. Other food items exhibit varying amounts of available protein (0.0%–9.3%), with six of the top foods exhibiting little protein (including the top food, the buds of *Bakerella clavata* var 1). These findings largely contradict the existing idea that large-bodied folivores feed on leaves to gain protein necessary for survival and reproduction that is not available in fruits (Hladik, 1978); in this study the highest-protein food was a fruit and many leaves delivered very little protein. However, it is important to keep in mind that many of our methods are based on marsupial physiology and therefore may not address how Indriidae digestive physiology may be adapted to tannins in some way that allows them to persist on a high tannin diet.

The limited data available suggest that CTs may be particularly important in sifaka diets relative to other primates. For example, Mountain gorillas (*Gorilla beringei*) consume foods containing tannins, but these foods make up a small fraction of their diet (4% of foods); their staple leaves lack tannins but their commonly-eaten fruits contain tannins (Rothman et al. 2011). Rothman et al. (2011) found gorillas tended to over-ingest protein to meet their energetic needs, thereby prioritizing their non-protein energy (NPE) intake, therefore tannins may be of little concern to gorillas as they accumulate plenty of protein, and may even function as protein absorbers to void excess protein (Rothman et al., 2011). In contrast, Spider monkeys (*Ateles chamek*) appear to overeat items high in energy to meet a minimum protein intake (Felton et al., 2009); in this way they would likely avoid tannin-rich fruits to be able to maintain their protein intake while overeating NPE.

Sifakas exhibit a more variable NPE and available protein (AP strategy); rather than showing a spider monkey (NPE maximization) or gorilla strategy (AP maximization), sifakas exhibit a more balanced strategy (AP and NPE are tightly correlated and both decrease in the lean season; Irwin et al., 2015). This variable strategy is possibly driven by the detrimental effects of CTs. During the lean season, sifakas exhibit increasing dietary diversity and decreasing foraging intakes, and this may be largely driven by mitigating the impacts of CTs (Marsh et al., 2006). In this way, sifakas may be prioritizing CT management over the optimization of macronutrients, resulting in their more balanced strategy (Irwin et al., 2015). However, further research is needed to examine both seasons in terms of CT intake, and if sifakas are actively selecting high-tannin or low-tannin foods as well as the effects of tannins on other macronutrients and on micronutrients (Bryant et al., 1991; Hassan et al., 2003; Mehansho et al., 1987). Finally, it is possible that toxins (e.g., alkaloids), which have been documented in other sifaka populations (Powzyk & Mowry, 2003) but have not yet been measured at Tsinjoarivo, are limiting food intakes in the lean season (Marsh et al., 2006).

4.3 | Future directions and recommendations

Although this study has broadly shown that diademed sifakas feed on tannin-rich foods and that protein intakes are low, there are still several unanswered questions. Future studies should focus on four key areas. First, it is important to identify if the sifakas have physiological adaptations to counteract the effects of CTs, and how effective these counterstrategies are. If present and effective, the sifakas may be absorbing much more protein than suggested by the results presented here (closer to the "Available Protein with PEG" values in Table 4). Although diademed sifakas have been observed consuming soils, Semel (2015) suggested that they do not consume soil to neutralize tannins but rather they may consume soil to neutralize toxins, decrease parasites or supply minerals. However, further studies are needed to investigate the impact of soil consumption with these new methods. Second, it is important to identify interactions with other PSMs that may impact the CT biological activity as well as more holistically looking at total tannins (Marsh et al., 2020). For example, Makkar et al. (1995) found that interactions between tannins and saponins had an additive effect on decreasing rate of digestion and true digestibility. Third, additional sampling is needed; this could include expanding the temporal scope, by comparing CT concentrations and effectiveness in foods eaten in different forests during the abundant season; this will contextualize the lean season strategy by understanding the contrasts among seasons. It could also include sampling more rarely-consumed foods; although our analyses captured the majority of feeding time, it is possible that more rarely-eaten foods are qualitatively different (e.g., high protein but high in toxins). Fourth, it will be useful to determine whether CTs play a role in food selection (i.e., if sifakas avoid or prefer foods based on CTs); this will inform studies of niche separation in the wild as well as the formulation of captive diets.

It is also important to keep improving lab methods and continue evaluating whether the lab methods we are using are appropriate. Our study corroborates Rothman et al. (2009) showing clear differences between the sephadex versus the quebracho method (see Table S1), in both inferred CT concentrations and in the ordering of

sample in CT concentrations. However, future improvements should be sought. It's also important to continue improving lab methods and evaluating whether the lab methods we are using are appropriate. For example, the AvailN method was designed primarily for leaves. Sifakas may be receiving protein from pollen similar to lorikeets (*Trichoglossus haematodus* and *Glossopitta pophyrocephala*) that rely on pollen to fulfill their protein requirements for daily maintenance as well as breeding (White, 1993; Roulston & Cane, 2000). However, pollen is somewhat resistant to degradation, so sifakas may be gaining protein by feeding on the pollen of flowers and buds of *Bakerella clavata* var 1 that we have been unable to detect for methodological reasons. Specifically, the nitrogen may be detected in the crude protein analysis, but the apparently "zero" protein in both PEG and non-PEG treatments in AvailN assay may reflect the fact that the reagents used are not breaking apart pollen grains. Further research is needed to confirm if the flower buds and flowers consumed contain pollen, and if the pollen of *Bakerella clavata* supplies protein in a form accessible to sifakas.

Finally, it would be interesting to determine if sifakas exhibit an active temporal separation of high- and low-tannin foods to maximize protein absorption. The fact that only very few of the selected foods contain appreciable levels of available protein suggest that there is a risk to sifakas if these foods are consumed shortly before or after CT-rich foods (as CTs from other foods may block the breakdown of other proteins with which it mixes in the gut). If active separation is occurring, sifakas would have more temporal separation between ingestion of high tannin and low tannin foods than expected due to chance alone, "protecting" the protein in the low-CT foods. This could also help address how Indriidae may differ from marsupials (much of the literature and assays are based on these species) in terms of physiological digestive counter-adaptations. By doing so, they may be using high-CT foods for non-protein macronutrients separately from using low-CT foods for protein acquisition.

5 | CONCLUSION

The impacts of PSMs are critically important in studying primate feeding ecology; it is becoming increasingly clear that understanding macronutrients alone is not enough. Our study suggests that diademed sifakas may be consuming foods with very little available protein, in large part due to the impacts of tannins, contradicting the commonly-held assumption that leaves universally provide protein in primate diets (Hladik, 1978); sifakas may actually be selecting leaves for other macronutrients due to the lack of available protein. However, further studies are necessary to determine if sifakas have behavioral or physiological counteradaptations to mitigate the impacts of tannins, as well as if they avoid or select CT-rich foods. By including analyses of CTs, a better understanding of food selection by primates can be achieved, which will be critically important both in understanding the evolution of different feeding strategies, and effectively managing wild and captive populations.

ACKNOWLEDGMENTS

The authors are grateful to Madagascar's Ministère de l'Environnement, de l'Environnement, d'Écologie et des Forêts, and CAFF/CORE for research permission, to SADABE (especially Jean-Luc Raharison) and Université d'Antananarivo (Mention Anthropobiologie et Développement Durable) for research facilitation and collaboration, and to local communities and research assistants based at Tsinjoarivo. They thank Bruno Ramorasata for field support and guidance, and Amanda Hamrick, Chazal Vololontsoa and Zo Rijamanalina for field collaborations. They would also like to thank J. Rothman for invaluable methodological advice and W. Foley for valuable conversations on pollen. Funding was provided by Northern Illinois University, IdeaWild, Global Wildlife Conservation and Primate Conservation, Inc.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

ORCID

Emma G. Thurau http://orcid.org/0000-0001-9554-2010
Mitchell T. Irwin http://orcid.org/0000-0003-2088-0028

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How to cite this article: Thurau EG, Rahajanirina AN, Irwin MT. Condensed tannins in the diet of folivorous diademed sifakas and the gap between crude and available protein. *Am J Primatol*. 2021;e23239. https://doi.org/10.1002/ajp.23239