Estrogenic Plant Foods of Red Colobus Monkeys and Mountain Gorillas in Uganda

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ABSTRACT Phytoestrogens, or naturally occurring estrogen-mimicking compounds, are found in many human plant foods, such as soybeans (*Glycine max*) and other legumes. Because the consumption of phytoestrogens may result in both health benefits of protecting against estrogen-dependent cancers and reproductive costs of disrupting the developing endocrine system, considerable biomedical research has been focused on the physiological and behavioral effects of these compounds. Despite this interest, little is known about the occurrence of phytoestrogens in the diets of wild primates, nor their likely evolutionary importance. We investigated the prevalence of estrogenic plant foods in the diets of two folivorous primate species, the red colobus monkey (*Procolobus rufomitratus*) of Kibale National Park and mountain gorilla (*Gorilla beringei*) of Bwindi Impenetrable National Park, both in Uganda. To examine plant foods for estrogenic activity, we screened 44 plant items (species and part) comprising 78.4% of the diet of red colobus monkeys and 53 plant items comprising 85.2% of the diet of mountain gorillas using transient transfection assays. At least 10.6% of the red colobus diet and 8.8% of the gorilla diet had estrogenic activity. This was mainly the result of the red colobus eating three estrogenic staple foods and the gorillas eating one estrogenic staple food. All estrogenic plants exhibited estrogen receptor (ER) subtype selectivity, as their phytoestrogens activated ERβ, but not ERα. These results demonstrate that estrogenic plant foods are routinely consumed by two folivorous primate species. Phytoestrogens in the wild plant foods of these two species and many other wild primates may have important implications for understanding primate reproductive ecology. Am J Phys Anthropol 148:88–97, 2012.

Naturally occurring estrogenic compounds (i.e., phytoestrogens) are found in more than 300 plant species (Dixon, 2004), including a number of human plant-based foods (e.g., soy [*Glycine max*]), (Kurzer and Xu, 1997). The consumption of phytoestrogens may result in both health benefits of protecting against estrogen-dependent cancers and menopausal disorders and reproductive costs of disrupting the developing endocrine system and affecting fertility. Consequently, considerable biomedical research has been focused on understanding the physiological and behavioral effects of phytoestrogens primarily using captive rodents and primates as models (Messina, 2010; Whitten and Patisaul, 2001). Examination of the phytoestrogen–animal relationship outside the laboratory has focused largely on domesticated livestock. For example, an estrogen mimic found in an introduced species of clover, *Trifolium subterraneum*, caused extensive female infertility in domesticated sheep of western Australia (i.e., "clover disease"), (Bennetts, 1946; Adams, 1990, 1995). Despite strong interest in the influence of phytoestrogens on human and livestock health and fertility, little is known about the ecological or evolutionary implications of feeding on estrogenic plants for wild animals (Wynne-Edwards, 2001). This is especially true for primates, which routinely take most of their diet from tropical plant foods (Milton, 1999).

Recently, field researchers have become interested in this topic, likely due to methodological advances that allow questions about the steroidal properties of plants and their effects on wild animals to be addressed using a mixed field and laboratory approach. Three recent primate field studies, leaf monkeys (*Trachypithecus phayrei*) in Thailand (Lu et al., 2010), common chimpanzees (*Pan troglodytes*) in Tanzania (Emery Thompson et al., 2008), and olive baboons (*Papio anubis*) in Nigeria (Higham et al., 2007), have examined a related class of hormone-mimicking plant compounds, phytoprogester-

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ones (i.e., naturally occurring progesterone-mimicking plant compounds). These studies suggest that consumption of plant parts from the phytoprogesterone-containing genus *Vitex* has a negative effect on female reproduction. However, these studies did not examine the steroidal activity of the plants proposed to affect reproduction. Furthermore, based on these studies it is not possible to evaluate the ecological or evolutionary implications of hormone-mimicking plants in the diets of wild primates, as these field studies focused on only the consumption and effects of a single plant species and did not concurrently collect systematic feeding data. Quantifying the relative proportion that each dietary item contributes to the overall diet within a given time frame is critical because the effects of a steroidal plant may only be seen when it is consumed above a certain threshold. Further, because wild primates often consume many plant food items each day, and since more than one of these items may contain phytosteroids (e.g., phytoestrogens, phytoprogesterones), it is important to examine a large proportion of the diet for hormonal activity to gain an accurate assessment of the influence of these compounds on wild primates. The synergistic interactions among steroidal plant compounds in different plants are likely just as important, if not more so, than the effects of just one compound (see Hayes et al., 2006 for similar issue with endocrine-disrupting pesticides). We previously showed that MF101, which is crude extract that contains 21 plants, contains multiple estrogenic compounds (Cvoro et al., 2007), and it is likely that they act synergistically to produce physiological effects, such as the prevention of hot flashes in postmenopausal women (Grady et al., 2009).

There are a number of ways that a plant compound could alter the endocrine functioning of a primate, with significant subsequent effects on reproductive physiology and behavior, and important implications for ecology and evolution through differential survival and reproduction. Phytoestrogens can disrupt the activity of endogenous estrogens by interacting with estrogen receptors (ERs), interfering with enzymes responsible for hormone metabolism (e.g., aromatase converts androgens to estrogens), or binding to the sex hormone binding globulins responsible for transporting sex hormones (i.e., estrogens and androgens) throughout the body (Whitten and Patisaul, 2001). The most well studied phytoestrogens are those that bind to ERs and compete with endogenous estrogens to promote estrogenic activity (i.e., agonists) or block it (i.e., antagonists) (Leitman et al., 2010). A specific phytoestrogen can act as an agonist or antagonist depending upon the dose of the compound consumed, the level of endogenous estrogens in the animal, and the tissue type. Therefore, determining the mechanism of action and physiological effects of ingesting these compounds in a field setting can be daunting. However, an initial screening of plant food items for compounds that bind to one of the two ERs (ERα and ERβ) and alter the activity of estrogen dependent genes is a very useful starting point. Based on what is known about phytoestrogens in human plant foods (e.g., genistein and daidzein), it is likely that the wild plant foods of primates containing compounds that bind to and activate the ERs will have important effects on primate physiology and behavior through their competition with endogenous estrogens. Further, based upon studies of humans, captive primates, and rodents that have shown various phytoestrogens to circulate in the blood after ingestion, it can be assumed that phytoestrogens in wild plant foods are bioavailable to wild primates feeding on them (Adlercreutz et al., 1986; Sfakianos et al., 1997; Watanabe et al., 1998). This claim can be further validated by screening blood samples from a given primate species for circulating phytoestrogens, but this was beyond the scope of this study.

The objective of this study was to determine the prevalence of phytoestrogens (defined here as compounds that bind to and promote estrogenic activity through the ERs) in the diets of two folivorous African primates (red colobus monkey [*Procolobus rufomitratus*] of Kibale National Park and mountain gorilla [*Gorilla beringei*] of Bwindi Impenetrable National Park, both in Uganda). Because we know little about the presence of such compounds in the plant foods of primates, these two species, one ape and one monkey, provide a new window into their prevalence in the diets of folivorous catarrhines.

METHODS

Study sites and species

Kibale National Park and red colobus monkey. Kibale National Park (KNP; 795 km^2), a mid-altitude, moist evergreen forest in western Uganda (0 13′–0 41′ N and 30 19′–30 32′ E) located in the foothills of the Rwenzori Mountains, is home to the highest recorded biomass of primates in the world with 13 species represented (Chapman and Lambert, 2000). One of these species, and the one comprising most of this biomass, is the Ugandan red colobus monkey (*Procolobus rufomitratus*), (Struhsaker, 1997). The red colobus is a forestomach-fermenting obligate folivore that lives in multimale–multifemale groups with an average group size of 65 individuals (Snaith et al., 2008). On average, males weigh 9.8 kg (n = 9) and females weigh 7.9 kg (n = 14; Chapman and Goldberg, unpublished data). *Procolobus rufomitratus* is considered vulnerable, with the Kibale red colobus likely the only remaining viable population of its subspecies (i.e., *tephrosceles*), (Struhsaker, 2005). As a morphologically specialized folivorous primate dependent upon its symbiotic gut bacteria (Bauchop and Martucci, 1968; Milton, 1980; Lambert, 1998), they are an ideal study species for examining the presence of phytoestrogens in the diet of wild primates. If the "plant defense hypothesis," which suggests that plants produce phytoestrogens as a defense against mammalian herbivory (Hughes, 1988; Harborne, 1993; Wynne-Edwards, 2001), has merit, then phytoestrogens would most likely occur in a colobine's diet of leaves and seeds (Milton, 1998; Chapman et al., 2002) since these parts are most vital to a plant's energy production and reproduction. Further, phytoestrogen defense would be an appropriate strategy for colobine food plants because these compounds are often more active after bacterial metabolism (Gultekin and Yildiz, 2006; Setchell and Clerici, 2010), while many other plant toxins are likely detoxified by their gut bacteria (Milton, 1998).

Bwindi Impenetrable National Park and mountain gorilla. Bwindi Impenetrable National Park (BINP; 330 km^2), another closed-canopy forest of western Uganda (0 53′–1 08′ S and 29 35′–29 50′ E), is home to one the last remaining populations of mountain gorillas (*Gorilla beringei*), with ~302 individuals (Guschanski et al., 2009). The mountain gorilla is a much larger primate than the red colobus (adult females ~100 kg, adult

males ~200 kg), and consequently spends most of its time on the ground (Rothman et al., 2008). Its body size is relevant to understanding its dietary niche, as gorillas do not have particularly strong morphological specializations to diet similar to those of colobines. Rather, large body size allows gorillas to be opportunistically frugivorous, with a diverse diet of leaves, bark, pith, stems, and fruit (Rothman et al., 2006a), and also to depend heavily on folivorous plant material during periods of fruit scarcity to meet their energetic needs (Rothman et al., 2008, 2011). As a caeco-colic fermenting folivorous ape that specializes on herbaceous vegetation (Lambert, 1998; Rothman et al., 2007), they may likewise face a "phytoestrogen defense" from their plant foods. Examining the presence of phytoestrogens in the mountain gorilla diet provides initial insight into the importance of digestive morphology (i.e., forestomach vs. caeco-colic fermenter), forest strata (tree vs. herbaceous vegetation), and phylogeny (monkey vs. ape) to phytoestrogen exposure in the folivorous primate diet.

Assessment of primate diet

To determine the diet of red colobus, behavioral data were collected on one group of monkeys (group size ~70 individuals) located near the Makerere Biological Field Station (Kanyawara) in KNP from August 13, 2007 to June 27, 2008 (258 days of sampling), for a total of 1327 h. To determine the annual diet of the mountain gorillas, behavioral data were collected on one group over a period of 319 days in 2002–2003 for a total of 1318 h (Rothman et al., 2008, 2007).

For red colobus, data were collected 6 days per week from 0800 to 1300 h using scan samples of five individuals every 30 min. When feeding, the plant species and parts being consumed were identified. We first calculated the percent of diet for each item at the weekly level by summing the number of observations of feeding on each plant item, regardless of time spent feeding on that item, and dividing this by the total number of feeding observations for the entire week. The mean of these weekly percent values (n = 45 weeks) was then calculated and used as the percent of total diet for each particular plant item. Thus, the mean percent time feeding on a particular plant item is used as a relative index of the importance of that food item in the diet. See Rothman et al. (2008, 2007) for detailed description of behavioral data collection and determination of diet for mountain gorillas.

Assessment of plant estrogenic activity

To examine the prevalence of phytoestrogens in the diets of red colobus and mountain gorillas, samples of their plant foods were collected, processed, and screened for estrogenic activity. Dietary items of gorillas were collected in 2002–2003, stored at Cornell University, and shipped in 2008 to University of California-Berkeley (UCB) for determination of estrogenic activity (see Rothman et al., 2008, 2007 for plant collection protocol for gorillas). For the red colobus, dietary items were collected using a tree-pruning pole or the skills of a trained tree-climber in 2007–2008. Plant items were collected fresh in the same stages of development as the primate ingested and dried using either a food dehydrator at low temperature or at ambient temperature out of direct sunlight. Dried plant material was stored in sealed plastic bags until transported to UCB for assessment of estrogenic activity via transient transfection assays. Although there was a fairly long lag time between the collection of plant samples and analyses for estrogenic activity, years of storage do not considerably reduce total phytoestrogen content (Lee et al., 2003). Further, as we were only interested in the presence or absence of estrogenic activity, any changes in phytoestrogen content over time were not likely to alter the results of our transfection assays.

Once at UCB, plant samples were stored in a refrigerator (4°C) until ground (0.85 mm mesh screen, Wiley Mill). Ground samples were then stored in a refrigerator (4°C) until analyzed. For analysis, 10 g of each sample were mixed with 100 ml HPLC grade methanol. The plant-methanol solution sat for 3 days at ambient out of direct light, allowing time for potentially estrogenic compounds to dissolve into the methanol. Then, the supernatant with potential estrogenic compounds was separated from the plant material using drip filtration and Whatman #1 filter paper (125 mm). The methanol was evaporated off using a rotary evaporator and the plant extract was redissolved in dimethyl sulfoxide (DMSO) at concentration of 0.1 g per 1 ml. For the plant extract to be at a concentration that was not toxic to the human osteosarcoma cells (U2OS) used in the transient transfection assays, the 0.1 g plant extract per 1 ml DMSO solution was diluted 1:10 in 100% ethanol. This solution was stored in a 4°C refrigerator until screened in the transient transfection assays.

Two different transient transfection assays were run to determine activity at both estrogen receptors (ER): ERα and ERβ (see Vivar et al., 2010 for details of transient transfection methodology). U2OS cells were cultured, collected, transferred to a cuvette, mixed with 5 μg of ERE-tk-Luc (estrogen response element [ERE] linked to luciferase gene) and 3 μg of an ERα or ERβ expression vector, and electroporated with a gene pulser to incorporate the ERE and ER into the cells. We then added either 1.5 μl of each plant extract in DMSO per 100% ethanol solution, 10 nM estradiol (final concentration; positive control), or nothing (blank control) to the transfected cells in triplicate and allowed the cells to incubate overnight. After 18 h the cells were lysed and the amount of light emitted (relative light units [RLU]) was measured using a luminometer. To determine if a plant extract had estrogenic activity, the mean RLU of the sample run in triplicate was compared to the mean RLU of the positive control and blank, also run in triplicate. This assay allows for determination of estrogenic activity based upon the product of the luciferase gene, a gene found in fireflies and marine copepods that is responsible for their bioluminescence. In the transfected cells, this gene is activated, thus producing light, when a compound binds to the ER and subsequently to the ERE. Thus, if a given plant extract has a compound which binds to the ER and subsequently promotes binding to ERE (i.e., a phytoestrogen), then the amount of light produced by the transfected cells approaches the amount produced by adding estradiol (i.e., an endogenous estrogen) to the cells (i.e., the positive control).

Analyses

All samples and controls were standardized for interassay comparability. To do so, the fold increase in RLU was calculated for all samples and positive controls

TABLE 1. Staple dietary items (i.e., foods comprising >1% of total diet) accounting for 79.3% of total diet of one group of red colobus monkey in Kibale National Park, Uganda, from August 2007 to June 2008, with estrogenic plants in bold

Plant species	Family	Part	% of Diet	ERβ estrogenic activity?
Newtonia buchananii	Fabaceae (Mimosoideae)	Young leaves	10.2	No
Trilepsium madagascariense	Moraceae	Young leaves	9.3	No
Prunus africana	Rosaceae	Young leaves	7.3	No
Albizia grandibracteata	Fabaceae (Mimosoideae)	Young leaves	6.1	No
Millettia dura	**Fabaceae (Papilionoideae)**	**Young leaves**	**5.1**	**Yes, all parts tested**
Acacia spp.	Fabaceae (Mimosoideae)	Young leaves	4.5	No
Dombeya kirkii	Sterculiaceae	Young leaves	4.5	No
Celtis africana	Ulmaceae	Young leaves	3.8	No
Celtis durandii	Ulmaceae	Young leaves	3.6	No
Eucalyptus grandis	**Myrtaceae**	**Bark**	**3.4**	**Yes, only part tested**
Prunus africana	Rosaceae	Mature leaves	3.1	No
Parinari excelsa	Chrysobalanaceae	Young leaves	2.7	No
Macaranga sp.	Euphorbiaceae	Young leaves	2.4	No, but yes for mature leaves
Bridelia sp.	Euphorbiaceae	Young leaves	1.7	No
Hypocreata sp.	Unknown	Young leaves	1.7	No
Ficus natalensis	**Moraceae**	**Young leaves**	**1.5**	**Yes, all parts tested**
Mestrazylon sp.	Unknown	Young leaves	1.5	Not tested
Strombosia scheffleri	Olacaceae	Young leaves	1.3	No
Prunus africana	Rosaceae	Bark	1.2	Not tested, but no for mature and young leaves
Alangium chinese	Alangiaceae	Young leaves	1.1	Not tested, but no for mature leaves
Funtumia africana	Apocynaceae	Young leaves	1.1	No
Mimusops bagshawei	Sapotaceae	Young leaves	1.1	Not tested
Urella sp.	Unknown	Young leaves	1.1	Not tested
Total			79.3%	10.0% of diet from estrogenic staples

using the mean RLU of the three triplicates divided by the mean RLU of the triplicate blanks run in their particular assay. For ERα assays (n = 3), the positive control of estradiol had a mean fold increase in luciferase activity of 33.18 (SEM = 6.55), while in ERβ assays (n = 9), the positive control had a mean fold increase of 4.39 (SEM = 0.62). Based upon the relative luciferase activity of the positive controls, estrogenic activity for plant samples was defined as any sample with a mean fold increase of at least twofold for ERα and ERβ. In total, 44 plant items from 29 species making up 78.4% of the diet of the red colobus and 53 plant items from 42 species making up 85.2% of the diet of the mountain gorilla were screened for estrogenic activity at ERβ. For ERα, 50 plant items from 39 species making up 77.4% of the diet of the mountain gorilla and 14 plant items from 11 species making up 12.6% of the diet of the red colobus were screened. Fewer items were screened for activity at ERα due to the lack of activity at ERα found for plants that were shown to have ERβ activity in this study (0/8 ERβ active plants), as well as the rarity of plant compounds having activity at this receptor (Leitman et al., 2010).

Our objective was to identify phytoestrogen-containing plant items (i.e., species and part) and calculate the percent of diet coming from such estrogenic plant items for both primate species. We used the transient transfection data to determine which plants had estrogenic activity and determined the prevalence of estrogenic plants in the diet of each primate by summing the percent diet from all estrogenic plant items.

RESULTS

Red colobus diet and estrogenic plant foods

The red colobus fed on 169 dietary items: 167 items from 73 plant species, as well as soil and insects. However, most of their diet (79.3%) came from 23 staple dietary items (defined here as foods comprising >1% of diet, Table 1). All other items are considered to be rare foods (i.e., <1% of diet). Considering the prominence of phytoestrogens in legumes (i.e., Fabaceae), it is interesting to note that four of the top ten food items were from this family.

Forty-four plants were screened by cotransfecting U2OS cells with ERE-tk-Luc and either ERα or ERβ. None of the 14 items tested had estrogenic activity with ERα, whereas 8 of the 44 items tested had estrogenic activity with ERβ (Table 2; Fig. 1). These estrogenic items were from five species and three plant families: Fabaceae (two species), Moraceae (two species), and Myrtaceae. Three of the eight estrogenic items were staple foods: *Millettia dura* young leaves, *Ficus natalensis* young leaves, and *Eucalyptus grandis* bark. These three foods comprised 10.0% of the red colobus diet (Table 1). The other five estrogenic items were rare foods: *Erythrina abyssinica* young leaves and flowers, *Ficus sansibarica* unripe fruit and young leaves, and *Ficus natalensis* unripe fruit. These five foods comprised 0.6% of the red colobus diet (Table 2). In total, at least 10.6% of the red colobus diet came from estrogenic plants.

Gorilla diet and estrogenic plant foods

Fifteen dietary staples made up 96.1% of the diet of the mountain gorilla group studied in 2002–2003 by JR (Table 3). Of 53 dietary items tested, representing 85.2% of annual diet, two had ERβ activity, while none of the 50 items had ERα activity (Table 4). These estrogenic items were from two species representing two plant families: Convolvulaceae and Monimiaceae. One of these items was rarely fed on (*Xymalos monospora* bark), while the other (*Ipomoea involucrata* leaves) was the second most fed on item, comprising 8.8% of the annual diet (Tables 3 and 4). Thus, at least 8.8% of the gorilla diet was comprised of estrogenic plants.

TABLE 2. Transient transfection assay data for red colobus monkey plant foods showing which items (species/part) had activity at ERα and/or ERβ

Plant species	Family	Part	% Diet	ERα relative luciferase activity[a]	ERβ relative luciferase activity[b]
Hypocreata sp.	Unknown	YL	1.7	Not tested	0.97
Alangium chinese	Alangiaceae	ML	0.2	Not tested	0.68
Funtumia africana	Apocynaceae	YL	1.1	Not tested	1.06
Funtumia africana	Apocynaceae	ML	<0.1	Not tested	0.68
Markhamia lutea	Bignoniaceae	YL	0.2	1.23	0.93
Markhamia lutea	Bignoniaceae	PT	0.7	1.34	1.92
Parinari excelsa	Chrysobalanaceae	YL	2.7	Not tested	0.88
Diospyros abyssinica	Ebenaceae	YL	<0.1	0.88	1.51
Bridelia sp.	Euphorbiaceae	YL	1.7	Not tested	1.16
Macaranga sp.	Euphorbiaceae	YL	2.4	Not tested	0.91
Acacia spp.	Fabaceae	YL	4.5	Not tested	0.59
Acacia spp.	Fabaceae	ML	0.1	Not tested	1.18
Albizia grandibracteata	Fabaceae	YL	6.1	Not tested	0.85
Albizia grandibracteata	Fabaceae	ML	0.1	Not tested	0.91
Erythrina abyssinica	**Fabaceae**	**YL**	**<0.1**	0.90	**2.62**
Erythrina abyssinica	**Fabaceae**	**FL**	**0.1**	1.29	**3.65**
Millettia dura	**Fabaceae**	**YL**	**5.1**	0.99	**3.79**
Newtonia buchananii	Fabaceae	ML	0.9	Not tested	1.14
Newtonia buchananii	Fabaceae	YL	10.2	Not tested	1.05
Trilepsium madagascariense	Moraceae	YL	9.3	Not tested	0.74
Trilepsium madagascariense	Moraceae	ML	<0.1	Not tested	0.96
Ficus sansibarica	**Moraceae**	**UF**	**0.1**	1.06	**3.92**
Ficus sansibarica	**Moraceae**	**YL**	**0.3**	1.29	**2.72**
Ficus natalensis	**Moraceae**	**YL**	**1.5**	1.37	**2.43**
Ficus natalensis	**Moraceae**	**UF**	**0.1**	Not tested	**3.97**
Ficus thonningii	Moraceae	YL	0.2	0.88	1.45
Eucalyptus grandis	**Myrtaceae**	**BA**	**3.4**	Not tested	**2.01**
Strombosia scheffleri	Olacaceae	YL	1.3	Not tested	0.85
Strombosia scheffleri	Olacaceae	ML	0.1	Not tested	0.91
Strombosia scheffleri	Olacaceae	DW	0.6	Not tested	0.99
Olea capensis	Oleaceae	YL	0.2	1.06	1.88
Prunus africana	Rosaceae	ML	3.1	Not tested	1.06
Prunus africana	Rosaceae	YL	7.3	Not tested	1.60
Fagara angolensis	Rutaceae	YL	0.2	Not tested	1.16
Teclea nobilis	Rutaceae	YL	0.5	0.78	1.46
Pouteria altissima	Sapotaceae	YL	0.1	Not tested	0.99
Chrysophyllum sp.	Sapotaceae	ML	<0.1	Not tested	1.15
Chrysophyllum sp.	Sapotaceae	YL	0.1	Not tested	0.84
Dombeya kirkii	Sterculiaceae	YL	4.5	Not tested	0.66
Dombeya kirkii	Sterculiaceae	ML	<0.1	Not tested	0.74
Celtis africana	Ulmaceae	YL	3.8	Not tested	1.02
Celtis africana	Ulmaceae	ML	<0.1	Not tested	1.56
Celtis durandii	Ulmaceae	YL	3.6	1.00	0.98
Chaetacme aristata	Ulmaceae	YL	0.1	0.94	1.50

YL = young leaves, ML = mature leaves, UF = unripe fruit, FL = flower, BA = bark, PT = petiole, DW = dead wood.
Estrogenic items shown in bold.
[a] For ERα assays, relative luciferase activity for positive control (E2) = 33.18 (±6.55), (n = 3); estrogenic activity defined as more than twofold increase as compared to the blank (absence of ligand).
[b] For ERβ assays, relative luciferase activity for positive control (E2) = 4.39 (±0.62), (n = 9); estrogenic activity defined as more than twofold increase as compared to the blank (absence of ligand).

DISCUSSION

We demonstrated that two folivorous primates from two different phylogenetic groups, one an ape (i.e., Hominoid) and one an Old World monkey (i.e., Cercopithecoid), regularly consumed estrogenic plants (red colobus: 10.6% of diet, mountain gorilla: 8.8%). For the red colobus, most of their consumed phytoestrogens came from three staple dietary items: *Millettia dura* young leaves, *Ficus natalensis* young leaves, and the introduced species, *Eucalyptus grandis* bark. For the mountain gorilla, most of their consumed phytoestrogens came from a single staple food: *Ipomoea involucrata* leaves. Furthermore, all plants with estrogenic activity were active with ERβ, but not with ERα.

One of our most interesting results, in addition to the discovery that both primates did feed on estrogenic plants, is that all of the estrogenic plants showed estrogen receptor subtype selectivity for ERβ. This finding is significant for a number of reasons. The original estrogen receptor (ER) was the first steroid receptor to evolve in vertebrates and is conserved across all vertebrate species (Thornton, 2001). This receptor later evolved into two different forms, ERα and ERβ (Thornton, 2001), long before the Order Primates evolved. From studies of knockout mice lacking either one of the two ERs it is known that each ER has different, nonredundant roles in the nervous, immune, cardiovascular, and skeletal systems, as well as opposing actions on cell proliferation across numerous tissues, including the uterus, ovary,

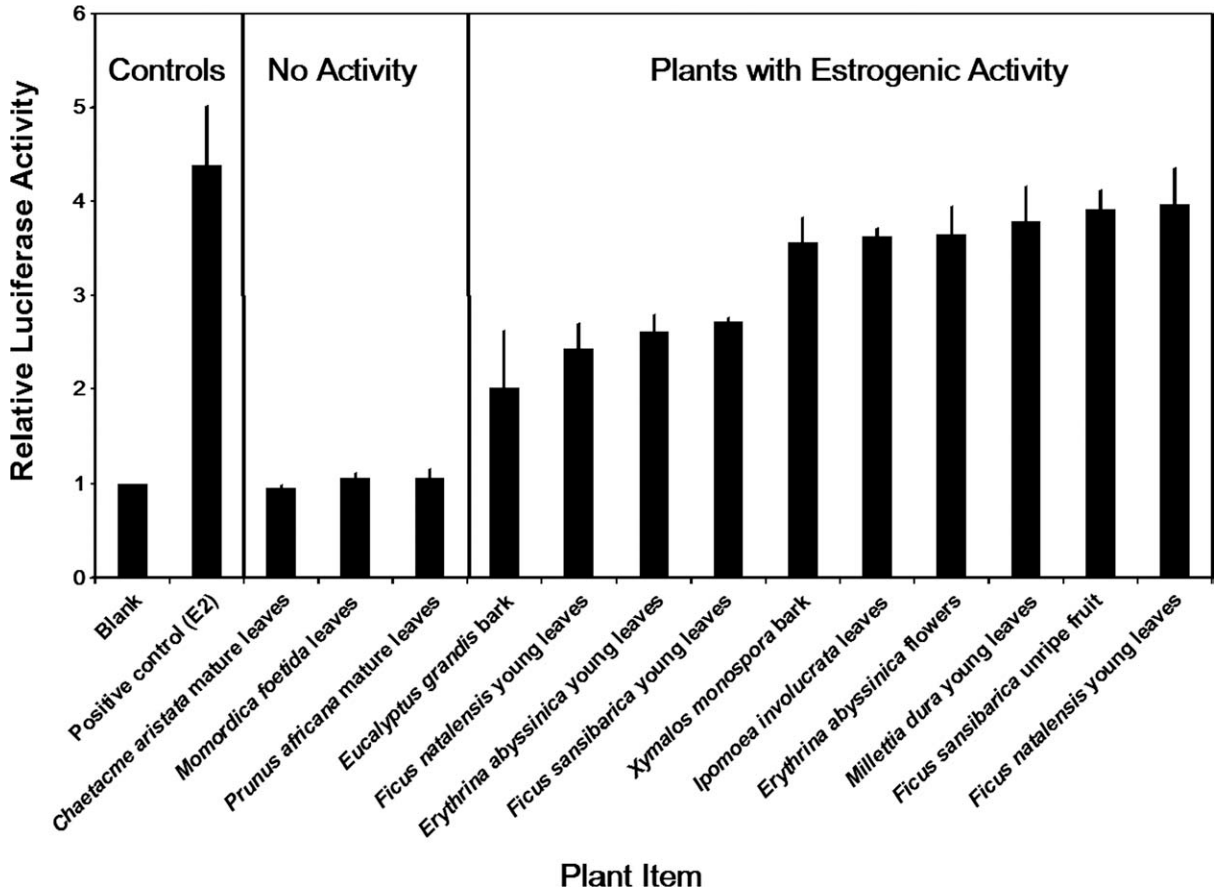

Fig. 1. The red colobus and mountain gorilla plant foods that selectively activated ERE (estrogen response element) transcription through ERβ (estrogen receptor beta), thus having estrogenic activity. Estrogenic activity was defined as any sample showing at least a twofold increase in relative luciferase activity as measured by the amount of light given off (i.e., relative light units [RLU]) from transiently transfected U2OS cells. Cells were treated with either nothing (blank control), 1.5 μl 10 nm E2 (positive control), or 1.5 μl of plant extract, and luciferase activity was measured. Each data point is the average of triplicate determinations ± standard error of the mean. Three samples with no activity are shown as an example (there were many others).

and brain (Heldring et al., 2007). Generally, it is ERβ that promotes cell growth arrest, which makes plants with ERβ selectivity of interest for treating or preventing estrogen-dependent cancers in humans (Heldring et al., 2007). Such plants are also of interest for hormone replacement therapy in menopausal women and for preventing osteoporosis, as they promote many of the actions of endogenous estrogens without the added risk of cancer promotion found in compounds with ERα activity (An et al., 2001; Cvoro et al., 2007). Consumption of estrogenic plants with ERβ selectivity may be one of many factors that help explain the low incidence of cancer in primates in general, with modern humans the one exception (Greaves, 2007). Variation in phytoestrogen metabolism due to differences in gut microbial communities causes some species to produce more active estrogenic compounds in the gut (Adlercreutz et al., 1987; Atkinson et al., 2005). For example, captive chimpanzees are known to excrete much greater amounts of the more bioactive phytoestrogen metabolite equol in their urine than humans (Adlercreutz et al., 1987; Musey et al., 1995). However, consuming ERβ selective plants may also lower fertility through disruption of cellular growth and tissue development in the reproductive systems of both females and males. As is often the case, there is likely a tradeoff between survival and reproduction (Wingfield and Sapolsky, 2003).

Another potential target of the ERβ selective plants might be the brain, as ERβ is found in high abundance in localized regions here and ERβ agonists can act directly on human neurons (Zhang et al., 2010). By acting on brain neurons it is possible that these plants might influence reproductive and other behaviors or reproductive function. To test this possibility for the red colobus, we concurrently collected data on their hormone levels and reproductive behavior along with the prevalence of estrogenic plants in their diet. We are currently examining these data for possible effects of phytoestrogens on physiology and behavior. In addition, variation in the consumption of estrogenic plants across seasons and age/sex classes of individuals will be an important area of future research to elucidate important phytoestrogen effects.

Additional insight into the possible significance of consuming estrogenic plants for these two primates is suggested by the ethnobotanical use of these or closely related plants (see Huffman, 2001 for similar argument for determining occurrence of self-medication in wild primates). Studies have isolated nonsteroidal isoflavones, plant compounds with a similar chemical structure to estrogens and known to have estrogenic activity, from *Millettia dura* bark (Derese et al., 2003) and seed pods (Yenesew et al., 1996). A related species, *M. griffoniana*,

TABLE 3. Staple dietary items (i.e., foods comprising >1% of total diet) accounting for 96.1% of total diet of one group of mountain gorillas in Bwindi National Park, Uganda, from 2002 to 2003, with estrogenic plant in bold

Plant species	Family	Part	% of diet	ERβ estrogenic activity?
Urera hypselodendron	Urticaceae	Leaves	19.2	No
Ipomoea involucrata	**Convolvulaceae**	**Leaves**	**8.8**	**Yes, for leaves only**
Myrianthus holstii	Moraceae	Ripe fruit	8.6	No
Momordica foetida	Cucurbitaceae	Leaves	8.0	No
Basella alba	Basellaceae	Leaves	7.8	No
Mimulopsis solmsii	Acanthaceae	Leaves	7.1	No
Myrianthus holstii	Moraceae	Leaves	6.6	No
Triumfetta tomentosa	Tiliaceae	Leaves	5.4	No
Urera hypselodendron	Urticaceae	Peel	5.4	No
Carduus kikuyorua	Asteraceae	Leaves	4.2	No
Mimulopsis arborescens	Acanthaceae	Pith	4.1	No
Decaying wood pieces		Wood	3.9	Not tested, but no for two species of decaying wood
Chrysophyllum albidum	Sapotaceae	Fruit	3	Not tested, but no for decaying wood
Cyathea manniana	Cyatheaceae	Pith	2.2	Not tested
Maesa lanceolata	Myrsinaceae	Fruit	1.8	Not tested, but no for leaves
Total			96.1%	8.8% of diet from estrogenic staples

Dietary data from Rothman et al. (2007).

is used traditionally in Cameroon to treat sterility, amenorrhea, and menopausal disorders (Ketcha Wanda et al., 2006). *Ficus natalensis* is used traditionally by the Gikuyu of Kenya during a ritual in which women smear the tree's milky sap over their bodies and men sleep on the tree's leaves to increase fertility (Karangi, 2008). The Gikuyu also believe that when animals feed on the leaves and seeds of *F. natalensis* their fertility increases. Related species with ethnobotanical use in Africa have been shown to have estrogenic activity, including *F. asperifolia* (Watcho et al., 2009) and *F. religiosa* (Ray and Pal, 1966; Jondhale et al., 2009). Although not native to or used medicinally in Africa, *Eucalyptus grandis* is an important fuel wood species there and an important source of paper products throughout the world. Studies of mill effluents have shown a related species, *E. globulus*, to have estrogenic activity, and this is suggested to relate to the feminization of male fish living downstream from such factories (Chamorro et al., 2010). Considering that *E. grandis* is a nonnative tree species growing along the edges of both KNP and BINP, and that numerous primate species, including black-and-white colobus monkeys (*Colobus guereza*), (Harris and Chapman, 2007), mountain gorillas (Rothman et al., 2006b), red colobus monkeys (MW, *personal observation*), and Guatemalan black howler monkeys (*Alouatta pigra*) (Bonilla-Sanchez et al., 2012), are thought to seek it out for its high sodium content (Rode et al., 2003; Rothman et al., 2006b), future studies should examine the possibility that *E. grandis* may act as a source of endocrine disruption for primates. The estrogenic staple food of the mountain gorilla, *Ipomoea involucrata*, is used in traditional medicine in Rwanda to treat infections (Sindambiwe et al., 1999) and in Nigeria to treat dysentery (Olukoya et al., 1993); the leaves are eaten by the Lele of Guinea because they are thought to increase fecundity (Wallace et al., 1998). Interestingly, ERβ knockout mice exhibit subfertility or infertility indicating that ERβ is important for reproduction.

Although both primate species fed on plants with estrogenic activity at ERβ and these or closely related plant species are used ethnobotanically, their effects on reproduction and health may differ between the two primates in this study. Both are folivorous, but differ in their foraging strategies, particularly with regard to gut morphology and prevalence of fruit in the diet. The mountain gorilla is a caeco-colic fermenting ape that prefers fruit when available and lacks any dramatic morphological specializations of the digestive tract for their folivorous diet (Lambert, 1998; Milton, 1999; Remis, 2000; Rothman et al., 2008). Thus, gorillas are much more similar to other noncolobine primate taxa that consume leaves (e.g., howler monkeys [*Alouatta*]) than is the red colobus with its specialized digestive morphology. As a forestomach-fermenting obligate folivore, the red colobus monkey is dependent upon its symbiotic gut bacteria for gaining nutrients from its diet consisting largely of tree leaves (Milton, 1980; Lambert, 1998; Chapman et al., 2002). These two different dietary strategies may result in important differences in the physiological effects of ingesting phytoestrogens for these two primates, as phytoestrogen metabolism is likely to differ depending upon the number, type, and location of the gut bacteria. Interspecific differences in the production of equol exist, likely due to differences in gut microbial communities (Adlercreutz et al., 1986; Setchell and Clerici, 2010). Because colobines have taken the mutualistic relationship with gut bacteria to a new level among primates, the physiological effects of consuming phytoestrogens may be greater for them than less digestively specialized primates, as has been documented for foregut-fermenting livestock (e.g., "clover disease" in sheep; Bennetts, 1946; Adams, 1990, 1995).

These results likely have important implications for primates beyond the colobines and gorillas, as two of the estrogenic staple foods of the red colobus, *Millettia dura* and *Ficus natalenis*, as well as two other estrogenic species rarely fed on by the red colobus, *Erythrina abyssinica* and *Ficus sansibarica*, are members of the two most important plant families for primates pan-tropically, Fabaceae and Moraceae. Leguminous (Fabaceae) foliage is often used by primates as a source of protein (Chapman et al., 2002) and species of the genus *Ficus* (Moraceae) are commonly used as a source of fruit and leaves by a wide variety of primate species in both the Old and New World tropics, especially during periods of food scarcity (Milton, 1991). It is well known that phytoestrogens are most prevalent in the Fabaceae (e.g., *Millettia*), and particularly in the subfamily Papilionoideae, while at least 18 different potentially estrogenic isoflavonoids have been identified in the Moraceae (e.g., *Ficus*), (Reynaud et al., 2005). Thus, it is likely that many forest primates are regularly consuming phytoestrogens in staple foods, regardless of geography or

TABLE 4. Transient transfection assay data for mountain gorilla plant foods showing which items (species/part) had activity at ERα and/or ERβ

Plant species	Family	Part	% Diet[a]	ERα relative luciferase activity[b]	ERβ relative luciferase activity[c]
Justicia glabra	Acanthaceae	L	<1.0	0.67	0.93
Mimulopsis arborescens	Acanthaceae	PI	4.1	1.07	0.91
Mimulopsis solmsii	Acanthaceae	L	7.1	1.26	0.82
Achyranthes aspera	Amaranthaceae	L	<1.0	1.44	0.82
Carpodinus glabra	Apocynaceae	L	<1.0	1.45	0.84
Schefflera sp.	Araliaceae	L	<1.0	Not tested	0.71
Periploca linearifolia	Asclepiadaceae	L	<1.0	0.98	0.72
Carduus kikuyorua	Asteraceae	L	4.2	0.56	0.74
Basella alba	Basellaceae	L	7.8	Not tested	1.55
Salacia elegans	Celastraceae	L	<1.0	0.95	0.63
Vernonia pteropoda	Compositae	BA	<1.0	0.88	1.05
Vernonia tuffnellae	Compositae	BA	<1.0	1.05	1.09
Ipomoea involucrata	**Convolvulaceae**	**L**	**8.8**	1.28	**3.62**
Ipomoea involucrata	Convolvulaceae	ST	<1.0	1.07	0.92
Ipomoea involucrata	Convolvulaceae	BA	<1.0	0.97	1.17
Momordica foetida	Cucurbitaceae	F	<1.0	0.87	1.06
Momordica foetida	Cucurbitaceae	L	8.0	1.13	0.89
Cyperus renschii	Cyperaceae	GS	<1.0	0.99	0.91
Drypetes sp.	Euphorbiaceae	RF	<1.0	1.33	0.67
Desmodium repandum	Fabaceae	L	<1.0	1.21	0.93
Englerina woodfordioides	Loranthaceae	ST	<1.0	0.98	0.95
Englerina woodfordioides	Loranthaceae	L	<1.0	1.26	0.73
Xymalos monospora	**Monimiaceae**	**BA**	<1.0	1.53	**3.56**
Xymalos monospora	Monimiaceae	L	<1.0	1.05	0.78
Ficus ingens	Moraceae	BA	<1.0	1.25	1.37
Ficus sp.	Moraceae	L	<1.0	0.96	0.97
Ficus sp.	Moraceae	RF	<1.0	0.99	0.89
Myrianthus holstii	Moraceae	RF	8.6	1.33	0.91
Myrianthus holstii	Moraceae	L	6.6	1.19	1.04
Myrianthus holstii	Moraceae	UF	<1.0	0.82	0.91
Maesa lanceolata	Myrsinaceae	L	<1.0	1.77	1.12
Syzygium guineense	Myrtaceae	RF	<1.0	0.66	0.80
Olea capensis	Oleaceae	BA	<1.0	0.97	0.69
Olinia usambarensis	Oliniaceae	RF	<1.0	0.95	0.93
Adenia gummifera	Passifloraceae	L	<1.0	1.04	0.94
Piper capense	Piperaceae	PI	<1.0	1.18	1.08
Cassipourea rwenzoriensis	Rhizophoraceae	DW	<1.0	1.04	0.95
Rubus sp.	Rosaceae	F	<1.0	1.40	0.96
Rubus sp.	Rosaceae	L	<1.0	0.80	1.00
Galiniera coffeoides	Rubiaceae	RF	<1.0	0.90	0.86
Galium thumbergianum	Rubiaceae	L	<1.0	1.71	0.70
Rytigynia kigenziesis	Rubiaceae	L	<1.0	1.11	0.74
Rytigynia kigenziesis	Rubiaceae	RF	<1.0	0.94	0.66
Teclea nobilis	Rutaceae	RF	<1.0	Not tested	0.89
Allophlylus abyssinicus	Sapindaceae	L	<1.0	0.95	0.98
Chrysophyllum albidum	Sapotaceae	DW	<1.0	1.33	0.89
Smilax anceps	Smilacaceae	L	<1.0	1.06	1.17
Triumfetta tomentosa	Tiliaceae	L	5.4	1.42	1.09
Droguetia iners	Urticaceae	L	<1.0	1.11	0.97
Urera hypselodendron	Urticaceae	PL	5.4	1.66	0.64
Urera hypselodendron	Urticaceae	L	19.2	0.91	0.91

Estrogenic items shown in bold.
L = leaves, ST = stem, DW = dead wood, GS = grass stem, RF = ripe fruit, UF = unripe fruit, BA = bark, PI = pith, F = fruit, PL = peel.
[a] % of diet data from Rothman et al. (2007).
[b] For ERα assays, relative luciferase activity for positive control (E2) = 33.18 (±6.55), (n = 3); estrogenic activity defined as more than twofold increase as compared to the blank (absence of ligand).
[c] For ERβ assays, relative luciferase activity for positive control (E2) = 4.39 (±0.62), (n = 9); estrogenic activity defined as more than twofold increase as compared to the blank (absence of ligand).

phylogeny. However, variation in the prevalence of estrogenic plants in the diets of these primates, the physiological and behavioral consequences of their ingestion, and what this means for primate ecology and evolution remains to be determined.

Numerous laboratory-based studies have demonstrated changes in hormone levels, cell growth, fertility, and behavior in captive animals, including primates, due to phytoestrogen consumption (Patisaul and Jefferson, 2010). If found in wild primates, these physiological and behavioral effects would likely result in differential survival and reproduction. Thus, endocrine interactions with plant compounds may be an important, yet almost totally overlooked, selective pressure influencing primate

evolution. Whether phytoestrogens increase or decrease primate fitness is still unclear. Future research should attempt to clarify if plants benefit from producing phytoestrogens by reducing primate herbivory through suppression of fertility (Hughes, 1988; Harborne, 1993; Wynne-Edwards, 2001) or if primates benefit from consuming phytoestrogens through increased survival (i.e., health benefits similar to those stated for humans) or reproductive success (Leopold et al., 1976; Glander, 1980; Strier, 1993; Huffman, 1997). Either way, estrogenic plants likely play important roles in primate ecology and evolution.

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