Title
Feeding on Phytoestrogens: Implications of Estrogenic Plants for Primate Ecology

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Publication Date
2011

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Implications of Estrogenic Plants for Primate Ecology

by

Michael David Wasserman

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Katharine Milton, Chair
Professor Isao Kubo
Professor Tyrone Hayes
Professor Colin A. Chapman

Fall 2011

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Abstract

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As most primates depend heavily on plant foods, the chemical composition of edible plant parts, both nutritional and detrimental, are of key importance in understanding primate ecology and evolution. One class of plant compounds of strong current interest due to their potential ability to alter the fertility, fecundity, and survival of both males and females are phytoestrogens. These plant compounds mimic the activity of vertebrate estrogens mainly through binding with the estrogen receptors, which results in altered physiology and behavior. Considerable evidence of interactions between phytoestrogens and the vertebrate endocrine system comes from research conducted on the potential health benefits and reproductive costs of phytoestrogens in human foods, especially soybeans (*Glycine max*) and other legumes. Despite this interest, little is known about the occurrence of estrogenic plants in the diets of wild primates. If wild primates do consume phytoestrogens, then the physiological and behavioral effects documented in captive and laboratory studies may promote differential survival and reproduction of individuals in a natural setting. Consequently, estrogenic plants would have an important, thus far neglected, role in primate ecology and evolution.

To examine the occurrence of estrogenic plants in the diets of wild primates, I screened plant foods for estrogenic activity in two strongly 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 if the consumption of phytoestrogens relates to physiological changes in a wild primate, I determined the seasonal pattern of estrogenic plant consumption and its relationship to hormone levels of the red colobus in Kibale during an 11-month field study. I screened 44 plant items 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 bound to and activated ERβ, but not ERα. Climatic factors were important for understanding variation in the proportion of diet coming from estrogenic plants for the red colobus, particularly for the consumption of *Millettia dura* young leaves. Although red colobus did not feed more heavily on *M. dura* young leaves when they were more available, they did feed more heavily on them during months of higher rainfall. Both fecal estradiol and fecal cortisol levels were positively related to the percent of diet from estrogenic *M. dura* young leaves. Thus, it appears that climatic factors may influence the intake of estrogenic plant foods by red colobus and that the consumption of estrogenic plant foods influences the hormone levels of these monkeys. These results show that phytoestrogens occur in the wild plant foods of at least two Ugandan primate species and suggest that consumption of estrogenic plants by red colobus monkeys may have important implications for their health and fitness through interactions with the endocrine system. Future studies should build upon these results by examining the prevalence of estrogenic plants in the diets of other primate species, especially frugivores, and by determining if the hormonal changes documented here translate into important physiological and behavioral changes that affect reproduction and survival. Phytoestrogens in the diets of wild primates may have important implications for understanding primate ecology and may provide insight into both non-human and human evolution.

This dissertation is dedicated to Julie and Kaia.
Their adaptability and love made this research possible.

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ACKNOWLEDGEMENTS

I would like to thank everyone that has helped with my research and teaching since I arrived at Berkeley in 2004. At times it felt like I was managing three different projects, and without the help of everyone in the field at the Makerere University Biological Field Station in Kibale National Park, Uganda, in the lab at UC-Berkeley (UCB), or at the National Primate Research Center in Madison, Wisconsin, I would not have been able to complete this dissertation project. Numerous others provided professional advice, suggestions, and feedback from early on in the proposal phase through the final edits of this dissertation, while many friends and family provided personal support and encouragement. I truly hope each person, including those I undoubtedly will forget to mention, knows how much I appreciate every minute of their time they kindly contributed to my professional and personal development since arriving at Berkeley.

First and foremost, I would like to thank my lab, including Professor Katharine Milton, Andrew Ritchie, Mariah Hopkins, Alexandra Taylor-Gutt, and Tarek Milleron. I was lucky to have had such great biologists to bounce ideas off of over the years. Alexandra helped with preparation of plant samples for analyses of estrogenic activity and was supported by a SPUR grant from the College of Natural Resources at UCB. Andrew Ritchie played an important role early on by tossing a copy of the Wynne-Edwards paper about phytoestrogens as endocrine disruptors on my desk that sparked my interest in the topic (as well as many one-on-one games that kept my head clear during the first few years of grad school). I could not have found a better PhD advisor than Prof. Milton. She has taught me much about primate ecology and evolution and played a central role in connecting my work to humans. She also has greatly improved my writing, teaching, and speaking skills. I am particularly grateful for our countless conversations over the years and her ability to clearly communicate the central importance of nutrition to understanding natural systems.

At Berkeley, I would like to thank Dr. Isao Kubo, Dr. Len Bjeldanes, Candice Herber, Holly Hantz, and Dr. Dale Leitman for providing guidance and feedback on the phytoestrogen analyses. Dr. Leitman generously opened the doors of his already busy lab so I could learn and conduct the analyses vital to answering my questions about the presence of estrogenic plants in the diets of wild primates. Dr. Tyrone Hayes and his seminar on endocrine disruptors inspired me to study phytoestrogens and how endocrine disruptors affect wild primates. Dr. Justin Brashares provided guidance since I first arrived at Berkeley, particularly as the chair of my qualifying exam. Dr. Darlene Francis provided many interesting discussions about stress physiology and also played an important role in my qualifying exam. The Primate Biology Group, run by Drs. Leslea Hlusko and Katharine Milton, has been a great venue to share my research ideas and results with over the years. I have made many great friends during my time at Berkeley, but I would particularly like to thank Paul Falso, Graham Bullock, Tim DeChant, Pauline Kamath, Cole Burton, Dan Fahey, Sang Lee, and Diego Funes. Finally, I would like to thank my dissertation committee: Drs. Katharine Milton, Colin Chapman, Isao Kubo, and Tyrone Hayes. The interdisciplinary nature of this dissertation was possible because of their guidance and expertise.

In Uganda I would like to thank Dr. Patrick Omeja, Dr. Dennis Twinomugisha, Baguma Charles, Musunguzi Hillary, Mutegeki Richard, Katusabe Swaibu, and Kaganzi Clovis for providing assistance in the field at Kibale. Charles, Hillary, and Richard were very dependable field assistants that kept things running while I was away, and Swaibu provided much needed assistance in the lab. My fieldwork depended on their skills and dedication. Patrick and Dennis

are invaluable project managers of the Kibale Fish and Monkey Project, and they were always willing to help with the red tape. Clovis has been a true friend since our first meeting in 2003, and his hospitality always make me feel at home when I am in Uganda. Dr. Jessica Rothman has become both a great friend and insightful colleague since we spent our first field season together in Kibale during the summer of 2006. She also provided the mountain gorilla plant food samples analyzed in this dissertation. Drs. Lauren Chapman and Erin Reardon took me under their wing early on as an undergraduate at the University of Florida, taught me much about physiological ecology, and were an important part of my first trip to Kibale in 2003. Dr. Colin Chapman has played such a large part in my professional development as a scientist, starting from the time I returned from Costa Rica wanting to pursue a career as a tropical ecologist. He has been an amazing mentor, and I cannot thank him enough for all he has done over the past ten years. Most notably, he suggested Berkeley and Prof. Milton for graduate school, and I am glad I was wise enough to listen to his advice. Dr. Chapman also provided the red colobus plant samples and rainfall, temperature, and phenological data. I would also very much like to thank the Uganda Wildlife Authority and Uganda National Council for Science and Technology for providing permission to conduct this research.

Dr. Toni Ziegler and Dan Wittwer were welcoming hosts at the Wisconsin National Primate Research Center. They have provided insight into primate endocrinology since my first project on the use of cortisol as an indicator of stress in the red colobus. I am truly thankful for all the time they invested in teaching me the various methods to measure steroid hormones and for allowing me to run so many samples during my time visiting their lab. I would also like to thank Dr. Karen Strier for inviting me to the biological anthropology group and the various venues to present my research while visiting the University of Wisconsin-Madison.

A special thank you goes to my wife, Julie Kearney Wasserman, for taking all the great primate pictures in Kibale that are vital to my lectures and research talks. She has been a very patient and adaptable partner throughout this project, moving between Berkeley, Kibale, and Madison. I would also like to thank my daughter for her adaptability, although she is likely still too young to remember most of our adventures. Next stop, Montreal. In addition, the support and encouragement from the Wassermans, Kearneys, and Monaghans were vital to the completion of this project.

Finally, I would like to acknowledge my funding sources for this research: a National Science Foundation (NSF) Doctoral Dissertation Improvement Grant (#0823651), the NSF Graduate Research Fellowship Program, the International Primatological Society, the University of California, Berkeley (UCB) Department of Environmental Science, Policy, and Management, UCB Center for African Studies, and the UCB Chang-Lin Tien Scholars Program (funded by the Philomathia Foundation).

INTRODUCTION

Plant species containing chemicals that alter the vertebrate endocrine system are consumed by both humans and non-human primates. However, the importance of these naturally occurring plant chemicals, including the phytoestrogens, to human and non-human primate ecology and evolution remains unclear and largely unstudied. Phytoestrogens, defined as plant compounds that mimic endogenous vertebrate estrogens, are present in domesticated food crops of many cultures and have been shown to alter both physiology and behavior in captive rodents and primates. Yet, little information exists on the prevalence of phytoestrogens in the plant foods of wild primates, nor their potential influence on wild primate physiology and behavior. Because the majority of human history has relied on a hunter-gatherer subsistence strategy mainly dependent on wild plant-based foods, investigating the prevalence of phytoestrogens in wild plants is necessary to improve our understanding of human origins. Furthermore, the nutritional environment in which human ancestors evolved can be best reconstructed by studying living non-human primates in a natural setting, especially East African primates with plant-based diets. This dissertation takes the necessary first step in examining the relationship between these plant compounds and the primates ingesting them by addressing three main questions:

1. *What are the hypothetical mechanisms through which phytoestrogens could influence primate ecology and evolution?*

2. *How prevalent are estrogenic plants in the diets of wild folivorous primates (i.e., red colobus monkey [Procolobus rufomitratus] and mountain gorilla [Gorilla beringei])?*

3. *If red colobus monkeys do consume estrogenic plants, do phytoestrogens influence their physiology via altered hormone levels?*

These questions are addressed using theory and methodology from the fields of biological anthropology, behavioral ecology, endocrinology, and plant chemistry. Chapter One provides a review of ecological studies focused on phytoestrogens, the role of sex steroid hormones in primate physiology and behavior, and how phytoestrogens can interfere with physiology and behavior. It concludes with a hypothetical model for how phytoestrogen interactions with the primate endocrine system could have significance for primate ecology and evolution. Chapter Two examines the prevalence of estrogenic plants in the diets of two wild primates, the red colobus monkey (*Procolobus rufomitratus*) of Kibale National Park and the mountain gorilla (*Gorilla beringei*) of Bwindi National Park, both in Uganda. Chapter Three examines how seasonal variation in the consumption of estrogenic plants by red colobus monkeys relates to their steroid hormone levels. Chapter Four concludes with an overview of the next steps in my research that will build upon the results found during my dissertation research.

I collected the field data used to address my main dissertation questions during an 11-month field study of the red colobus in Kibale National Park, Uganda. I then used transient transfection assays at the University of California, Berkeley to screen various plant species for estrogenic activity and immunoassays at the National Primate Research Center in Madison, Wisconsin, to quantify the steroid hormone levels of individually recognized red colobus adults from fecal samples collected during the field study. To my knowledge, this is the first field study to collect such data concurrently.

Using a novel approach to examine the relationship between primates and the plants they depend on provides important knowledge thus far neglected in studies of primate biology. From an ecological standpoint, discussions of primate ecology may be neglecting a critical factor influencing health, fertility, diet choice, and abundance. From an anthropological perspective, since non-human primates are the closest living relatives of modern humans and live in an environment more similar to that of pre-agricultural human ancestors, data on the primate-phytoestrogen relationship provides an improved evolutionary and ecological context for human origins. In addition, this dissertation provides background information on the prevalence of endocrine disruption in a natural setting. Anthropogenic endocrine disruptors are currently thought to pose great risk to various vertebrate species, including humans and non-human primates. It is therefore of key importance to better understand the relationship between naturally-occurring endocrine disruptors and primate feeding ecology and physiology to improve our ability to evaluate and predict the consequences of increased endocrine disrupting compounds entering the environment. Studies of wild primates and phytoestrogens are also increasingly important because the human food and botanical treatment landscape now contains many products derived from estrogenic plants, either specifically for their reported benefits (e.g., botanical treatments for menopausal symptoms) or simply as a byproduct (e.g., soy-based foods). Although much debate is currently taking place over the role of these compounds in the modern human diet and their possible effects on human physiology and health, a more comprehensive understanding of these issues will not become clear until an evolutionary medical approach is taken.

CHAPTER 1:

The potential roles of estrogenic plants in primate ecology and evolution

ABSTRACT:

As most primates depend heavily on plant foods, the chemical composition of edible plant parts, both nutritional and detrimental, are of key importance in understanding primate ecology and evolution. One class of plant compounds of strong current interest due to their potential ability to alter the fertility, fecundity, and survival of both males and females are phytoestrogens. These plant compounds mimic the activity of vertebrate estrogens mainly through activity at the estrogen receptors, with potentially important ramifications for physiology and behavior. It is very possible that hormonal interactions between plants and primates have played an important, thus far neglected, role in their ecological and evolutionary relationships. However, empirical data on the presence of estrogenic plant foods in the diets of wild primates have not been collected. Nonetheless, numerous hypotheses regarding the phytoestrogen-primate relationship have been postulated, including the “plant defense hypothesis”, “self-medication hypothesis”, and “neutral hypothesis”. On the other hand, phytoestrogens in human plant-based foods and the health effects of consuming such foods is the focus of much research. In this review I summarize what little is known about phytoestrogens from an ecological and evolutionary perspective, consider the role of sex steroid hormones in primate physiology and behavior and how phytoestrogens can interfere with this, and present a hypothetical model for how phytoestrogen interference with the primate endocrine system could have significance for primate ecology and evolution. If wild primates do consume phytoestrogens, then the potential physiological and behavioral effects seen in captive and laboratory studies likely promote differential survival and reproduction of individuals. Consequently, phytoestrogens would have an important, albeit neglected, role in primate ecology and evolution. This is an important area of research in need of both field and lab-based studies for furthering our understanding of primate ecology and evolution, as well as improving our ability to evaluate the costs and benefits of phytoestrogen consumption for humans.

KEYWORDS: environmental endocrinology, endocrine disruption, plant-animal interactions, herbivory, self-medication

INTRODUCTION:

The Importance of Plant Chemistry to Primate Ecology and Evolution

Most primates depend heavily on plant foods, such as the leaves, fruits, and flowers of tropical trees (Milton 1979). As a result, the chemical composition of edible plant parts, both nutritional and detrimental, are of key importance in understanding primate ecology and evolution. For example, research on leaf-eating primates has shown that the availability of high protein, low fiber leaves is related to food choice and biomass (Milton 1979, Oates et al. 1990, Chapman et al. 2002, Wasserman & Chapman 2003). However, the significance of plant secondary metabolites to primates is much less clear. Plants produce certain compounds (e.g., alkaloids, cyanogenic glycosides) as a defense mechanism against insect herbivory (Coley & Barone 1996), but evidence that such compounds function to deter mammalian herbivores, such as primates, is not as strong (Milton 1979, Milton 1998, Burgess & Chapman 2005). Mammalian herbivores have the ability to deal with many disruptive compounds either by avoiding them through selective, generalist feeding strategies (i.e., behavioral adaptations for avoiding or minimizing their consumption) or by evolving detoxification mechanisms (i.e., physiological adaptations) (Freeland & Janzen 1974). This is especially true for primates because many taxa have specialized symbiotic microbes that can likely detoxify many defensive compounds (e.g., the forestomach fermenting colobines; Bauchop & Martucci 1968, Milton 1980, Milton 1998). In addition, all primates also have the behavioral plasticity of a large-brained animal that allows for a highly selective, opportunistic diet. Indeed, only one recent study has effectively shown a plant secondary compound (i.e., tannins) to reduce mammalian reproductive success (i.e., in the common brushtail possum [*Trichosurus vulpecula*]) through the negative effects of tannins on nitrogen availability (DeGabriel et al. 2009).

Nonetheless, it is hypothesized that primates must deal with minimizing consumption of plant secondary metabolites when selecting their foods (Glander 1979, McKey et al. 1981). Milton (1998) discussed two types of plant compounds that primates are faced with: 1) those that are toxic to the feeder or the feeder's gut microbes and 2) those that inhibit digestion or absorption of nutrients. An overlooked third type of problematic plant secondary metabolite also exists, and it can be defined by its ability to significantly alter long-term internal processes of the feeder, such as endocrine functioning. Phytoestrogens, naturally occurring plant compounds that mimic the activity of vertebrate estrogens, are a prime example of this third type of secondary metabolite. Because such compounds mimic endogenous vertebrate compounds and often remain bioactive after passing through the stomach, their consumption may have important repercussions for primates. For example, daidzein, a phytoestrogen found in soy (*Glycine max*), is converted to the more bioactive compound equol via gut microorganisms (Patisaul et al. 2009). Further, phytoestrogens are known to disrupt the fertility of mammals feeding on plants that produce them (Hughes 1988, Wynne-Edwards 2001). As there are also plant compounds that mimic androgens (e.g., testosterone), progestins (e.g., progesterone), and glucocorticoids (e.g., cortisol) (Heftmann 1975, Beck et al. 2003, Janeczko & Skoczowski 2005, Iino et al. 2007), it is likely that hormonal interactions between plants and primates has played an important, thus far neglected role in their ecological and evolutionary relationships.

The Importance of Phytoestrogens to Primate Ecology and Evolution

Interest in phytoestrogens stems mainly from their presence in human plant-based foods, including soybeans, chickpeas, flaxseed, peanuts, barley, and broccoli (Mazur 1998). Because of their ubiquity in many widely-consumed human foods and interest in the health effects of consuming such foods, many *in vitro* and *in vivo* laboratory studies using human cell assays and captive rodents and monkeys have been conducted, along with human clinical and epidemiological studies, on the physiological and behavioral effects of phytoestrogen consumption (Whitten & Patisaul 2001). Two opposing points of view have resulted from this research: a) phytoestrogens provide health benefits, such as cancer prevention (e.g., Leitman et al. 2010), or b) phytoestrogens act as endocrine disruptors and threaten reproductive health (e.g., Cederroth et al. 2010a). However, an ecological and evolutionary perspective that considers both why a plant produces such compounds and why a primate would consume a plant producing them would advance our understanding of the consequences of phytoestrogen consumption more than simply framing the issue in dichotomous terms. Yet, to date, little is known about the ecological or evolutionary context of phytoestrogen consumption for humans, primates, or any vertebrate animal for that matter (Wynne-Edwards 2001), as very few studies on wild animals have focused on this topic.

Additionally, a focus on the occurrence of phytoestrogens in modern human foods has skewed our understanding of the ecological and evolutionary relationship between phytoestrogens and primates as attention here is focused on the study of cultivated plant foods that have existed for a relatively short period of time in comparison to their wild counterparts. Although much is known about phytoestrogens in cultivated plants, especially soy, little is known about their presence in wild plants used by humans for food prior to agriculture. The majority of human history has relied on a hunter-gatherer subsistence strategy strongly dependent on wild plant-based foods and human biology has changed little since that time (Milton 2000). For these reasons, an investigation of the prevalence of phytoestrogens in wild plants and their effects on primate dietary ecology and physiology could greatly improve our understanding of the possible effects of these compounds on human health and reproductive parameters and, consequently, their potential role in human evolution. This context to the phytoestrogen debate is of current importance as human foods and botanical supplements containing products derived from estrogenic plants (e.g., botanical treatments for menopausal symptoms, soy-based foods) are increasingly in use.

To address the role of phytoestrogens in the ecology and evolution of both human and nonhuman primates, a comparative data set that includes the prevalence of phytoestrogens in the wild tropical plant foods of primates and the physiological and behavioral effects of their ingestion are needed. Further, because non-human primates are a well-studied group with much known about their feeding behavior and are modern human's closest living relatives, the study of non-human primates is well suited to improving our understanding of the costs and benefits of phytoestrogen consumption for humans and other vertebrate herbivores. In this review I summarize what little is known about phytoestrogens from an ecological and evolutionary perspective, consider the role of sex steroid hormones in primate physiology and behavior and how phytoestrogens can interfere with these hormones, and present a hypothetical model for how phytoestrogen interference with the primate endocrine system could have significance for primate ecology and evolution.

THE ECOLOGY AND EVOLUTION OF PHYTOESTROGENS:

Estrogenic Plant-Animal Interactions: A Significant Ecological Relationship?

Numerous hypotheses about the ecological and evolutionary significance of phytoestrogens to vertebrates have been postulated. The "plant defense hypothesis" suggests that plants benefit from producing these compounds by reducing vertebrate herbivory through suppression of fertility (i.e., consuming phytoestrogens is costly for vertebrate herbivores; Hughes 1988, Harborne 1993, Wynne-Edwards 2001). Further, steroid production is a cheap, low-risk defense mechanism for plants because it requires only minor modifications to existing biosynthetic pathways (Wynne-Edwards 2001). Alternatively, the "self-medication hypothesis" suggests that vertebrate herbivores benefit from consuming phytoestrogens through increased survival (i.e., health benefits) or reproductive success, while the plant produces such compounds for reasons other than herbivore deterrence (Leopold et al. 1976, Berger et al. 1977, Glander 1980, Strier 1993, Huffman 1997, Fidler et al. 2008, Forbey et al. 2009). A third "neutral hypothesis" suggests that there are no significant effects of consuming phytoestrogens for wild vertebrates. However, a lack of relevant, concurrent data on feeding behavior, estrogenic activity of the plant, and physiological or behavioral effects on the feeder has prevented a critical evaluation of these hypotheses.

Although the ecological relationship between estrogenic plants and wild vertebrates is unclear, it is well established that certain phytoestrogens can have dramatic physiological effects. This was first documented in western Australia where it was discovered that formononetin and biochanin A, phytoestrogens found in a species of clover (*Trifolium subterranean*) that domesticated sheep were feeding on, caused an epidemic of infertility that led to greatly reduced numbers of lambs and considerable economic loss to farmers (Bennetts & Underwood 1951, Cornwell et al. 2004).

One rare example of an ecological study on phytoestrogens examined the possibility that increased phytoestrogen consumption by California quail (*Callipepla californica*) during drier years inhibited their reproduction. It was postulated that the quail possibly used the phytoestrogen content of their plant foods as a cue to limit production of offspring to wetter years when food was more available (Leopold et al. 1976). This "chemical cue hypothesis" was reiterated for mountain voles (*Microtus montanus*), where such compounds may have been used to time reproduction to periods of high quality food availability (Berger et al. 1977, Berger et al. 1981). Soon after, it was suggested that Costa Rican howler monkeys (*Alouatta palliata*) were possibly consuming certain plant species to affect their own reproductive timing (Glander 1980; Table 1.1). Later, seasonality in chimpanzee (*Pan troglodytes*) reproductive parameters at Gombe National Park, Tanzania, was hypothesized to result from annual variations in rainfall and diet, with possible mediation by phytoestrogens (Wallis 1997). It should be noted that all of these suggestions concerning the potential role of phytoestrogens in reproductive seasonality are highly speculative with little to no supporting data, and alternative hypotheses are not adequately considered.

Recently, researchers have again become interested in this phenomenon, 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. For birds, Fidler et al. (2008) proposed a hypothetical mechanism for how phytoestrogens could mediate reproductive timing of the kakapo (*Strigops habroptilus*), a New Zealand parrot that produces offspring during mast fruiting years (i.e., every 3-5 years). For primates, three recent field studies (i.e., of Phayre's 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 suggested that consumption of plant parts from the phytoprogesterone-containing genus *Vitex* may affect female reproduction through altered cycle length, probability of conception, or receptivity. However, these primate studies all focused on only one of the many plant genera (i.e., often more than 80 genera) that primates feed on. Lu et al. (2010) have stressed the need for field studies to combine data on intake rates and chemical analyses of a substantial portion of a primate's diet to gain a more accurate understanding of the importance of steroidal plants to primates.

A Botanical Perspective: Phytoestrogens as Plant Adaptations

Although phytoestrogens may play a role in defending plants against vertebrate herbivory through interference with the vertebrate endocrine system, there have yet to be any studies to demonstrate this benefit for plants. Steroids (i.e., estrogens, androgens, progestins, glucocorticoids) and steroid-like compounds (e.g., isoflavonoids) are found in many plants as secondary metabolites in biosynthetic pathways (Janeczko & Skoczowski 2005), but their similarities to endogenous vertebrate steroids and biological effects in these animals may simply be a coincidence of chemistry. Cholesterol is widely distributed in plants, and plants produce steroids from cholesterol using metabolic pathways similar to those of vertebrates (Heftmann 1975).

The strongest support for a plant defense explanation of steroid production is found with insect herbivory (Kubo et al. 1983, Harborne 1993). Some plants convert cholesterol to phytoecdysteroids (i.e., insect-molting hormone mimics), which disrupt the development of herbivorous insects that feed on such plants and can even lead to mortality (Kubo et al. 1983). Plant steroids which are similar to vertebrate hormones may in fact have primary roles in plant physiology, affecting plant germination, growth, flowering, and sex expression (Heftmann 1975, Janeczko & Skoczowski 2005). For example, experiments that applied vertebrate steroid hormones to plants showed that these plants had biological responses to these steroids (Janeczko & Skoczowski 2005). More specifically, endogenous progesterone occurs widely in plants and many plant species contain progesterone-binding proteins (Iino et al. 2007). Progesterone may play an endogenous physiological role in the plant, although this is not yet fully understood (Iino et al. 2007). In support of this, I have found that plant steroids or steroid-like compounds may be fairly ubiquitous, as all thirteen plant items I tested from Kibale National Park, Uganda, contained compounds that bound to various steroid hormone antibodies (Table 1.2), with most showing progesterone-like structures (Fig. 1.1). However, binding to an antibody only infers structure and says nothing about activity. Thus, many of these steroidal plant compounds may show little to no biological effects in the plants themselves or in animals consuming them. *In vitro* and *in vivo* studies are needed to show steroidal activity of such plant compounds.

Considering only phytoestrogens, over 160 plant compounds found in more than 300 plant species from 32 plant families have thus far been shown to exhibit estrogenic activity (Dixon 2004, Reynaud et al. 2005). These phytoestrogens are divided into two main families of phenolic compounds based upon chemical structure: isoflavonoids (e.g., isoflavones, coumestans) and stilbenes (e.g., resveratrol) (Cornwell et al. 2004, Reynaud et al. 2005). Lignans are also considered phytoestrogens, but must first be converted by gut microbes to mammalian lignans to show estrogenic activity (Cornwell et al. 2004). The isoflavonoids are the most abundant phytoestrogens and are predominately found in the subfamily Papilionoideae of the legume family (Fabaceae) (Dixon 2004, Reynaud et al., 2005).

The endogenous role of phytoestrogens for plants remains unclear, but they do appear to play a role as a recruitment signal for soil microbes, thus promoting symbiosis between plants and nitrogen-fixing bacteria (Fox et al. 2004). This interspecific communication role likely explains the abundance of isoflavonoids in the legumes, which are known for their mutualistic relationships with nitrogen-fixing bacteria. Phytoestrogens are also known to protect plants against fungal and bacterial pathogens (Fox et al. 2004). Additionally, flavonoids (a broader group of secondary metabolites that includes the estrogenic isoflavonoids) play a role in protecting plants against harmful ultraviolet light and in altering the wavelength of light to appropriate physiological levels (Mazur & Adlercreutz 1998). Although we are only beginning to understand the role of phytoestrogens from the plant's perspective, it is becoming clear that their occurrence is due to a complex set of factors and they appear to provide important benefits to plants.

Despite the evidence for other functions of steroids and steroid mimics in plants and the possibility of their steroidal activity in vertebrates being simply a biochemical coincidence, the costs and benefits of such compounds arising from plant-animal interactions at both an ecological and evolutionary level cannot be discounted. If a secondary benefit to the plant from negative effects on vertebrate herbivores were to occur, increased production of such compounds could be selected for, even though such compounds were originally or are mainly produced for other functions. Therefore, the various roles of phytoestrogens for the plant are not mutually exclusive and potential mediation of plant-animal interactions could be one very important consequence of plant steroids and steroid mimics. Many interesting questions remain regarding their presence, including: could hormonal interactions between plants and vertebrates mediate ecological relationships in ways found between plants and bacteria or plants and insects? What are the evolutionary implications of these similarities in biochemistry?

THE ROLE OF STEROID HORMONES IN PRIMATE PHYSIOLOGY AND BEHAVIOR

Before addressing how phytoestrogens could influence the ecology and evolution of primates, an understanding of how endogenous vertebrate steroid hormones regulate physiology and behavior is needed. It is through the disruption of the physiological and behavioral endpoints of steroid hormones that phytoestrogens can alter the survival and reproduction of primates.

Molecular Mechanism of Action for Steroid Hormones

Hadley (2000) provides a detailed review of the vertebrate endocrine system. In brief, it is the body's internal long-term communication system (as compared to the quicker nervous system) including the brain and a series of ductless endocrine glands that secrete chemical signals (i.e., hormones) directly into the blood. These hormones travel through the body, bind to receptors in certain cell types, and alter the activity of those cells, mainly through direct interaction with the genetic material in those cells. Although there are over 80 different types of hormones in vertebrates, four classes of steroid hormones are particularly important for understanding the role of the endocrine system in vertebrate reproductive physiology and behavior: estrogens, androgens, progestins, and glucocorticoids. These steroid hormones are produced by various endocrine glands (i.e., mainly the gonads and adrenal glands) and secreted into the blood where they circulate, likely bound to a protein (e.g., sex hormone-binding globulin [SHBG]). Steroids passively diffuse through cell membranes, due to their hydrophobic nature, and bind to specific receptors found inside certain cell types. This binding event results in a conformational change to the receptor that allows the receptor-steroid complex to bind to the nuclear chromatin at response element genes. Consequently, these genes are switched on, altering the regulation of the transcription and translation of downstream steroid-dependent genes. Ultimately, changes in gene activity alter the production of proteins which results in changes in the physiology and behavior of the organism.

Of specific relevance to phytoestrogens, endogenous estrogens operate mainly through binding to estrogen receptors (ER) found inside certain cell types (e.g., brain, urogenital, bone, gonadal) which results in an estrogen receptor-estrogen complex (Heldring et al. 2007, Leitman et al. 2010). This complex binds to the estrogen response element on the intranuclear chromatin and regulates the transcription and translation of estrogen dependent genes (Heldring et al. 2007, Leitman et al. 2010). This overview is sufficient for the ecological and evolutionary concerns of this review; however, the molecular mechanisms of steroid hormones are actually much more complicated, including non-genomic estrogen signaling. For a review of the details of estrogen signaling, see Heldring et al. (2007) and Leitman et al. (2010).

It is important to note that there are two main forms of estrogen receptors in vertebrates, ERα and ERβ. The original steroid receptor evolved long ago (*c.* 400-500 mya; Thornton et al. 2003), and this ancestral receptor evolved into the five current types of steroid receptors found in vertebrates today (i.e., estrogen receptors, progesterone receptors, androgen receptors, glucocorticoid receptors, and mineralocorticoid receptors). The estrogen receptor arose first and is conserved across all vertebrates (Thornton 2001, Thornton et al. 2003). However, the ancestral ER evolved into two different forms, ERα and ERβ, at two points in the evolution of vertebrates; unique sets are found in the teleosts and the tetrapods (Thornton 2001). Due to the conservative nature of the endocrine system across tetrapods, as demonstrated by ER evolution, studies within this group can inform us about how estrogens function in primates.

From studies of knockout mice, we know that each ER has different roles in the nervous, immune, cardiovascular, and skeletal systems, as well as opposing actions on cell proliferation across numerous tissues, including the uterus, ovary, and brain (Heldring et al. 2007, Leitman et al. 2010). Generally, it is ERβ that arrests cell growth, while ERα promotes cell proliferation (Heldring et al. 2007). The distribution of ERα and ERβ differs across tissues and organs, as well as within them. For example, different parts of the brain differ in ER distribution, with each regulating different aspects of behavior (Patisaul et al. 2009). Specifically, ERα appears to mediate sexual behavior in males and females (Patisaul et al. 2004), while ERβ mediates levels of aggression and anxiety (Patisaul and Bateman 2008).

Physiological and Behavioral Endpoints: How Sex Steroid Hormones Regulate Reproduction

For a detailed review of the role of sex steroid hormones in vertebrate reproduction see Hadley (2000); the following is a brief summary of this text.

By directly regulating the transcription of certain genes and consequently the synthesis of specific proteins, steroid hormones alter the physiology and behavior of an organism starting *in utero* and continuing throughout life. These hormonal effects are either organizational, which cannot be reversed and usually occur while an organism is developing, or activational, which can be reversed and occur during adulthood. The organizational nature of sex steroid hormones is most obvious in reproductive development and sex differentiation. Individuals can be classified according to their genetic, gonadal, and phenotypic sex, but it is only the phenotypic sex of an individual that is under great influence of circulating sex steroid hormones (i.e., estrogens and androgens). A genetic and gonadal male can be transformed to a phenotypic female by estrogens, while a genetic and gonadal female can be transformed to a phenotypic male by androgens, especially during the embryonic stage. More specifically, the main androgen produced by the male testes is testosterone, which plays a critical role in the development of the male phenotype, including the differentiation and development of the male sex organs. The main estrogen produced by the female ovaries is estradiol, but estradiol does not play a critical role in the development of the female phenotype until puberty because all individuals become female unless they are exposed to a high enough level of androgens. Exemplifying the importance of dose and timing of sex steroid hormones in development, one injection of testosterone into a female rat at a critical time of a few days after birth caused those females to never cycle as adults, while males given estrogen will assume the female mating posture and will not mount females.

During adulthood, the activational effects of androgens and estrogens on the development of secondary sexual characteristics, reproductive functioning, and germ production occur. The hypothalamo-pituitary gonadal hormone axis (HPG) is critical to understanding this role played by the sex steroids, as it regulates both estrogen and testosterone production. The hypothalamus releases gonadotropin-releasing hormone (GnRH), which travels to the pituitary and stimulates the production and release of the gonadotropins (luteinizing hormone [LH] and follicle-stimulating hormone [FSH]). These gonadotropins then circulate through the blood and bind to receptors in the gonads and stimulate the production and release of the sex hormones. The axis is controlled through a negative feedback loop where increasing levels of androgens or estrogens travel to the brain and bind to receptors there causing less GnRH to be produced from the hypothalamus and less gonadotropins from the pituitary. Consequently, the production of additional sex steroid hormones is reduced.

In males, LH and FSH are responsible for the production of testosterone and sperm production and maturation, while testosterone plays a vital role in the production of sperm, development of secondary sexual characteristics, and promotion of sexual behavior. Estrogens are also produced by and important to males: they play a role in the production and viability of sperm, aggressive and sexual behaviors, bone growth, and cardiovascular function. Androgens are converted to estrogens via the enzyme aromatase, and thus, levels of these two sex steroid hormones directly influence one another.

In females, LH and FSH are responsible for estrogen production and follicle production and maturation, while estrogens are important for the promotion of female secondary sexual characteristics, regulation of the ovulatory cycle, promotion of sexual behavior, maturation of fertilized ova, growth of the uterine wall, and implantation of fertilized ova in the uterus. Mirroring the male situation, androgens are also produced by and important to females, mainly because estrogens are produced by converting androgens via aromatase activity. The ovary mainly secretes estradiol during the follicular phase (i.e., before ovulation) of the ovulatory cycle and mainly secretes progesterone, a key progestin, during the luteal phase (i.e., after ovulation) and pregnancy. Estrogen levels gradually increase during the follicular phase, until they spike, along with LH and FSH, causing ovulation. Then progesterone levels gradually rise and stay elevated if the ovum is fertilized and the female becomes pregnant (progesterone maintains pregnancy). Otherwise, progesterone levels fall at the end of the cycle. If pregnant, estradiol and progesterone are produced by the placenta from precursor hormones produced by the fetal adrenal gland or circulating in maternal blood. At the time of parturition, progesterone levels drop and estrogen levels, which enhance uterine excitability, rise, leading to labor. Afterwards, lactation results in low estrogen levels until after weaning. The female HPG differs from males in that estradiol is required at the pituitary in order for GnRH to promote gonadotropin production and secretion, and therefore, the ovary controls cycle length and ovulation through estrogen production. Initial levels of estrogens during early follicular phase exert a negative feedback, but once levels rise and remain elevated, a positive feedback is triggered, resulting in the LH and FSH surge that signals ovulation.

Based upon the various roles of sex steroid hormones in vertebrate male and female development, physiology, and behavior, it is clear that interference with estrogens, androgens, and/or progestins can have important consequences for reproduction in both sexes.

PHYTOESTROGEN EFFECTS: MOLECULAR, PHYSIOLOGICAL, AND BEHAVIORAL INTERFERENCE

Relevance of Phytoestrogens to Primate Reproduction and Health

There are a number of factors that influence steroid hormone levels in primates, including social factors (e.g., dominance hierarchies, dispersal events) and ecological factors (e.g., parasitism, predation; Fig. 1.2). These sources of variation can have important consequences for primate reproduction and health. One of the most direct links between the environment and primate endocrine system occurs through the diet, especially through interactions with plant compounds that mimic endogenous vertebrate hormones. Due to this mimicry, phytoestrogens have been the focus of numerous medical studies interested in the potential benefits of these compounds to prevent cancer or treat menopausal symptoms, as well as the potential costs of disrupting the developing endocrine system (e.g., from soy formula fed to infants) and reducing the fertility of both males and females (Whitten & Patisaul 2001). As with many aspects of biology, a tradeoff between survival and reproduction will determine the cost-benefit ratio of consuming phytoestrogens (Wingfield & Sapolsky 2003).

Additionally, the costs and benefits of phytoestrogen consumption will be influenced by the age and sex of the consumer and the amount, type, and mechanism of action of the phytoestrogen consumed. Phytoestrogens are expected to exert their strongest effects on pregnant females and their developing offspring, as well as infants, juveniles, and subadults, who are still developing (i.e., through organizational effects). However, activational changes in adult males and females are also expected. Because adult male sex steroid levels do not fluctuate as much as those of the other sex/age classes, detecting phytoestrogen-induced changes in adult male hormonal status or the physiological and behavioral effects of these changes may be easier than for other sex/age classes. Further, any effect found in adult males is likely just as significant for other sex/age classes.

Molecular Mechanism of Action: How Phytoestrogens Interfere with Sex Steroids

In general, any costs or benefits of phytoestrogen consumption result from interactions with the vertebrate endocrine system. Phytoestrogens can disrupt the activity of endogenous estrogens by interacting with estrogen receptors (ERs, Krishnan et al. 1993), interfering with enzymes responsible for hormone metabolism (e.g., aromatase, Hayes et al. 2002), or binding to the sex-hormone binding globulin (SHBG) responsible for transporting sex hormones throughout the body (Whitten & Patisaul 2001). In this discussion, I will focus only on those compounds that bind to ERs, as this appears to be the prominent mechanism of action (Whitten & Patisaul 2001).

Most phytoestrogens operate through their selective activity at ERβ (Kuiper et al. 1998). Selective binding to ERβ has important ramifications for physiology and behavior, as ERβ regulates different genes than ERα, although there is some overlap between the two ERs (Leitman et al. 2010). Through their interaction with ERβ and competition with endogenous estrogens for binding to these receptors, phytoestrogens can act as either estrogen agonists (i.e., promoting estrogenic activity) or antagonists (i.e., blocking estrogenic activity) depending on the dose ingested, strength of the specific compounds, and endogenous hormonal state of the feeder (Almstrup et al. 2002, Leitman et al. 2010). At low doses, phytoestrogens tend to decrease estrogenic activity, while at high doses they increase it (Almstrup et al. 2002). Thus, endogenous estrogenic activity can either increase or decrease depending on the dose and strength of the phytoestrogen consumed.

For a phytoestrogen to bind to ERβ and alter the activity of genes regulated by this receptor-ligand complex, an estrogenic plant must first be ingested by a primate. This plant is digested and nutrients and other plant chemicals are absorbed at various points along the gastrointestinal tract. Once phytoestrogens pass from the GI tract into the bloodstream, with the amount absorbed dependent upon both species and individual (due to differences in gut morphology and microbial community), they travel throughout the body. As they pass into cell types containing ERβ, including reproductive organs and the brain (Whitten & Patisaul 2001), they form a ligand-ERβ complex (Leitman et al. 2010). This complex travels to the nucleus of the cell and binds to the estrogen response element (ERE) on intranuclear chromatin or causes cellular changes through non-genomic mechanisms (Leitman et al. 2010). It is well established that phytoestrogens bind to ERβ, activate cellular processes, and alter biological functioning at the molecular level. More importantly for the ecology and evolution of primates is whether these molecular effects are great enough to manifest themselves in physiological and behavioral changes.

Physiological and Behavioral Endpoints: How Phytoestrogens Interfere with Reproduction

Not all phytoestrogens binding to and activating ERβ produce the same endpoints, so generalizations about likely physiological and behavioral effects are difficult (Whitten & Patisaul 2001). To further complicate matters, such compounds have tissue-specific effects and the concentration in the blood, concentration of endogenous estrogen which will compete for binding to the ERs, and the concentration of ERs and SHBG will all affect the outcome. Ideally, each estrogenic plant and its phytoestrogen(s) need to be examined separately, as well as the synergistic effects of such compounds ingested together. In general, understanding the biological effects of phytoestrogen consumption is complex and context dependent. Nonetheless, a few generalizations about probable physiological and behavioral effects are possible. Here, I will focus on how phytoestrogens alter hormone levels, cell growth, fertility, and sexual, aggressive, and anxiety-related behaviors.

Hormonal Changes:

At the organizational level, *in utero* exposure to phytoestrogens appears to directly influence the phenotype of developing animals through DNA methylation, with sex hormone levels being one of the affected traits (Cline & Wood 2009). As far as activational effects in adults, a number of studies have demonstrated hormonal changes due to phytoestrogen consumption. In humans eating their usual diet, consumption of phytoestrogens was related to an increase in SHBG levels and a decrease in plasma estradiol levels (Adlercreutz et al. 1987).

In captive adult female cynomolgus monkeys fed the estrogenic *Pueraria mirifica*, urinary FSH and estradiol levels were suppressed and menstruation ceased when they consumed this plant daily at the highest dose given, while no effect was detected when fed only a single dose (Trisomboon et al. 2007). This reduction in FSH and estradiol was thought to occur through the action of phytoestrogen on ERs in the hypothalamus and pituitary, thus triggering the negative feedback mechanism of HPG. Effects of phytoestrogens on endogenous hormone levels are likely to occur through disruption of the negative feedback loop of the HPG. It is not only estradiol production that can be shut off, but also testosterone production, which can be reduced or shut off with increasing levels of estrogens (Hadley 2000). Exemplifying this, genistein, a phytoestrogen in soy, has been shown to suppress the HPG, with testosterone and LH levels reduced in adult male mice exposed to this compound (Whitten & Patisaul 2001). Alternatively, no effects of isoflavone consumption on testosterone or estradiol levels were found in captive cynomolgus monkeys (Simon et al. 2004).

Cell growth:

Because ERβ has an anti-proliferative effect on cell growth, while ERα promotes cell growth, plants containing phytoestrogens with ERβ selectivity are of particular interest for treating or preventing estrogen-dependent cancers, hormone replacement therapy in menopausal women, and preventing osteoporosis (An et al. 2001, Cvoro et al. 2007, Heldring et al. 2007, Leitman et al. 2010). Such plant compounds appear to promote many of the actions of endogenous estrogens without the added risk of cancer promotion found in compounds with ERα activity. However, the effects of phytoestrogens on cancer depend on more than just ER selectivity. If exposed in utero, genistein, a phytoestrogen found in soy, increases the risk of breast cancer, while exposure from the neonatal through adult stages decreases risk, with greatest benefits coming from earliest age of exposure (Cline & Wood 2009). However, if pre-cancerous cells are already present, genistein increases growth of these cancerous regions. Further, low doses of phytoestrogen tend to promote cell growth, while high doses inhibit it (Whitten & Patisaul 2001).

For primates, mediation of cell growth by phytoestrogens and its implications for cancer susceptibility is an area of research well worth exploring. Non-human primates are particularly immune to the carcinogenic effects of estrogens, as indicated by the rarity of breast cancer in captive monkey and ape colonies even when exposed to potent carcinogens (Adlercreutz et al. 1986, Musey et al. 1995). This may, in part, be due to diets high in phytoestrogens and the presence of gut microorganisms that can convert these phytoestrogens to more bioactive forms, such as equol (Adlercreutz et al. 1986, Musey et al. 1995). Non-human primates may benefit from the cancer protective mechanisms of ERβ selective phytoestrogens.

Fertility:

If phytoestrogens act as agonists and increase estrogenic activity, adult male fertility (through sperm production) can decrease and feminization can occur (Guillette 2000, Hayes 2005, Cederroth et al. 2010a). For example, Cederroth et al. (2010b) found a 25% reduction in sperm counts in male mice fed a high soy diet starting *in utero* and a 21% reduction in litter size compared to mice fed a soy free diet. In addition, growth (i.e., smaller body mass) and reproductive development (i.e., decreased anogenital distance) of male rats were affected by exposure to genistein through the maternal diet during gestation and lactation as compared to a geinstein-free maternal diet, suggesting an irreversible organizational effect from phytoestrogens on morphology important to reproduction (Ball et al. 2010). For females, changes in the timing of ovulation, and even failure to ovulate, have been documented, likely mediated by disruption of negative feedback of HPG axis. This is a mechanism similar to birth control where exogenous estrogens and progestins block the LH/FSH surge necessary for ovulation through the negative feedback mechanism (Hadley 2000). More specifically, genistein was found to affect female reproductive development (i.e., earlier vaginal opening and smaller ovaries) and cause longer estrous cycles (Kouki et al. 2003). Additionally, resveratrol, a phytoestrogen found in grapes that binds to both ER subtypes, reduced body weight, disrupted the estrous cycle, and increased ovarian weight in adult female rats (Henry & Witt 2002).

Behavioral Changes:

Phytoestrogens, including genistein, have been found to decrease mating behavior in adult female rats (Kouki et al. 2003, Hartley et al. 2003, Patisaul et al. 2004). In captive adult male cynomolgus monkeys (*Macaca fascicularis*), a 15-month soy-based high-isoflavone diet resulted in an increase in aggressive behaviors (i.e., 67% more frequent compared to individuals fed an isoflavone-free diet), an increase in submissive behaviors (i.e., 203% more frequent), and a decrease in affiliative behaviors (i.e., 68% less time in body contact and 30% more time alone; Simon et al. 2004). It was postulated that these effects were likely due to the weaker action of isoflavones on ERβ than estradiol, thus reducing the inhibition of the aggression–promoting action of ERα (Simon et al. 2004). Male rats fed a high-isoflavone diet spent less time in social interactions and had higher corticosterone response to stress than rats fed an isoflavone-free diet (Hartley et al. 2003). Overall, the isoflavone fed rats had greater anxiety related physiological and behavioral measures than isoflavone free rats. Similarly, the ERβ agonist equol increased aggression and anxiety in male rats that were exposed neonatally (Patisaul & Bateman 2008). However, other studies found no effect of equol on anxiety-related behaviors in adult male rats (Patisaul et al. 2009). Behavioral effects may be most prominent when exposure occurs early in life or greatly influenced by dose or timing of exposure.

Summary of Phytoestrogen Effects:

This review covers only a small proportion of the laboratory-based studies that have examined the physiological and behavioral effects of phytoestrogens in a captive setting, including changes in hormone levels, cell growth, fertility, and behavior. Nonetheless, it is clear that these are significant changes that would likely result in differential survival and reproduction if found in primates in a natural setting. Field studies of wild primates should begin examining relationships between phytoestrogen consumption and hormone levels, survival rates, fitness, and behavior to determine if these laboratory-based effects occur in natural systems.

SCALING UP: THE POTENTIAL IMPORTANCE OF PHYTOESTROGENS FOR PRIMATE ECOLOGY AND EVOLUTION

Phytoestrogens as a Selective Pressure on Primates?

The physiological and behavioral effects discussed in the previous section have the ability to influence the survival and reproduction of an individual, and thus phytoestrogens may act as a selective pressure through promotion of differential survival and reproduction. For example, lowered fertility and fecundity, as well as altered behavior leading to altered survival rates, could produce suboptimal fitness for individuals, groups, populations, or species that ingest more of these estrogenic compounds, as suggested by the “plant defense hypothesis”. Alternatively, the consumption of phytoestrogens could increase fertility, fecundity, and survival as is suggested by the “chemical cue/self-medication hypotheses”. Either way, effects of phytoestrogen ingestion may play an important, thus far neglected, role in primate ecology and evolution through differential survival and reproduction.

Furthermore, because primates are generally a highly herbivorous group, most primate species likely encounter phytoestrogens to varying degrees in their diets. Supporting this, phytoestrogens are most prevalent in legumes (i.e., Fabaceae, Reynaud et al. 2005) and leguminous plant foods are often an important part of the diet for many primates, particularly folivores, because of their protein content (Chapman et al. 2002). However, certain groups of primates likely encounter phytoestrogens more often in the diet due to the types of plant parts consumed. Because leaves are photosynthetic organs essential to a plant’s energy production, they are commonly defended from herbivory through the production of certain types of chemicals (e.g., tannins, alkaloids) (Coley & Barone 1996). In contrast, fruits exist mainly as a means for seed dispersal, and thus, ripe fruits are not expected to contain chemical defenses. Therefore, if phytoestrogens are often produced as a plant defense, then it is expected that they are more prevalent in leaves than fruits, and thus more prevalent in the diets of folivorous species (e.g., colobines) than the diets of frugivorous species (e.g., cercopithecines, chimpanzees). Regardless of whether it is the plant or the primate that is obtaining an evolutionary advantage from phytoestrogens, the heavily plant-dependent diet of most primates suggests that consumption of phytoestrogens does occur to some degree. To my knowledge, how heavily and frequently wild primates feed on such compounds has not yet been addressed.

Variation in exposure to phytoestrogens and the magnitude of phytoestrogen effects likely occurs at both the intraspecific and interspecific levels for reasons other than dietary niche. It has been found that both interspecific and intraspecific variation exists in phytoestrogen metabolism, with individuals and species differing in number and types of microorganisms that convert phytoestrogens to more active estrogenic compounds in the gut (Adlercreutz et al. 1987, Patisaul et al. 2009). Chimpanzees are known to excrete much greater amounts of the more bioactive equol in their urine than humans, possibly explaining their rarity of estrogen-dependent cancers, even when exposed to exogenous estrogens or potent carcinogens (Adlercreutz et al. 1987, Musey et al. 1995). Chimpanzees apparently convert diet-based phytoestrogens into more bioactive forms more effectively than humans do, possibly through different gut microorganism activity or endogenous metabolism (Musey et al. 1995).

Although evidence for physiological and behavioral effects of phytoestrogen consumption with potential importance to differential survival and reproduction, as well as evidence for sources of variation in exposure to phytoestrogens at both the intraspecific and interspecific levels, exists, almost no empirical data have been collected to evaluate the role of phytoestrogens in primate ecology and evolution. This is an important area of research in need of both field and lab-based studies.

Overview

In this review, I have attempted to summarize what is known about phytoestrogens from a range of perspectives, including their ecological and evolutionary roles and physiological and behavioral effects. In addition, I have provided a summary of how steroid hormones function at the molecular, physiological, and behavioral levels to demonstrate how plant chemicals can interact with these processes. In doing so, my ultimate objective has been to convince the reader that phytoestrogens have the potential to play an important role in primate ecology and evolution, especially since most primate species depend heavily on a plant-based diet that likely contains such hormone-mimicking compounds. This is an exciting area of inquiry for primatologists with many questions in need of study.

Questions at the ecological level include: Which primate species consume estrogenic plants in their diets? Do primates actively select for estrogenic plants, avoid them, or are they simply indifferent to their presence? How are various phytoestrogens metabolized by gut microorganisms and to what extent do inter- and intraspecific differences in gut microbial ecology alter the bioactivity of these compounds? What are the physiological and behavioral effects of consuming estrogenic plants for wild primates? Do phytoestrogens result in less cell growth, suboptimal fertility, increased aggression, or decreased sexual behavior as seen in captive studies? Questions at the evolutionary level include: If altered physiology and behavior are seen in wild primates, are the magnitudes of these changes great enough to affect survival and reproduction such that fitness differs between individuals or populations feeding on such plants versus those that do not? Which of the three hypotheses, the "plant defense", "self-medication", or "neutral" hypothesis, best explains the relationship between primates and estrogenic plants? Is this a general trend or does it vary by species and context? Do phytoestrogens in wild plant foods act as important selective forces and therefore play a role in the evolution of primates? Comparative studies of primates and their plant foods are needed to address these questions.

As a final thought, it is worth considering that phytoestrogens could be an overlooked, important factor influencing the continued survival of endangered primate species, where optimal reproduction is vital to their persistence. Combined with the threat of deforestation, logging, hunting, and climate change, the potential of increased estrogenic plants in the environment could set up a situation where isolated wild populations could be at further risk of extinction due to suboptimal recruitment, especially for long-lived species with relatively few offspring, such as primates.

FIGURE LEGEND

Figure 1.1. Percent of total phytosteroids from each specific phytosteroid found in plant samples collected from Kibale National Park, Uganda, and measured using immunoassays for each of four steroid hormones (cortisol, androgens, progesterone, and estradiol). YL = young leaves, ML = mature leaves

Figure 1.2. Potential factors that can influence the steroid hormone levels of wild primates.

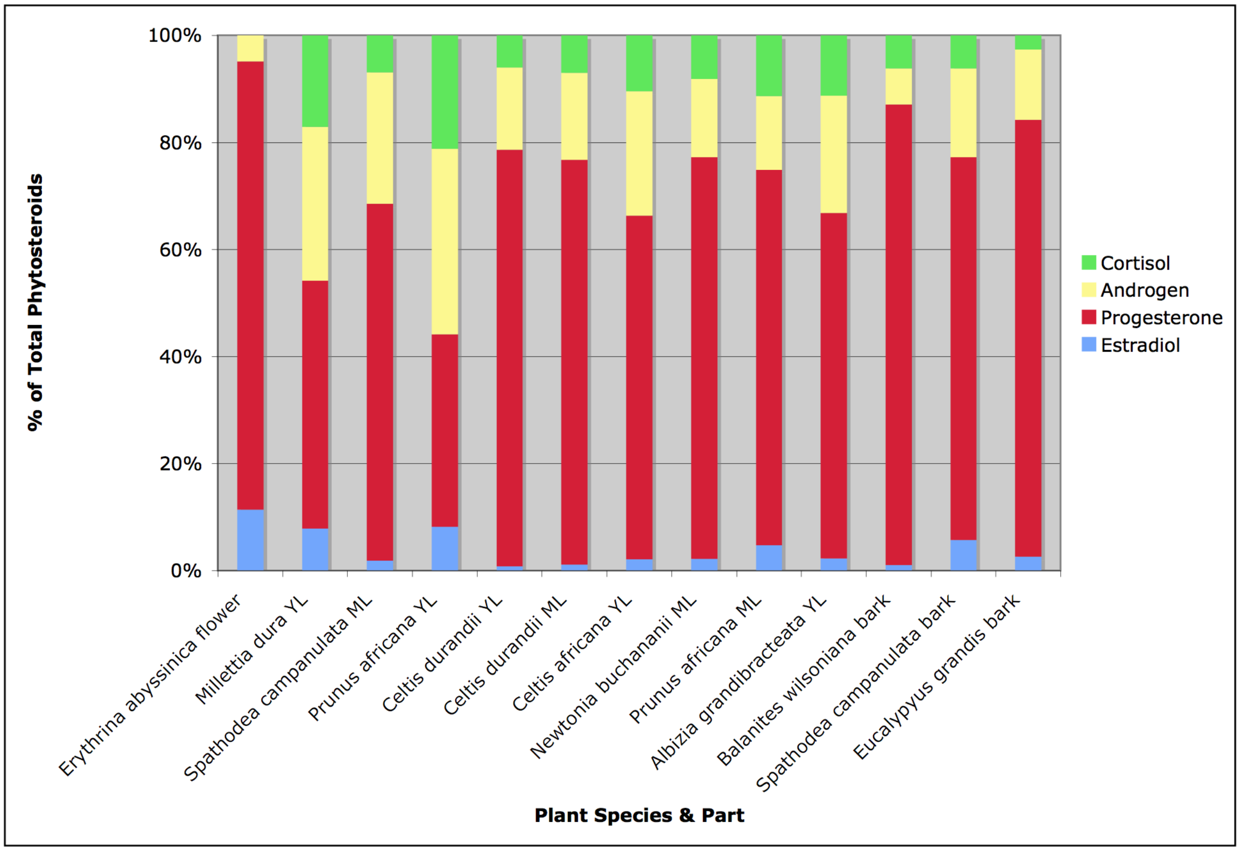

100%
80%
60%
40%
20%
0%
% of Total Phytosteroids
Cortisol
Androgen
Progesterone
Estradiol
Erythrina abyssinica flower
Millettia dura YL
Spathodea campanulata ML
Prunus africana YL
Celtis durandii YL
Celtis durandii ML
Celtis africana YL
Newtonia buchananii ML
Prunus africana ML
Albizia grandibracteata YL
Balanites wilsoniana bark
Spathodea campanulata bark
Eucalypyus grandis bark
Plant Species & Part

Figure 1.1

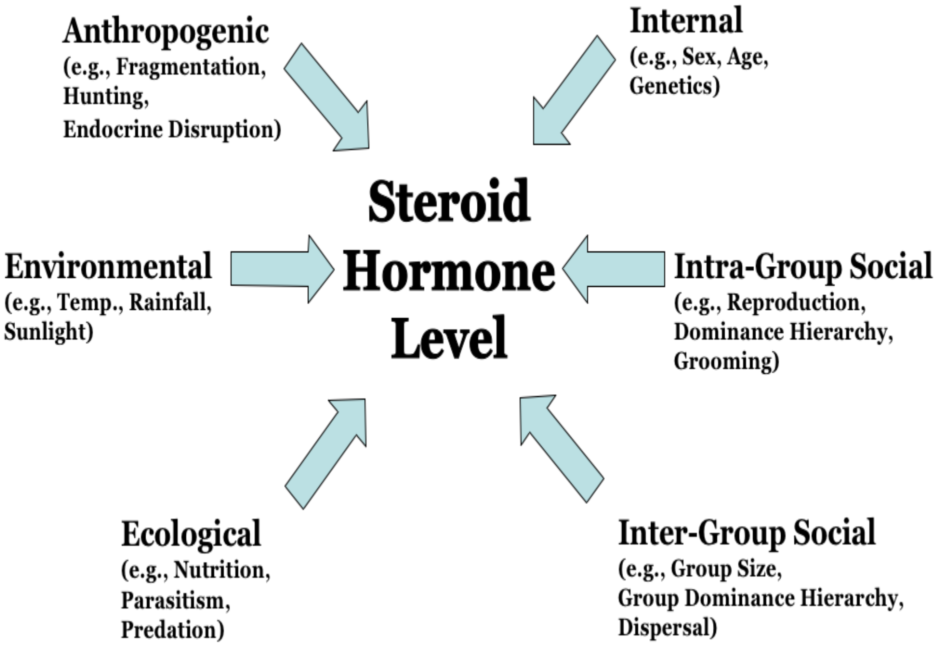

Anthropogenic
(e.g., Fragmentation,
Hunting,
Endocrine Disruption)
Internal
(e.g., Sex, Age,
Genetics)
Steroid
Hormone
Level
Environmental
(e.g., Temp., Rainfall,
Sunlight)
Intra-Group Social
(e.g., Reproduction,
Dominance Hierarchy,
Grooming)
Ecological
(e.g., Nutrition,
Parasitism,
Predation)
Inter-Group Social
(e.g., Group Size,
Group Dominance Hierarchy,
Dispersal)

Figure 1.2

TABLE 1.1. Summary of field studies that either examined or suggested the possibility of phytosteroid-containing plants, including estrogenic plants, to influence primate ecology.

Primate Species	Plant Species	Evidence	Reference
Phayre's leaf monkey (*Trachypithecus phayrei*)	*Vitex spp.* fruit and leaves (Lamiaceae)	hormonal, behavioral, ethnobotanical	Lu et al., 2010
Common chimpanzee (*Pan troglodytes*)	*Vitex fisheri* fruit (Lamiaceae)	hormonal, behavioral, ethnobotanical	Wallis, 1997; Emery Thompson, 2008
Olive baboon (*Papio hamadryas*)	*Vitex doniana* ripe fruit and young leaves (Lamiaceae)	hormonal, behavioral, morphological, immunoassay for plant hormones, ethnobotanical	Higham et al., 2007
Modern human (*Homo sapiens*)	many, e.g., soy (*Glycine max*) (Fabaceae)	ethnobotanical, hormonal, *in vitro* and *in vivo* assays, phytochemical	Many, for a review see Wynne-Edwards, 2001
Howler monkey (*Alouatta palliata*)	unknown – none suggested	hypothesized – no evidence presented	Glander, 1980
Vervet monkey (*Cercopithecus aethiops*)	*Acacia elatior* flowers (Fabaceae)	hypothesized - behavioral, *in vitro* bioassay	Whitten, 1983; Garey, 1993
Kenyan galago (*Galago senegalensis*)	*Acacia drepanolobium* gum (Fabaceae)	hypothesized - behavioral, phytochemical	Nash & Whitten, 1989
Muriquis (*Brachyteles arachnoides*)	*Enterolobium contortisiliquum* fruit (Fabaceae)	hypothesized - behavioral, phytochemical	Strier, 1993

Table 1.2. Amount of plant compounds that bound to four different steroid hormone antibodies in immunoassays as measured for various plant species/part items. Phytosteroid levels are ng / g of dry plant material.

Plant species	Plant Part	Estradiol	Progesterone	Androgen	Cortisol	Total Binding
Celtis durandii	young leaves	10.9	1054	207.3	81.1	1353.3
Celtis durandii	mature leaves	10.2	675	145.1	62.2	892.5
Spathodea campanulata	mature leaves	15.1	526	193.5	54.4	789.0
Balanites wilsoniana	bark	6.1	503	39.5	35.9	584.5
Celtis africana	young leaves	9.7	295	106.6	47.9	459.1
Newtonia buchananii	mature leaves	7.8	270	52.5	29.2	359.6
Albizia grandibracteata	young leaves	7.4	208	70.6	36.2	322.2
Millettia dura	young leaves	18.8	110	68.3	40.7	237.8
Erythrina abyssinica	flower	21.8	160	9.2	0.0	191.0
Prunus africana	mature leaves	7.6	112	22.1	18.1	159.8
Prunus africana	young leaves	11.4	50	48.1	29.5	139.0
Eucalyptus grandis	bark	2.9	89	14.4	2.8	109.1
Spathodea campanulata	bark	5.4	67	15.5	5.7	93.6

CHAPTER 2:

The estrogenic plant foods of red colobus monkeys and mountain gorillas in western Uganda

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. I investigated the prevalence of estrogenic plant foods in the diets of two strongly 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, I screened 44 plant items 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 bound to and activated ERβ, but not ERα. These results confirm that estrogenic plant foods are routinely consumed by two highly folivorous African 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.

KEYWORDS: primate, phytoestrogens, feeding ecology, environmental endocrinology, Kibale National Park, Bwindi Impenetrable National Park

INTRODUCTION:

Naturally occurring estrogen-mimicking compounds (i.e., phytoestrogens) are found in many plant species (Dixon 2004), including a number of human plant-based foods (e.g., soy; Kurzer & Xu 1997). Because 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, considerable biomedical research has been focused on understanding the physiological and behavioral effects of phytoestrogens primarily using captive rodents and primates as models (Whitten & Patisaul 2001, Messina 2010). The examination of the phytoestrogen-animal relationship outside the laboratory has focused largely on domesticated livestock. For example, an estrogen mimic found in a non-native 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 (i.e., 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 hamadryas*] in Nigeria [Higham et al. 2007]) have examined a related class of hormone-mimicking plant compounds, phytoprogesterones (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 thought to affect reproduction. Furthermore, it is impossible to yet 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. To fully understand the influence of a single plant species on a wild primate’s hormone levels, data on the relative proportion that dietary item contributes to the overall diet within a given time frame is critical. 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 primate biology. The synergistic interactions among steroid-mimicking 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).

There are a number of ways that a plant compound could alter the endocrine functioning of a primate, with significant downstream 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 & Patisaul 2001). The most well studied phytoestrogens are those that compete with endogenous estrogens to bind to ERs and then either promote estrogenic activity (i.e., agonists) or block it (e.g., antagonists) (Leitman et al. 2010). Often, a specific phytoestrogen can act as either depending upon the dose of the compound consumed and the levels of endogenous estrogens in the animal. 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 upon 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.

To quantify the prevalence of phytoestrogens (defined here as compounds that bind to and promote estrogenic activity through the ERs) in the diets of two highly 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), I screened the majority (i.e., > 75% of diet) of their plant foods for estrogenic activity using transient transfection assays. Since 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 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 & 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 9 kg forestomach-fermenting obligate folivore that lives in multimale-multifemale groups with an average group size of 65 individuals (Snaith et al. 2008). *Procolobus rufomitratus* is considered vulnerable, with the only viable population remaining in KNP (Struhsaker 2005). As a morphologically-specialized folivorous primate dependent upon its symbiotic gut bacteria (Bauchop & Martucci 1968, Milton 1980, Lambert 1998), they are an ideal study species for examining the presence of phytoestrogens in the wild primate diet. 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 validity, 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 & Yildiz 2006, Setchell & 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 approximately 302 individuals (McNeilage et al. 2001*;* 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 (Rothman et al. 2008). 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 approximately 70 individuals) located near the Kanyawara research station in KNP from August 13, 2007 to June 27, 2008 (258 days of sampling), for a total of 1327 hrs. 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 hrs (Rothman et al. 2007; 2008).

For red colobus, data were collected six days per week from 0800 to 1300 h using scan samples of five individuals every 30 minutes. When feeding, the plant species and parts being consumed were identified. I 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. (2007, 2008) for detailed description of behavioral data collection and determination of diet for the 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 to University of California-Berkeley (UCB) for determination of estrogenic activity in 2008 (see Rothman et al. 2007, 2008 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 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 determination of estrogenic activity via transient 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 three 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 osteocarcoma cells (U2OS) used in the transient transfection assays, the 0.1 g plant extract / 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). Cells (U2OS) 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 either ERα or ERβ expression vectors, and electroporated with a gene pulser so that the ERE and ER would be incorporated into the cells. I then added either 1.5 μl of each plant extract in DMSO / 100% ethanol solution, 1.5 μl 10nM estradiol (positive control), or nothing (blank control) to the transfected cells in triplicate and allowed the cells to incubate overnight. The following day, the cells were lysed and the amount of light given off (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 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 16 for ERα and 2 for 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, 14 plant items from 11 species making up 12.6% of the diet of the red colobus and 50 plant items from 39 species making up 77.4% of the diet of the mountain gorilla were screened. Fewer items were screened for activity at ERα due to the rarity of plant compounds having activity at this receptor (Leitman 2010, *personal communication*) and the lack of activity at ERα found for plants that were shown to have ERβ activity in this study (0 / 8 ERβ active plants).

My 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. I 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 (79.4%) came from 23 staple dietary items (i.e., foods comprising > 1% of diet, Table 2.1). 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.

None of the 14 items screened had activity at ERα; eight of the 44 items screened had activity at ERβ (Table 2.2; Figure 2.1). These estrogenic items were from five species and three plant families: Fabaceae [2 species], Moraceae [2 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 2.1). The other five estrogenic items were rare foods: *Erythrina abyssinica* young leaves and flowers, *Ficus brachylepis* unripe fruit and young leaves, and *Ficus natalensis* unripe fruit. These five foods comprised 0.6% of the red colobus diet (Table 2.2). 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 2.3). Of 53 dietary items tested, representing 85.2% of annual diet, two had activity at ERβ, while none of the 50 items had activity at ERα (Table 2.4). 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 a staple dietary item comprising 8.8% of the annual diet (Tables 2.3, 2.4). Thus, at least 8.8% of the gorilla diet was comprised of estrogenic plants.

DISCUSSION:

This study demonstrated that two highly 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 one staple food: *Ipomoea involucrata* leaves. Further, all plants with estrogenic activity were only active at ERβ and not at ERα.

One of the most interesting results of this study, 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 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, we know that each ER has different, non-redundant roles in the nervous, immune, cardiovascular, and skeletal systems, as well as opposing actions on cell proliferation across numerous tissues, including the uterus, ovary, 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). Primate consumption of estrogenic plants with ERβ selectivity may explain the low incidence of cancer in this taxon, with modern humans the one exception (Greaves 2007). 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 & Sapolsky 2003). To determine the biological significance of consuming ERβ active plants for wild primates, future studies should examine the relationship between the consumption of these plants and the primate's physiology and behavior.

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 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 (Yenesaw et al. 1996). A related species, *M. griffoniana*, 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 & Pal 1966; Jondhale et al. 2009). Although not native to or used medicinally in

Africa, *Eucalyptus grandis* is an important fuelwood species there and an important source of paper products throughout the world. Studies of kraft 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 non-native tree species growing along the edges of both KNP and BINP, and that numerous primate species (e.g., black-and-white colobus monkeys [*Colobus guereza*, Harris & Chapman 2007], mountain gorillas [Rothman et al. 2006b], red colobus monkeys [MW, *personal observation*], Guatemalan black howler monkeys [*Alouatta pigra*, Serio Silva et al. 2006]) 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 convolucrata*, is used in traditional medicine in Rwanda (Sindambiwe et al. 1999) and Nigeria (Olukoya et al. 1993); the leaves are eaten by the Lele of Guinea because they are thought to increase fecundity (Wallace et al. 1998).

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 highly 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 highly folivorous diet (Lambert 1998, Milton 1999, Remis 2000, Rothman et al. 2008). Because of this, gorillas are much more similar to other primate taxa that consume leaves (e.g., howler monkeys [*Alouatta*]) than is the red colobus. As a forestomach fermenting obligate folivore, the red colobus monkey is dependent upon its specialized digestive morphology and 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. For instance, some phytoestrogens (e.g., formononetin, daidzein) are converted to the more bioactive compound, equol, via bacterial metabolism in numerous animal species (Setchell & Clerici 2010). Interspecific differences in the production of equol exist, as rodents and chimpanzees are more efficient equol-producers than humans or pigs, and it is suggested that these differences are due to differences in gut microbial communities (Adlercreutz et al. 1986, Setchell & 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 brachylepis*, 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 primate species both in the forests of western Uganda and elsewhere in Africa, Latin America, and Asia, are regularly consuming phytoestrogens in staple foods, regardless of geography or 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.

FIGURE LEGEND

Figure 2.1. 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 E_2 (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).

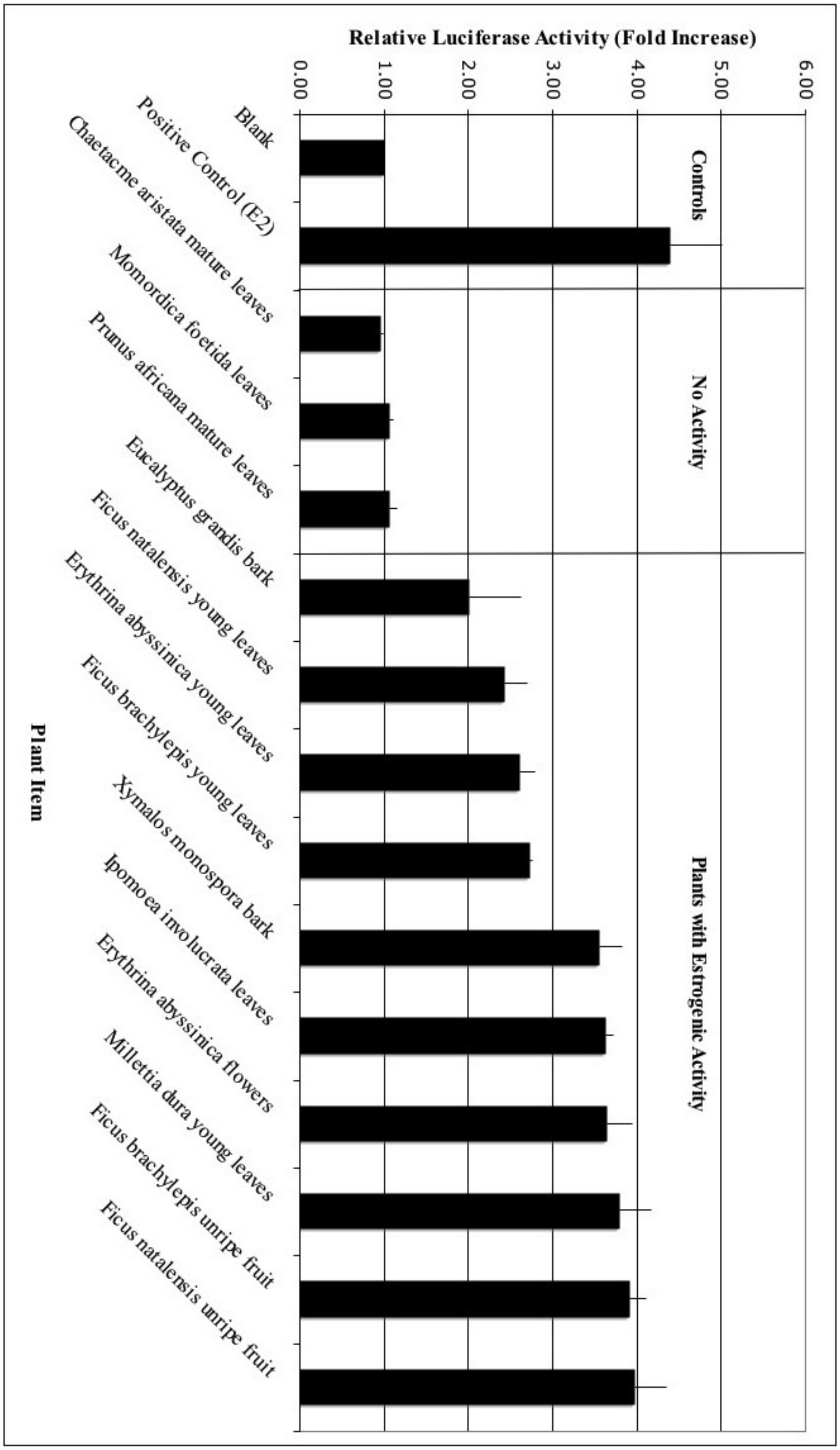

Relative Luciferase Activity (Fold Increase)
0.00
1.00
2.00
3.00
4.00
5.00
6.00
Controls
No Activity
Plants with Estrogenic Activity
Blank
Positive Control (E2)
Chaetacme aristata mature leaves
Momordica foetida leaves
Prunus africana mature leaves
Eucalyptus grandis bark
Ficus natalensis young leaves
Erythrina abyssinica young leaves
Ficus brachylepis young leaves
Xymalos monospora bark
Ipomoea involucrata leaves
Erythrina abyssinica flowers
Millettia dura young leaves
Ficus brachylepis unripe fruit
Ficus natalensis unripe fruit
Plant Item

Figure 2.1.

TABLE 2.1. Staple dietary items (i.e. foods comprising > 1% of total diet) accounting for 79.4% 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	ERβEstrogenic Activity?
Newtonia buchananii	Fabaceae (Mimosoideae)	young leaves	10.2	no
Bosqueia phoberos	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 mukole	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.		young leaves	1.7	no
Ficus natalensis	**Moraceae**	**young leaves**	**1.5**	**yes, all parts tested**
Mestrazylon sp.		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 latifolia	Apocynaceae	young leaves	1.1	no
Mimusops bagshawei	Sapotaceae	young leaves	1.1	not tested
Urella sp.		young leaves	1.1	not tested
Total			**79.4%**	**10.0% of diet from estrogenic staples**

TABLE 2.2. Transient transfection assay data for red colobus monkey plant foods showing which items (species/part) had activity at ERα and/or ERβ. Estrogenic items shown in bold.

Plant Species	Family	Part	% Diet	ERα Relative Luciferase Activity[a]	ERβ Relative Luciferase Activity[b]
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
Alangium chinese	Alangiaceae	ML	0.2	not tested	0.68
Aningeria altissima	Sapotaceae	YL	0.1	not tested	0.99
Bosqueia phoberos	Moraceae	YL	9.3	not tested	0.74
Bosqueia phoberos	Moraceae	ML	<0.1	not tested	0.96
Bridelia sp.	Euphorbiaceae	YL	1.7	not tested	1.16
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
Chrysophyllum sp.	Sapotaceae	ML	<0.1	not tested	1.15
Chrysophyllum sp.	Sapotaceae	YL	0.1	not tested	0.84
Diospyros abyssinica	Ebenaceae	YL	<0.1	0.88	1.51
Dombeya mukole	Sterculiaceae	YL	4.5	not tested	0.66
Dombeya mukole	Sterculiaceae	ML	<0.1	not tested	0.74
Erythrina abyssinica	**Fabaceae**	**YL**	**<0.1**	0.90	**2.62**
Erythrina abyssinica	**Fabaceae**	**FL**	**0.1**	1.29	**3.65**
Eucalyptus grandis	**Myrtaceae**	**BA**	**3.4**	not tested	**2.01**
Fagara angolensis	Rutaceae	YL	0.2	not tested	1.16
Ficus brachylepis	**Moraceae**	**UF**	**0.1**	1.06	**3.92**
Ficus brachylepis	**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
Funtumia latifolia	Apocynaceae	YL	1.1	not tested	1.06
Funtumia latifolia	Apocynaceae	ML	<0.1	not tested	0.68
Hypocreata sp.		YL	1.7	not tested	0.97
Macaranga sp.	Euphorbiaceae	YL	2.4	not tested	0.91
Markhamia platycalyx	Bignoniaceae	YL	0.2	1.23	0.93
Markhamia platycalyx	Bignoniaceae	PT	0.7	1.34	1.92
Millettia dura	**Fabaceae**	**YL**	**5.1**	0.99	**3.79**
Newtonia buchananii	Fabaceae	ML	0.9	not tested	1.14

TABLE 2.2 cont. Transient transfection assay.

Plant Species	Family	Part	% Diet	ERα Relative Luciferase Activity[a]	ERβ Relative Luciferase Activity[b]
Newtonia buchananii	Fabaceae	YL	10.2	not tested	1.05
Olea welwitchii	Oleaceae	YL	0.2	1.06	1.88
Parinari excelsa	Chrysobalanaceae	YL	2.7	not tested	0.88
Prunus Africana	Rosaceae	ML	3.1	not tested	1.06
Prunus Africana	Rosaceae	YL	7.3	not tested	1.60
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
Teclea nobilis	Rutaceae	YL	0.5	0.78	1.46

[a] For ERα assays, relative luciferase activity for positive control (E2) = 33.18 (+/- 6.55) (n = 3); estrogenic activity defined as > 16-fold 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 > 2-fold increase as compared to the blank (absence of ligand).

YL = young leaves, ML = mature leaves, UF = unripe fruit, FL = flower, BA = bark, PT = petiole, DW = dead wood

TABLE 2.3. 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	ERβEstrogenic 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).

TABLE 2.4. Transient transfection assay data for mountain gorilla plant foods showing which items (species/part) had activity at ERα and/or ERβ. Estrogenic items shown in bold.

Plant Species	Family	Part	% Diet[a]	ERα Relative Luciferase Activity[b]	ERβ Relative Luciferase Activity[c]
Achyranthes aspera	Amaranthaceae	L	<1.0	1.44	0.82
Adenia gummifera	Passifloraceae	L	<1.0	1.04	0.94
Allophlylus abyssinicus	Sapindaceae	L	<1.0	0.95	0.98
Arundinaria alpina	Poaceae	ST	<1.0	1.22	0.93
Basella alba	Basellaceae	L	7.8	not tested	1.55
Carpodinus glabra	Apocynaceae	L	<1.0	1.45	0.84
Carduus kikuyorua	Asteraceae	L	4.2	0.56	0.74
Cassipourea rwenzoriensis	Rhizophoraceae	DW	<1.0	1.04	0.95
Chrysophyllum albidum	Sapotaceae	DW	<1.0	1.33	0.89
Cyperus renschii	Cyperaceae	GS	<1.0	0.99	0.91
Desmodium repandum	Fabaceae	L	<1.0	1.21	0.93
Droguetia iners	Urticaceae	L	<1.0	1.11	0.97
Drypetes sp.	Euphorbiaceae	RF	<1.0	1.33	0.67
Englerina woodfordioides	Loranthaceae	ST	<1.0	0.98	0.95
Englerina woodfordioides	Loranthaceae	L	<1.0	1.26	0.73
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
Galiniera coffeoides	Rubiaceae	RF	<1.0	0.90	0.86
Galium thumbergianum	Rubiaceae	L	<1.0	1.71	0.70
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
Justicia glabra	Acanthaceae	L	<1.0	0.67	0.93
Maesa lanceolata	Myrsinaceae	L	<1.0	1.77	1.12
Mimulopsis arborescens	Acanthaceae	PI	4.1	1.07	0.91
Mimulopsis solmsii	Acanthaceae	L	7.1	1.26	0.82
Momordica foetida	Cucurbitaceae	F	<1.0	0.87	1.06

TABLE 2.4 cont. Transient transfection assay data.

Plant Species	Family	Part	% Diet[a]	ERα Relative Luciferase Activity[b]	ERβ Relative Luciferase Activity[c]
Momordica foetida	Cucurbitaceae	L	8.0	1.13	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
Olea capensis	Oleaceae	BA	<1.0	0.97	0.69
Olinia usambarensis	Oliniaceae	RF	<1.0	0.95	0.93
Periploca linearifolia	Asclepiadaceae	L	<1.0	0.98	0.72
Piper capense	Piperaceae	PI	<1.0	1.18	1.08
Rapanea rhododendroides	Myrsinaceae	L	<1.0	1.13	0.77
Rubus sp.	Rosaceae	F	<1.0	1.40	0.96
Rubus sp.	Rosaceae	L	<1.0	0.80	1.00
Rytigynia kigenziesis	Rubiaceae	L	<1.0	1.11	0.74
Rytigynia kigenziesis	Rubiaceae	RF	<1.0	0.94	0.66
Salacia elegans	Celastraceae	L	<1.0	0.95	0.63
Schefflera sp.	Araliaceae	L	<1.0	not tested	0.71
Smilax anceps	Smilacaceae	L	<1.0	1.06	1.17
Syzygium guineense	Myrtaceae	RF	<1.0	0.66	0.80
Teclea nobilis	Rutaceae	RF	<1.0	not tested	0.89
Triumfetta tomentosa	Tiliaceae	L	5.4	1.42	1.09
Urera hypselodendron	Urticaceae	PL	5.4	1.66	0.64
Urera hypselodendron	Urticaceae	L	19.2	0.91	0.91
Vernonia pteropoda	Compositae	BA	<1.0	0.88	1.05
Vernonia tuffnellae	Compositae	BA	<1.0	1.05	1.09
Xymalos monospora	**Monimiaceae**	**BA**	<1.0	1.53	**3.56**
Xymalos monospora	Monimiaceae	L	<1.0	1.05	0.78

[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 > 16-fold 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 > 2-fold increase as compared to the blank (absence of ligand).

L = leaves, ST = stem, DW = dead wood, GS = grass stem, RF = ripe fruit, UF = unripe fruit BA = bark, PI = pith, F = fruit, PL = peel

CHAPTER 3:

Seasonality in the consumption of estrogenic plant foods: Implications for red colobus monkey (*Procolobus rufomitratus*) hormonal status

ABSTRACT:

Though numerous studies have examined the detrimental effects of anthropogenic endocrine disrupting compounds (EDCs), little is known about the effects of naturally occurring plant-produced EDCs on wild vertebrates. This is especially true for wild primates, most of which depend heavily on plant foods to meet their nutritional needs. Phytoestrogens are one of the main types of plant-produced EDCs that occur in the wild primate diet. Both the availability and chemical content of plant foods containing phytoestrogens are influenced by climatic factors. To examine the seasonal pattern of phytoestrogen consumption and its relationship to hormone levels in a wild primate, I conducted an 11-month field study of red colobus monkeys (*Procolobus rufomitratus*) in Kibale National Park, Uganda. In a previous study, I identified a number of red colobus plant foods that contained phytoestrogens using transient transfection assays. In this study, I found the percent of diet coming from these estrogenic plant foods averaged 10.7% (n = 45 weeks; range: 0.7% to 32.4%). Climatic factors were important for understanding variation in the proportion of diet coming from estrogenic plants, particularly for the consumption of *Millettia dura* young leaves. Although red colobus did not feed more heavily on *M. dura* young leaves when they were more available, they did feed more heavily on them during months of higher rainfall. In stepwise regressions examining the relationship of both climatic factors and estrogenic plant consumption with male red colobus hormone levels, the weekly median fecal estradiol level was best predicted by the percent of diet from estrogenic *M. dura* young leaves, and the weekly median fecal cortisol level was also best predicted by the percent of diet from *M. dura* young leaves. Thus, it appears that climatic factors influence how much red colobus consume estrogenic plant foods and the consumption of these estrogenic plant foods influences hormone levels of red colobus monkeys. Further, these results suggest that consumption of estrogenic plants by red colobus monkeys may have important implications for their health and fitness through disruption of the endocrine system. It is likely that the ecology and evolution of wild primates have been influenced by plants that produce naturally occurring EDCs in ways not yet fully appreciated.

KEYWORDS: primate ecology, estradiol, cortisol, environmental endocrinology, wild plant foods, legumes

INTRODUCTION:

Endocrine disruption is a current concern among many scientists because a number of synthetic chemicals pose a threat to humans and wildlife through interference with the vertebrate endocrine system (Hayes 2005, Propper 2005). Much research has focused on the physiological and behavioral effects of anthropogenic endocrine disrupting compounds (EDCs), particularly those with estrogenic activity (Colborn et al. 1993, Guillette 2000, Hayes et al. 2002, Milnes et al. 2006). Such effects include modification of the developing reproductive system if exposed at early life stages (i.e., genital and gonadal deformation) and altered steroid hormone profiles, gamete production, and sex-typical behaviors if exposed as adults (Hayes et al. 2002, Milnes et al. 2006). Much less is known about the effects of consuming naturally occurring plant-produced estrogenic EDCs (i.e., phytoestrogens) on wild vertebrates (Wynne-Edwards 2001). Because most wild primates depend heavily on a diverse plant-based diet, the consumption of estrogenic plants may have important implications for their ecology and evolution. Further, as half of all primate species are at risk of extinction (Chapman & Peres 2001), a better understanding of the relationship between primates and naturally occurring phytoestrogens in their wild plant foods is needed because such compounds have the potential to alter fertility and mating behavior (Wynne-Edwards 2001, Whitten & Patisaul 2001, Simon et al. 2004, Cederroth et al. 2010a).

Phytoestrogen consumption has been shown to disrupt fertility and affect behavior in a number of laboratory and domesticated species, including rodents, monkeys, sheep, and cattle, as well as humans (Adams 1990, Adams 1995, Whitten & Patisaul 2001, Cederroth et al. 2010b). Due to the conservative nature of the endocrine system across vertebrates, including a lack of change in estrogen receptors, effects such as those found in laboratory models and domesticated livestock are expected in wild primates feeding on similar estrogenic plant compounds (Thornton 2001, Hayes 2005). In a previous study, I confirmed the presence of estrogenic plant foods in the diet of a wild primate, the red colobus monkey of Kibale National Park, Uganda, using transient transfection assays (Wasserman et al, *in prep*). Three staple foods (foods comprising > 1% of diet) made up most of the diet coming from estrogenic plant foods: *Millettia dura* [Fabaceae] young leaves, *Ficus natalensis* [Moraceae] young leaves, and the introduced *Eucalyptus grandis* [Myrtaceae] bark. Here, my main objective is to determine if the consumption of these estrogenic plant foods by red colobus monkeys has the potential to alter their physiology and behavior via interference with their endocrine system.

Two hormonal axes of the endocrine system are important for understanding the potential for phytoestrogens to alter fertility and behavior in wild primates. The first, the hypothalamo-pituitary gonadal axis (HPG), plays a central role in regulating the development and maintenance of a primate's reproductive system through the production of sex steroids (i.e., estrogens and androgens) and their downstream effects on reproductive physiology and behavior (Hadley 2000, Wingfield & Sapolsky 2003). This axis is controlled through a negative feedback loop in which increasing levels of sex steroids can shut down further sex steroid production, as well as the production of pituitary gonadotropins (Hadley 2000, Hayes 2005). Interference with the HPG can result in altered fertility in both males, due to the role of testosterone and gonadotropins in sperm production, and females, due to the role of estrogens and gonadotropins in their estrous cycle (Hadley 2000). The second hormonal axis, the hypothalamo-pituitary adrenal axis (HPA), plays a central homeostasis role in the face of external disturbances or internal physiological changes through the production of glucocorticoids (e.g., cortisol; Wingfield & Sapolsky 2003, Sapolsky 2005). Although, the HPA axis plays an important role in allowing an animal to deal with environmental problems (e.g., a predator attack), long-term activation of this stress response can lead to a series of detrimental effects that lowers an animal's ability to survive and reproduce (Sapolsky 2005). The HPA axis is also regulated via a negative feedback mechanism in which increased cortisol levels can shut down further glucocorticoid production (Hadley 2000). These two hormone axes interact: there is strong evidence that the HPA axis can suppress the HPG axis (i.e., stress can suppress reproduction; Wingfield & Sapolsky 2003) and recent evidence suggests that estrogens can suppress the negative feedback loop of the HPA axis, thus increasing production of glucocorticoids (Weiser & Handa 2009).

I examined patterns of phytoestrogen consumption and fecal hormone levels of red colobus monkeys (*Procolobus rufomitratus*) in Kibale National Park, Uganda, during an 11-month field study (Aug. 2007 – Jun. 2008). Specifically, I determined how weekly variation in the percent of time spent feeding on previously identified estrogenic plant foods related to fecal estradiol and cortisol levels in adult male red colobus monkeys. As both consumption of estrogenic plants and red colobus hormone levels may vary seasonally, the relationships of rainfall and temperature to these factors were also examined. Climate can mediate the relationship between phytoestrogen consumption and primate hormone levels through three main mechanisms that are not mutually exclusive: (1) its effects on food availability (i.e., plant phenology is linked to climatic variables and preferred parts of estrogenic plants are fed on when available; Struhsaker 1997, Chapman et al. 1999), (2) its effects on phytoestrogen levels (i.e., plants contain more or less of these compounds during certain times of the year and this affects when the red colobus feed on estrogenic plants; Leopold et al. 1976, Mazur & Adlercreutz 1998, Morrison et al. 2010), and (3) its direct effects on hormone levels (i.e., primates may suffer climate related stress under certain conditions; Wingfield et al. 1983, Wingfield 2005). These mechanisms were predicted to be important for understanding the relationship between climate, phytoestrogen consumption, and hormonal status in the red colobus at Kibale.

METHODS:

Study site and species

Kibale National Park (KNP; 795 km^2) is a mid-altitude, moist evergreen forest in the foothills of the Rwenzori Mountains of western Uganda (0 13' - 0 41' N and 30 19' - 30 32' E) (Chapman et al. 2010). This forest receives an average of 1698 mm of rainfall per year (1990 - 2008), with most falling during two rainy seasons (Chapman et al. 2010). Kibale contains the highest recorded biomass of primates in the world with 13 species represented (Chapman & Lambert 2000). This high level of biodiversity and complex community of primates, along with the rapid loss of forest outside the park (Howard et al. 2000), make understanding ecological relationships within this forest a particularly critical endeavor.

The Ugandan red colobus monkey (*Procolobus rufomitratus*) is considered vulnerable, with the only viable population remaining in KNP (Struhsaker 2005). The Kibale population consists of numerous multimale-multifemale groups with an average group size of 65 individuals (Snaith et al. 2008). As a forestomach-fermenting obligate folivore dependent upon symbiotic gut bacteria for securing nutrients from its highly folivorous diet (Bauchop & Martucci 1968, Milton 1980, Lambert 1998, Chapman et al. 2002), the red colobus is an ideal subject for examining the effects of phytoestrogen consumption on wild primates.

With their specialized forestomach fermentation system, colobines may be particularly susceptible to the estrogenic activity of plants, as has been documented for foregut-fermenting livestock (e.g., "clover disease" of sheep; Bennetts, 1946; Adams, 1990; 1995). Data show that phytoestrogens are often more active after bacterial metabolism (Gultekin & Yildiz 2006, Setchell & Clerici 2010). For example, a number of phytoestrogens (e.g., formononetin, daidzein) are converted to the more bioactive compound, equol, via bacterial metabolism in a number of animal species, including foregut fermenters (Setchell & Clerici 2010). Interspecific differences in the production of equol from less active dietary phytoestrogens have also been documented. For example, rodents and chimpanzees are more efficient equol-producers than humans or pigs, and these differences appear to be due to differences in gut microbial communities (Adlercreutz et al. 1986, Setchell & Clerici 2010). Because colobines have taken the mutualistic relationship with gut bacteria to a higher level among primates through the development of a complex partitioned forestomach, the effects of consuming phytoestrogens may be greater for them than for other highly folivorous primates lacking these gastric specializations (e.g., gorillas, howler monkeys) or for more frugivorous species (e.g., chimpanzee, spider monkeys).

Assessment of red colobus diet

To determine the red colobus diet, behavioral data were collected on one group (group size approximately 70 individuals) located near the Kanyawara research station in KNP from August 13, 2007 to June 27, 2008 (258 days of sampling), for a total of 1327 hrs. Data were collected six days per week from 0800 to 1300 h using scan samples of five individuals every 30 minutes. When feeding, plant species and the part being consumed were identified. I 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. Thus, the percent time feeding on a particular plant item each week is used as a relative index of the importance of that food item in the diet for that week. Using these weekly values, I calculated the percent of diet from all estrogenic plant foods, all staple estrogenic plant foods, and all rare estrogenic plant foods, as well as the percent of diet from each of the staple estrogenic foods for each week of the study ($n = 45$). A staple food was defined as any plant item that was fed on > 1% of the total study time, while a rare food was any plant item that was fed on < 1% of the total study time. Estrogenic activity of plant foods was previously demonstrated using transient transfection assays (Wasserman et al., *in prep*).

Assessment of climate

Rainfall data were collected each day and summed to calculate the total rainfall for each week of the study ($n = 45$ weeks). Daily maximum temperature was also recorded and is reported as the weekly mean maximum temperature. Both rainfall and temperature data were provided by C.A. Chapman, who maintains a continuous climatic data set from the Kanyawara research station in KNP (*unpublished data*).

Assessment of plant phenology

Phenological data from the forest at the Kanyawara research station in KNP were collected concurrently with this study (C.A. Chapman, *unpublished data*). Each month, trees were monitored for presence of ripe fruits, unripe fruits, mature leaves, young leaves, and flowers. Each tree was given a qualitative phenological score from 0 (none) to 4 (highest amount present) for each plant part category. Using this data set, I calculated the mean phenological score for the staple estrogenic plant foods of the red colobus for each month to provide an index of their availability. Young leaves of *Ficus natalensis* (n = 2 individual trees) and *Millettia dura* (n = 11 individual trees) were monitored, but *Eucalyptus grandis* bark was not because bark is not known to show phenological variation.

Assessment of fecal hormone levels

Although direct evidence of physiological effects of phytoestrogen consumption similar to those of laboratory studies are difficult to obtain in ecological studies, indirect measures can provide evidence of changes in either the HPG axis or HPA axis, indicating potential disruption of reproductive capabilities. These hormonal effects can be examined noninvasively using the measurement of excreted estradiol and cortisol metabolites in the feces. Steroid hormones are inactivated in the liver and excreted as metabolites in urine and feces. Fecal estradiol metabolites and fecal cortisol metabolites are the end products of the HPG and HPA axes, respectively, and they can be used as reliable indices of reproductive and stress physiology of wild primates (Wasser et al. 1988, Heistermann et al. 1993, Whitten et al. 1998, Touma & Palme 2005, Ziegler & Wittwer 2005).

Fecal samples were collected immediately upon defecation from 10 known adult males (i.e., individual identification based either on a unique collar color/tag shape combination or easily detectible scars and features [e.g., fur color pattern, bends in tail]). Adult males were selected because female reproductive state can greatly influence steroid hormone levels (Weingrill et al. 2004), thus complicating determination of effects of external factors on the endocrine system. Fecal samples were collected between 0830 and 1230 hrs to reduce the contribution of diurnal variation seen in the excretion patterns of fecal steroids (Sousa & Ziegler 1998, Wasserman unpublished data on red colobus at Kibale). The dry matter content of all fecal samples was calculated by drying 0.5g of each fecal sample to a constant weight to control for the influence of fiber content of diet on the amount of hormones measured in the fecal sample (Wasser et al. 1993).

My goal was to collect one sample per male per week (potential n = 450); however, this was not always possible due to logistics of working in dense forest cover with free-ranging subjects (actual n = 407). Fecal samples were immediately placed in sterile vials and stored in a handheld cooler with ice packs until brought back to the field station later that day, where they were stored in a -20^{o}C freezer. On the day of extraction, samples were taken from the -20^{o}C freezer and thawed. Each sample was homogenized using a spatula and 0.5g was weighed to a test tube. I added 5 ml of 5.0 pH citrate buffer and 5 ml 95% ethanol to each fecal sample, and this fecal material solution was mixed on a homogenizer for 24 hours. Steroid hormones were then separated from the fecal pellet using a centrifuge, and 2ml of supernatant were passed through a preconditioned solid phase extraction cartridge at a flow rate of 4ml / min. The steroid hormones were then stored in these cartridges with both ends capped. Capped samples were stored out of direct light until analysis via radioimmunoassay (RIA) for estradiol content and enzyme immunoassay (EIA) for cortisol content by MW at the Wisconsin National Primate Research Center (WNPRC). The final hormone values are given in ng of steroid hormone per g of dry feces.

At WNPRC, the cartridges were washed with 1ml of 5% methanol and the steroid hormones were collected using 2ml of 100% methanol passed through the cartridge at a 1ml / min flow rate. The methanol was then evaporated off and steroids hormones were reconstituted in 1 ml of 100% ethanol and stored in a 4^{o}C refrigerator until analyses. For the estradiol RIA, recovery was 107.44% ± 2.53%. Parallelism was demonstrated with no significant difference between slopes of the serial dilution of the sample pool and standard curve ($p > 0.05$). Interassay variation for the high pool was 14.62% and for the low pool was 9.6%, while intrassay variation was 4.53% for the high pool and 7.51% for the low pool. Recovery for the cortisol EIA was 125.27% ± 3.18%. Parallelism was demonstrated using serial dilution curves, with no significant difference between the sample pool and standards ($p > 0.05$). Interassay variation for the high pool was 18.83% and for the low pool was 16.62%, while intrassay variation was 6.24% for the high pool and 6.26% for the low pool.

Statistical analyses

To determine if the consumption of estrogenic plant foods by red colobus monkeys interfered with their endocrine system and if climate played an important mediating role in this relationship, I tested two main hypotheses: (1) changes in red colobus hormonal levels across the study were related to changes in phytoestrogen consumption and (2) climatic factors influenced red colobus hormone levels directly and/or indirectly though their effects on the timing of estrogenic plant availability and/or consumption. I used fecal estradiol level as an index of reproductive physiology and fecal cortisol level as an index of stress physiology. I used three descriptive statistics of both hormone data sets (i.e., mean, median, and standard error of the mean of weekly estradiol and cortisol) in my analyses to examine the influence of phytoestrogen consumption and climatic seasonality on both the central tendency and variation of hormone levels for each week of the study. I used six different indices of phytoestrogen consumption: (1) % of diet from all estrogenic plants, (2) % diet from staple estrogenic plants, (3) % of diet from rare estrogenic plants, and (4-6) % of diet from each of the estrogenic staples [*Millettia dura* young leaves*, Ficus natalensis* young leaves*,* and *Eucalyptus grandis* bark]. I used two indices of climatic seasonality: (1) weekly total rainfall and (2) weekly mean maximum temperature. Relationships between hormone levels, phytoestrogen consumption, and climatic variables were examined at the weekly level using Pearson correlations ($n = 45$).

To further clarify if seasonality influenced the consumption of various estrogenic plants, I also examined the relationship between rainfall and phenology of *Millettia dura* young leaves and *Ficus natalensis* young leaves, as well as the availability of these two estrogenic food items and the percent of time the red colobus fed on each, at the monthly level using Spearman rank correlations ($n = 11$). Feeding tradeoffs between the three staple estrogenic plant foods were examined using Pearson correlation to determine if the red colobus fed less on one estrogenic staple food when also feeding on another estrogenic staple food in the same week ($n = 45$).

To determine the relative importance of phytoestrogen consumption and climatic seasonality and their potential synergistic effects on red colobus hormonal status, I used stepwise regression including six predictor variables: (1) % of diet from rare estrogenic foods, (2-4) % of diet from each of the three staple estrogenic foods, (5) rainfall, and (6) mean maximum temperature, and two outcome variables: (1) fecal estradiol and (2) fecal cortisol. All variables were analyzed at the weekly level (n = 45). A model was created for the mean, median, and standard error of the mean for both estradiol and cortisol.

RESULTS:

Estrogenic plants in red colobus diet

The weekly percent of diet coming from estrogenic plant foods averaged 10.7% (n = 45 weeks), ranging from a low of 0.7% to a high of 32.4% (Fig. 3.1). This was mainly due to the consumption of three staple estrogenic plant foods; their contribution to the weekly diet averaged 10.1% (range: 0.7% - 31.0%). The red colobus fed on at least one of the three staple estrogenic plant foods during each week, but never fed on all three during the same week. Of these three estrogenic staples, *Millettia dura* young leaves were fed on most, with a weekly average of 5.1% of the diet and a range from none during 8 different weeks to 15.5% during the highest week. *Eucalyptus grandis* bark was fed on second most with a weekly average of 3.4% of the diet across the study and a range from none during 33 different weeks to 31.0% during the highest week. *Ficus natalensis* young leaves were fed on least with a weekly average of 1.5% of the diet across the study and a range from none fed on during 28 weeks to 11.8% during the highest week. As for the rare (non-staple) estrogenic plant foods, their contribution to the weekly diet averaged 0.6%, with a range from none fed on during 33 different weeks to 6.1% of the diet during the highest week. Feeding tradeoffs among the three species were found: the more the red colobus fed on *M. dura* the less they fed on both *F. natalensis* (r = -0.348, p = 0.019) and *E. grandis* (r = -0.0369, p = 0.013). No significant relationship was found between feeding on *F. natalensis* and *E. grandis* (r = -0.0189, p = 0.215).

Climatic variation

Rainfall was highly variable temporally, both between weeks during the dry seasons (weeks 1-3 [Aug.], 16-31 [Dec. – early Mar.], and 44-46 [June]) and wet seasons (weeks 4-15 [Sept. – Nov.] and 32-43 [late Mar. – May]) and across weeks within a particular season (Fig. 3.2). Total rainfall during the 45-week study was 1304.5 mm, with an average of 28.99 mm falling per week (range = 0 – 100.33 mm). Weekly mean maximum temperature averaged 25.7 ^{o}C (range = 23.1 - 30.3 ^{o}C) (Fig. 3.3).

Phenology of staple estrogenic plant foods

Young leaves of both *M. dura* and *F. natalensis* were available throughout the study. The mean of the monthly phenological scores for *M. dura* was 1.54 with a range from 0.73 to 2.11 and for *F. natalensis* was 1.27 with a range from 0.5 to 2 (Fig. 3.4). The availability of *M. dura* young leaves was significantly related to rainfall with a one-month time lag (i.e., rainfall in month 1 resulted in an increase in availability of *M. dura* young leaves in month 2; $r_s = 0.778$, $p = 0.008$). The availability of *F. natalensis* young leaves was unrelated to rainfall ($r_s = -0.075$, $p = 0.836$). Even though some young leaves of *F. natalensis* were available year-round, there were months when the red colobus did not feed on them, while *M. dura* young leaves were fed on in every month of the study. However, red colobus did not feed on *M. dura* young leaves more when phenological data showed that more were available ($r_s = 0.232$, $p = 0.492$), nor did they feed on *F. natalensis* young leaves more when they were more available ($r_s = -0.041$, $p = 0.905$). However, there was a significant positive relationship between the amount of rainfall in a given month and the percent of diet from *M. dura* young leaves ($r_s = 0.836$, $p = 0.001$).

Adult male red colobus fecal estradiol and cortisol levels

Weekly mean fecal estradiol levels averaged 80.03 ng / g dry feces, with a range from 27.32 to 297.69 ng / g (n = 45; Fig. 3.5). However, individual samples had much greater variation in estradiol levels, with a range from 19.62 to 2237.81 ng /g (n = 407). Due to one or two individuals having extremely high levels during a few weeks (e.g., 2237.81 ng /g), certain weeks have very high levels of variation, with a range in the weekly standard error of the mean (SEM) from 1.07 to 216.95 ng /g and an average of 38.66 ng / g. Five of the weeks had one sample > 1000 ng / g, while 14 weeks had one or two samples > 500 ng /g. These one or two extremely high samples were responsible for the high SEM during those weeks.

Weekly mean fecal cortisol levels averaged 71.58 ng / g dry feces across the study, with a range from 33.17 to 107.59 ng / g (n = 45; Fig. 3.6). Individual samples did not show as much variation for cortisol levels as they did for estradiol levels, with a range from 18.63 to 200.12 ng /g (n = 407). The average SEM was 8.67 ng /g, with a range from 1.86 to 23.12 ng / g.

Phytoestrogen consumption, climate, and fecal hormone levels

There was a positive relationship between percent of total feeding time spent feeding on estrogenic plants and both mean and SEM male estradiol levels (mean: $r = 0.432$, $p = 0.003$; SEM: $r = 0.495$, $p = 0.001$; Table 3.1, Fig. 3.7). Thus, the more red colobus consumed estrogenic plants, the higher their estradiol levels, due to the extreme levels mentioned above. The rare estrogenic plant foods added little to this relationship, as they did not show a significant relationship with fecal estradiol, while the staple estrogenic plant foods did (mean: $r = 0.428$, $p = 0.003$; SEM: $r = 0.479$, $p = 0.001$). Further, this relationship was best explained by one estrogenic staple food, *E. grandis* bark (mean: $r = 0.386$, $p = 0.009$; SEM: $r = 0.450$, $p = 0.002$); this significant positive relationship was even stronger when removing weeks that *E. grandis* was not fed on from the analysis (mean: $r = 0.697$, $p = 0.012$; SEM: $r = 0.696$, $p = 0.012$; Fig 3.8). The other two staple estrogenic plant foods showed significant relationships with median fecal estradiol, but in opposite directions (*M. dura*: $r = 0.602$, $p < 0.001$; *F. natalensis*: $r = -0.315$, $p = 0.035$). These relationships were also significant when only examining weeks in which each item was fed on (*M. dura*: $r = 0.574$, $p < 0.001$, Fig. 3.9; *F. natalensis*: $r = -0.500$, $p = 0.041$, Fig 3.10). As for climatic variables, rainfall showed a significant relationship with fecal estradiol (mean: $r = 0.322$, $p = 0.031$; median: $r = 0.298$, $p = 0.047$; SEM: $r = 0.332$, $p = 0.026$).

The percent of time spent feeding on *M. dura* young leaves was the best predictor of fecal cortisol levels (mean: $r = 0.402$, $p = 0.006$; median: $r = .358$, $p = 0.016$), and this positive relationship was even stronger when excluding weeks that it was not fed on (mean: $r = 0.475$, $p = 0.003$; median: $r = 0.461$, $p = 0.004$, Fig. 3.11). *E. grandis* bark showed a significant negative relationship with fecal cortisol (mean: $r = -0.309$, $p = 0.039$; median: $r = -0.298$, $p = 0.047$), but this relationship was not significant when removing weeks that it was not fed on from the analysis. Climatic variables did not show significant relationships with fecal cortisol levels.

Climatic variables did show significant relationships with the percent of time estrogenic plants were fed on. There was a significant positive relationship between rainfall and the percent of time feeding on *M. dura* young leaves ($r = 0.414$, $p = 0.005$). There was a significant positive relationship between the weekly mean maximum temperature and the percent of diet from all estrogenic food items ($r = 0.310$, $p = 0.038$), again due to the estrogenic staples ($r = 0.319$, $p = 0.033$). Further, this relationship was due to the percent of diet from *E. grandis* bark ($r = 0.252$, 0.095), which was significant only when removing weeks it was not fed on from the analysis ($r = 0.614$, $p = 0.034$).

Relative importance of phytoestrogen consumption and climate for fecal hormone levels

In stepwise regressions predicting fecal estradiol levels, the mean and SEM were both best predicted by percent of diet from *E. grandis* bark, rainfall, and mean maximum temperature (mean: $R^2 = 0.366$, $p < 0.001$; SEM: $R^2 = 0.434$, $p < 0.001$), while the median was best predicted by percent of diet from *M. dura* young leaves ($R^2 = 0.362$, $p < 0.001$). In stepwise regressions predicting fecal cortisol levels, the mean and median were both best predicted by percent of diet from *M. dura* young leaves (mean: $R^2 = 0.162$, $p = 0.006$; median: $R^2 = 0.128$, $p = 0.016$), while none of the predictor variables were significant for the standard error of the mean.

DISCUSSION:

I showed that the ingestion of estrogenic plant foods was related to adult male red colobus fecal estradiol and cortisol levels. However, to fully appreciate the relationship between estrogenic plant foods and red colobus monkeys, the effects of climatic seasonality on the availability of and patterns of feeding on estrogenic plant foods must also be considered.

Seasonality in consumption of estrogenic plants

Temporal variation in the percent of diet coming from estrogenic plants was high regardless of the index of phytoestrogen consumption used (i.e., all estrogenic plant foods, staple estrogenic plant foods, or each staple estrogenic plant food separately: *Millettia dura* young leaves, *Ficus natalensis* young leaves, *Eucalyptus grandis* bark). It is worth noting that red colobus fed on at least one staple estrogenic plant food each week, but never fed on all three staple estrogenic foods in a single week. This could indicate some type of threshold in phytoestrogen consumption, result from the spatial distribution of these species, or relate to relative food availability and red colobus food preference. However, neither *M. dura* nor *F. natalensis* young leaves were fed on more during periods of higher availability, and the availability of *E. grandis* bark is assumed to have been constant throughout the study. Further, all combinations of two of the three staples were fed on in at least one week of the study, limiting the argument for spatial distribution effects. A phytoestrogen threshold is the best possible explanation for this foraging pattern, but this hypothesis requires further testing.

Rainfall patterns were related to both availability of and red colobus feeding on *M. dura* young leaves, while patterns in temperature were related to red colobus feeding on *E. grandis* bark. Neither rainfall nor temperature patterns were related to availability of or red colobus feeding on *F. natalensis* young leaves. *Ficus natalensis* young leaves were likely used when other leafy foods were unavailable, due to their availability throughout the year (Terborgh 1983, Milton 1991). Supporting this, the more the red colobus fed on *M. dura* young leaves, the less they fed on *F. natalensis* young leaves.

Although the red colobus did not feed more heavily on *M. dura* young leaves when they were more available, they did feed more heavily on them during the weeks with more rain. This might mean that variation in the chemical content of these leaves in response to increased rainfall is more important than availability in determining when and how much red colobus will feed on

M. dura young leaves. These changes could be nutritional, as higher protein levels would be attractive to red colobus (Chapman et al. 2003), or non-nutritional, as increased phytoestrogen content may either be attractive as a form of self-medication (Glander 1980, Strier 1993, Huffman 1997, Forbey et al. 2009) or act as a feeding deterrent if phytoestrogen consumption above a certain threshold reduces fertility (Hughes 1988; Harborne 1993; Wynne-Edwards 2001). Increases in isoflavone concentration (i.e., phytoestrogens) with increased precipitation have been documented in soybeans (*Glycine max*), which are members of the same subfamily (Papilionoideae) of legumes as *M. dura* (Morrison et al. 2010). If this relationship with rainfall holds for *M. dura* young leaves, the premise of a phytoestrogen threshold which influences foraging decisions is further supported.

As for patterns of consumption of *E. grandis* bark, red colobus occasionally binged on this item, possibly as a source of sodium (Rode et al. 2003, Rothman et al. 2006b, Harris & Chapman 2007). Why red colobus fed more heavily on *E. grandis* bark during warmer weeks is harder to explain. The *E. grandis* grove, located on the border of the forest, receives more direct sunlight than other areas of the red colobus home range. It would be predicted that the red colobus would want to avoid such open areas during warmer weeks to reduce heat stress. However, this relationship may also be explained by considering the effects of climate on plant chemistry. Flavonoids (a broader group of secondary metabolites that includes the estrogenic isoflavonoids) play a role in protecting plants against harmful UV light and altering the wavelength of light to appropriate physiological levels (Mazur & Adlercreutz 1998). Thus, it is possible that a plant's phytoestrogen levels are highest with intense sunlight. If this is the case for *E. grandis*, then red colobus may be feeding on this plant when its phytoestrogens levels are highest, which could explain why the highest estrogen levels measured correlated with *E. grandis* consumption (see discussion below). The red colobus are either feeding on *E. grandis* during these times for their increased phytoestrogens or due to changes in micronutrient content regulated by temperature or solar insolation. Because *E. grandis* is fed on for its high sodium content, the possibility exists that sodium levels are highest during warmer periods due to increased transpiration and subsequent movement of water and sodium from the soil.

Future studies need to examine variation in phytoestrogen levels in both *M. dura* and *E. grandis* and determine how such variation relates to rainfall and solar insulation. Further, tradeoffs between phytoestrogen consumption and sodium or protein consumption should be examined to determine if the red colobus are feeding on these plants when phytoestrogen levels are expected to be highest because they are targeting the phytoestrogens themselves or simply as a consequence of a correlation between phytoestrogen content and nutritional content. Nonetheless, the effects of sunlight and rainfall on phytoestrogen levels may be critical to a primate's decision to feed on a specific plant and may explain the red colobus foraging patterns documented here with regard to estrogenic plants.

Hormonal effects of phytoestrogen consumption

There are a number of ways phytoestrogens can interfere with the functioning of the primate endocrine system. This study examined the effects of plants known to have compounds that bind to intracellular estrogen receptors (ERs) (Wasserman et al. *in prep*) and thus have the potential to interfere with both the physiological and behavioral endpoints promoted by estrogens and the negative feedback loop of the HPG axis (thus altering endogenous estrogen and testosterone production). Through their interaction with ER and competition with endogenous estrogens for binding to these receptors, phytoestrogens can act as either estrogen agonists (i.e., promoting estrogenic activity) or antagonists (i.e., blocking estrogenic activity) depending on the dose ingested, strength of the specific compounds, and endogenous hormonal state (Almstrup et al. 2002, Leitman et al. 2010). At low doses, phytoestrogens tend to decrease estrogenic activity, while at high doses they increase it (Almstrup et al. 2002). If they act as agonists and increase estrogenic activity, adult male fertility (through sperm production) can decrease and feminization can occur (Guillette 2000, Hayes 2005, Cederroth et al. 2010a). This may not only be due to increased estrogenic activity, but also to the suppression of the HPG axis through negative feedback and the resulting decrease in the production of testosterone.

On the other hand, since phytoestrogens tend to be much weaker than endogenous estrogens (Whitten & Patisaul 2001), competitive displacement of endogenous hormones may result in a reduction of estrogenic activity. This antagonistic effect could result in additional production of endogenous sex steroids through a dampening of the negative feedback mechanism. Indeed, increases in endogenous estrogen levels in male vertebrates exposed to anthropogenic endocrine disruptors have been documented (Milnes et al. 2006). However, this may largely be due to the conversion of androgens to estrogens via aromatase (Hayes et al. 2002). Because most phytoestrogens act as aromatase inhibitors (Almstrup et al. 2002), antagonistic effects would likely cause increased testosterone levels and reduced estrogenic activity. Thus, endogenous estrogenic activity can either increase or decrease depending on the dose and strength of the phytoestrogen consumed. Although the exact mechanism of action for how each of the three staple estrogenic plant foods would interact with the HPG and HPA axes is not known, finding correlative relationships between consumption of these plants and red colobus hormone levels suggests that interference did occur. Further studies will be needed to determine the biological significance of these relationships, but nonetheless, my results have identified the estrogenic plant species that are most likely to alter red colobus health and fertility via endocrine disruption.

Specifically, adult male red colobus fecal estradiol levels were related to the consumption of estrogenic plant foods. Mean estradiol level had a positive relationship with all estrogenic foods, but, upon closer examination, this relationship was due to increased variation when *E. grandis* bark was fed on. This increased variation was the result of one or two males that had much higher estradiol levels (i.e., up to 100 fold higher) when the red colobus binged on *E. grandis*. Because this relationship was driven by only one or two samples from only a few weeks of the study, caution should be used in interpreting its meaning. It could be that once a threshold is met, the male red colobus' endocrine system is disrupted by the phytoestrogens in the *E. grandis* bark, such that only one or two individuals during a few weeks of the study went above this threshold. On the other hand, the high samples may have been the result of sample collection timing, such that all individuals were ingesting a high amount of phytoestrogens when feeding on *E. grandis* bark but the effects of this could only be detected during a narrow window of time after consumption. In this scenario, if samples were collected at the appropriate time for all males, then high levels would be observed in all of them. Regardless, it appears that *E. grandis* bark consumption has a short term, high level effect on red colobus fecal estradiol levels, and one which differs from the effects of the other two staple estrogenic plant foods. Because *E. grandis* and other eucalypts are native to Australia and often planted in Africa and Latin America for use as timber and fuel wood, future studies should examine the possibility that eucalypts may disrupt the primate endocrine system. Threats from invasive plant species containing phytoestrogens may be an important, though thus far neglected, issue in conservation biology.

A more straightforward and expected outcome was found between the consumption of *M. dura* young leaves and median fecal estradiol levels of the red colobus. In this case, estradiol levels of all males increased in response to the consumption of *M. dura* young leaves. *Millettia dura* is a legume (i.e., Fabaceae) of the Papilionoideae subfamily, and the papilionoids are known to contain estrogenic isoflavonoids (Reynaud et al. 2005). For example, soy is also a member of this legume subfamily. Studies of livestock ingestion of papilionoids show that their consumption can dramatically impair reproduction (Bennetts & Underwood 1951, Adams 1990, Adams 1995), and similar detrimental effects on fertility may also occur in humans (Cederroth et al. 2010a). At times they may also provide health benefits such as cancer prevention or alleviation of menopausal disorders (Setchell & Cassidy 1999, Ososki & Kennelly 2003, Dixon 2004, Leitman et al. 2010). Based on these studies and the relationship found here, it is possible that *M. dura* exerts a strong influence on the red colobus endocrine system, with possible downstream effects on their behavior, health, and fitness. However, as red colobus are likely to have had a long evolutionary relationship with this native tree species, thus it is more likely that they have evolved adaptations to protect against any potential endocrine disruption caused by ingesting leaves of *M. dura* (Wynne-Edwards 2001). Thus, the different effects of *M. dura* and *E. grandis* consumption on red colobus estradiol levels may be explained in part by the length of the relationship between the primate and the plant (i.e., native versus exotic estrogenic plant effects).

The negative relationship between the consumption of *F. natalensis* young leaves and fecal estradiol levels is possibly due to red colobus feeding less or not at all on *M. dura* young leaves when they fed on *F. natalensis*, rather than any effects of ingesting phytoestrogens of *F. natalensis*. The multiple regression analyses supported this, as *F. natalensis* was not an important predictor in any of the models. Alternatively, this fig species may have had either high enough concentrations of phytoestrogens or phytoestrogens with strong enough activity to shut down the HPG axis through negative feedback, thus causing a reduction in estrogen levels. Many phytoestrogens can act as aromatase inhibitors at low doses (Almstrup et al. 2002), thus causing a reduction in estrogen and increase in testosterone levels. Chemical analyses of the phytoestrogens present in *F. natalensis*, *in vitro* analysis of their strength of action through ER binding, and *in vivo* analyses of their physiological endpoints are needed to clarify this possibility. Regardless, this species did not appear to be as important for understanding the effects of estrogenic plants on red colobus physiology as were *E. grandis* and *M. dura*.

Feeding on *M. dura* young leaves also had a positive relationship with fecal cortisol. It is likely that the phytoestrogens interacted not only with the HPG axis, but also with the HPA axis to alter cortisol production. Studies on amphibians have shown that anthropogenic endocrine disruptors in pesticides can lead to an increase in stress hormone production (Hayes et al. 2006). Further, estrogens are able to alter the negative feedback loop of the HPA axis, thus changing production of glucocorticoids (Weiser & Handa 2009). Therefore, it is possible for the phytoestrogens of *M. dura* young leaves to influence cortisol levels in red colobus.

This final relationship helps support the conclusion that the consumption of estrogenic plant foods affected red colobus physiology. The relationship with fecal estradiol may simply have been due to the RIA antibody binding to metabolites of the phytoestrogens passing through the monkeys. However, the additional relationship with cortisol provides strong evidence that the phytoestrogens were absorbed and affected the endocrine system through the HPG and HPA axes, since metabolites of phytoestrogens would not bind to the cortisol antibody. Increased cortisol production from phytoestrogen consumption may result in a synergistic threat to endangered primates which live in environments with unusually high proportions of estrogenic plants both through the suppression of their immune and reproductive systems by elevated cortisol (Sapolsky 2005) and by altered fertility through phytoestrogen interaction with ERs (Cederroth et al. 2010a).

Conclusions

Climate can affect red colobus phytoestrogen consumption and hormone levels through three main mechanisms: (1) effects on food availability, (2) effects on phytoestrogen levels, and (3) effects on hormone levels. Although these mechanisms are not mutually exclusive, my results suggest that the effect of rainfall on phytoestrogen levels of *M. dura* young leaves is the most important factor affecting the timing and magnitude of red colobus feeding on estrogenic plants. Subsequently, the amount of *M. dura* young leaves in the diet relates to changes in red colobus estradiol and cortisol levels. Effects of solar insolation on phytoestrogen levels of *E. grandis* bark may also influence red colobus estradiol levels, but this requires further study. Based upon numerous experimental studies of the physiological effects of phytoestrogen consumption on rodents and primates (Whitten & Patisaul 2001), the conservative nature of the endocrine system across vertebrates (Thornton 2001, Hayes 2005), and the significant correlative relationships found in this ecological study (despite the high levels of variation in fecal hormone levels due to many other environmental and social factors), it is likely that the consumption of estrogenic plant foods by red colobus has important implications for their health and fitness. The extent of these implications should be examined in future studies by either looking at the relationship between the ingestion of these plant foods and the monkeys' behavior (as phytoestrogens are known to alter aggressive, mating, and anxiety-related behaviors; Hartley et al. 2003, Simon et al. 2004, Kouki et al. 2003, Patisaul & Bateman 2008) or by examining the relationship between the ingestion of phytoestrogens and an index of fitness.

This study raises the possibility of additive effects of natural plant-based endocrine disruptors and aspects of climate change on wild primates, effects that might have the potential to threaten their long-term survival. Three findings warrant this: (1) Climate change in the form of increased rainfall (*c.* 300 mm more per year now as compared to the early 1900's) has been documented at this study site of KNP (Chapman et al. 2005), (2) red colobus were found to feed more heavily on *M. dura* young leaves during wetter weeks of this study, and (3) agricultural studies have documented increased levels of phytoestrogens with increased rainfall in *Glycine max* (soy), a species in the same subfamily as *Millettia*. Thus, the relationship between climate change and phytoestrogen consumption in wild primates appears to warrant further research. Concerns over altered levels of isoflavones in soy due to climate change and the effects this may have on the human food supply have already been raised (Caldwell et al. 2005), and similar concerns should apply to changes in the wild plant foods of non-human primates as well.

FIGURE LEGEND

Figure 3.1. Percent of total diet from each of the estrogenic staple plant foods, as well as a sum of the percent of diet from all rare estrogenic plant foods, of the Ugandan red colobus monkey for each week of the study (n = 45). The wet and dry seasons, as determined by monthly rainfall amounts during the study, are indicated for each week of the study.

Figure 3.2. Amount of rainfall (mm) at Kanyawara Research Camp, Kibale National Park, Uganda, for each week of the study (n = 45). Unpublished data from C. Chapman.

Figure 3.3. Mean maximum temperature at Kanyawara Research Camp, Kibale National Park, Uganda, for each week of the study (n = 45). Unpublished data from C. Chapman.

Figure 3.4. Average monthly phenological scores for young leaves of *Millettia dura* (n = 11) and *Ficus natalensis* (n = 2) across 11 months of the study (Aug. 2007 – June 2008) and their relationship to average monthly rainfall.

Figure 3.5 Mean (+/- SEM) fecal estradiol level from 10 adult male red colobus for each week of study (n = 45).

Figure 3.6. Mean (+/- SEM) fecal cortisol level from 10 adult male red colobus for each week of study (n = 45).

Figure 3.7. Relationship between percent of total diet from all estrogenic plant items and the mean fecal estradiol level of 10 adult male red colobus for each week of study (n = 45).

Figure 3.8. Relationship between percent of total diet from *Eucalyptus grandis* bark and the standard error of the mean fecal estradiol level of 10 adult male red colobus for each week *E. grandis* bark was fed on (n = 12).

Figure 3.9. Relationship between percent of total diet from *Millettia dura* young leaves and the median fecal estradiol level of 10 adult male red colobus for each week *M. dura* young leaves were fed on (n = 37).

Figure 3.10. Relationship between percent of total diet from *Ficus natalensis* young leaves and the median fecal estradiol level of 10 adult male red colobus for each week *F. natalensis* was fed on (n = 17).

Figure 3.11. Relationship between percent of total diet from *Millettia dura* young leaves and the mean fecal cortisol level of 10 adult male red colobus for each week *M. dura* was fed on (n = 37).

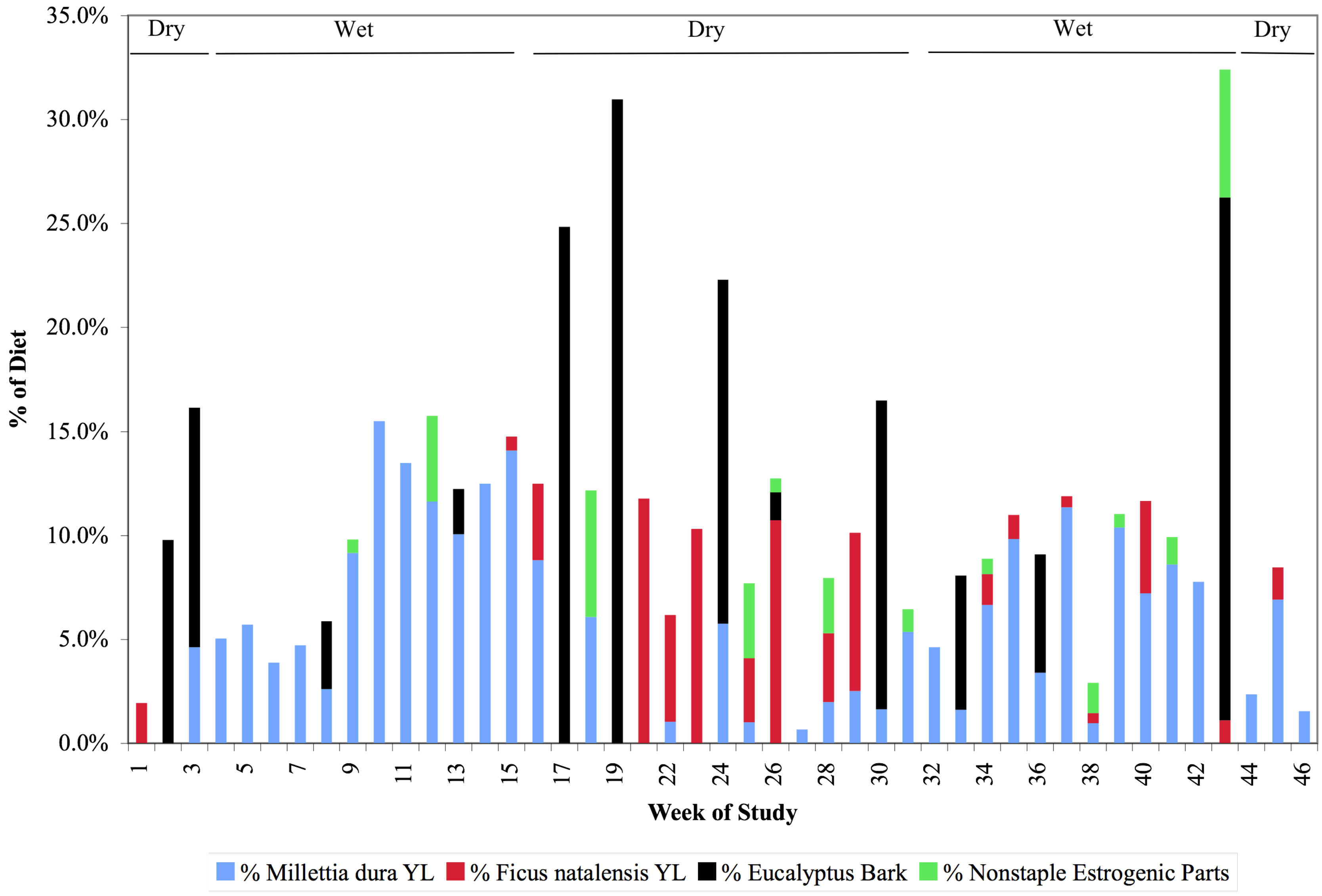
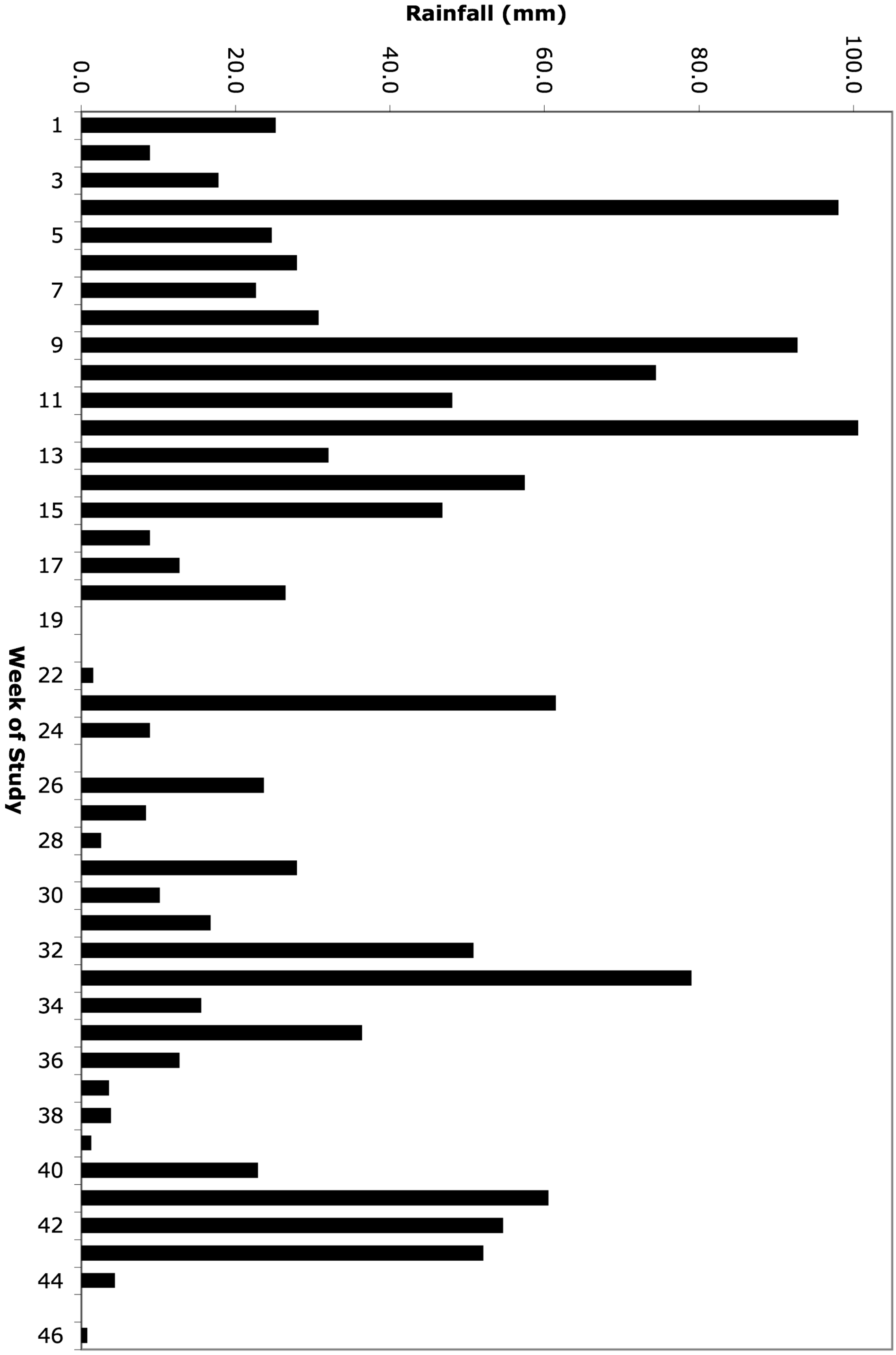

35.0%
30.0%
25.0%
20.0%
15.0%
10.0%
5.0%
0.0%
% of Diet
Dry
Wet
Dry
Wet
Dry
1
3
5
7
9
11
13
15
17
19
22
24
26
28
30
32
34
36
38
40
42
44
46
Week of Study
% Millettia dura YL
% Ficus natalensis YL
% Eucalyptus Bark
% Nonstaple Estrogenic Parts

Figure 3.1

Figure 3.2


Rainfall (mm)
0.0
20.0
40.0
60.0
80.0
100.0
Week of Study
1
3
5
7
9
11
13
15
17
19
22
24
26
28
30
32
34
36
38
40
42
44
46

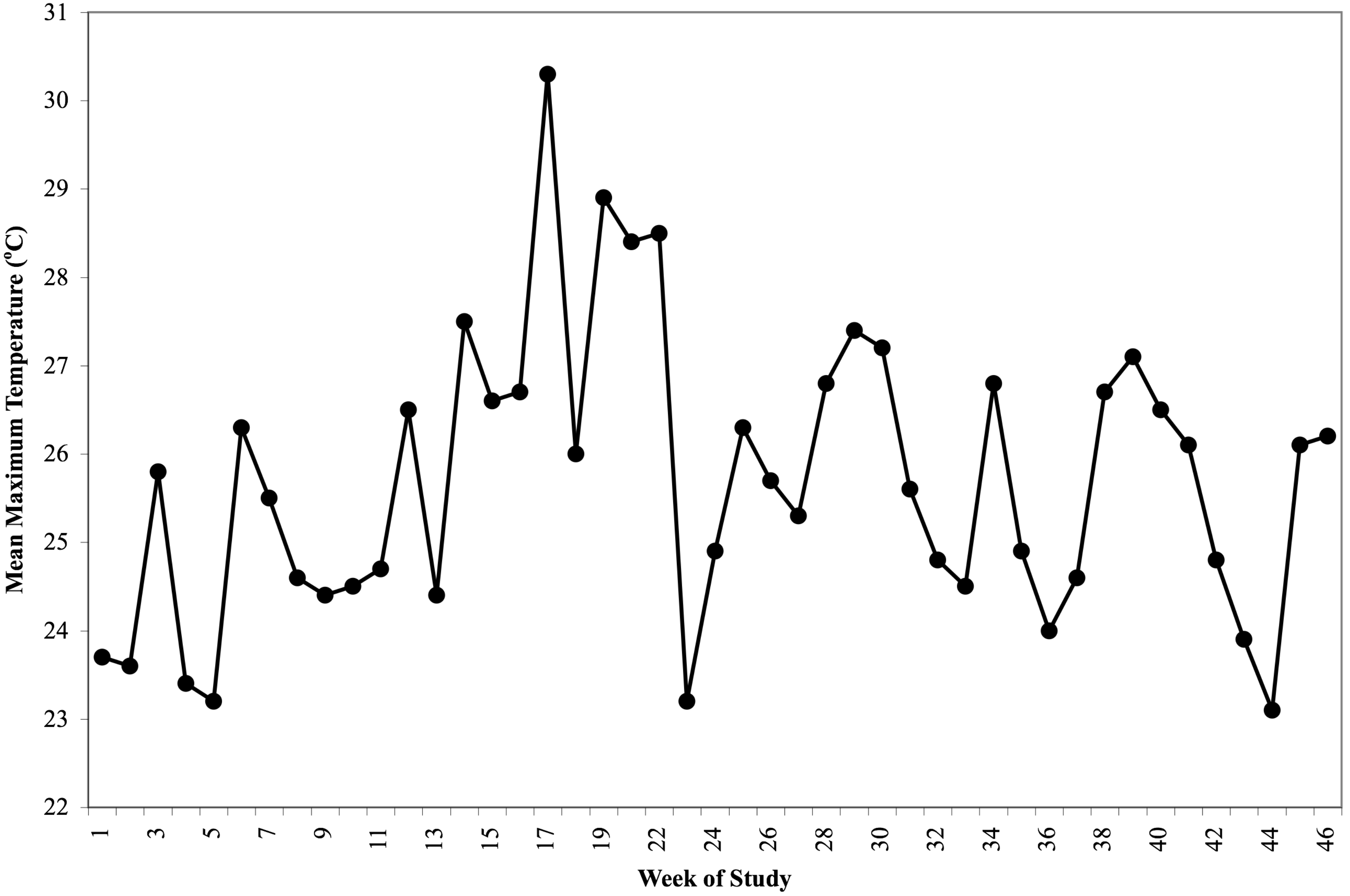

Mean Maximum Temperature (ºC)
31
30
29
28
27
26
25
24
23
22
1
3
5
7
9
11
13
15
17
19
22
24
26
28
30
32
34
36
38
40
42
44
46
Week of Study

Figure 3.3

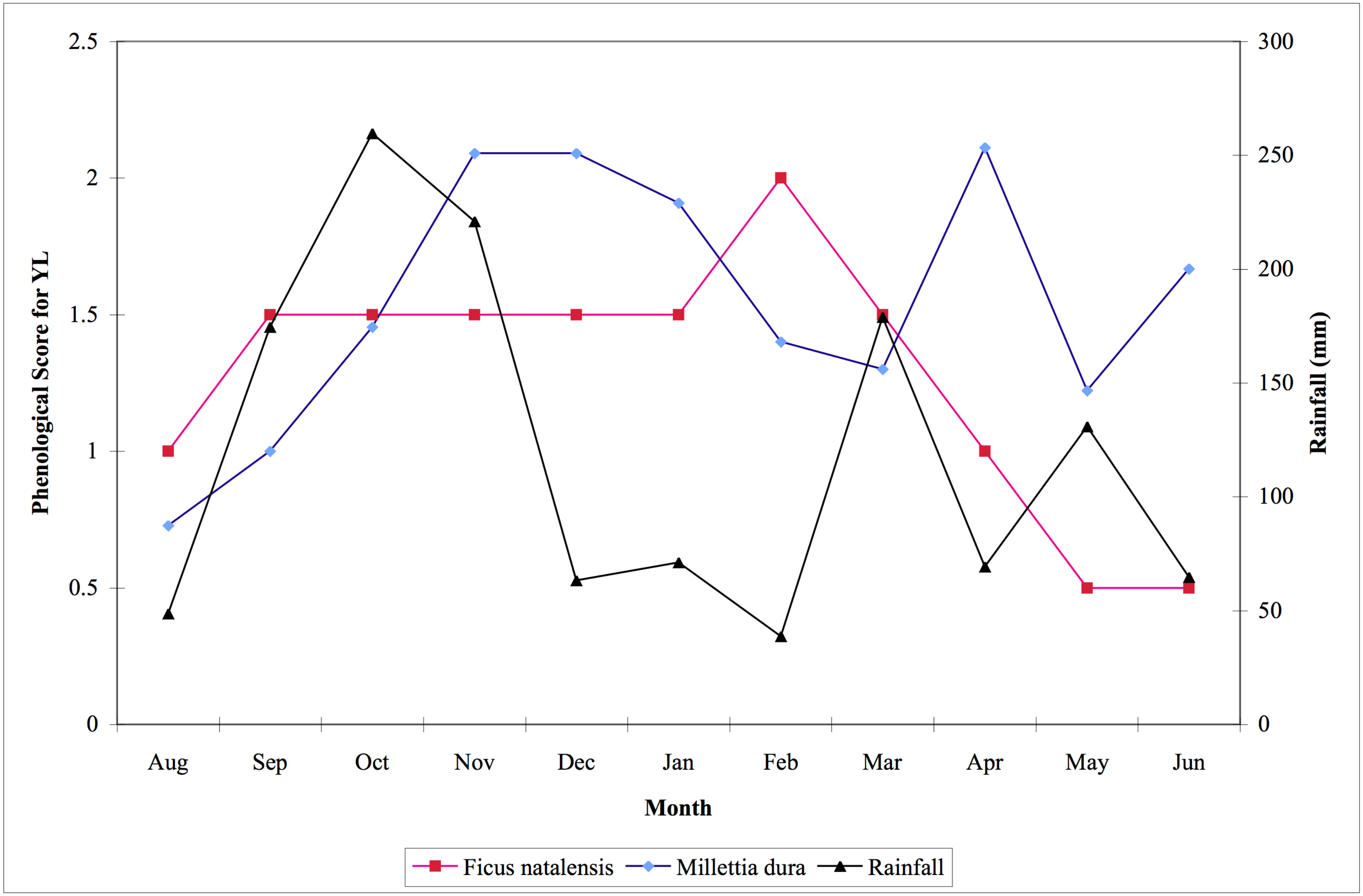

Phenological Score for YL
2.5
2
1.5
1
0.5
0
Rainfall (mm)
300
250
200
150
100
50
0
Aug
Sep
Oct
Nov
Dec
Jan
Feb
Mar
Apr
May
Jun
Month
Ficus natalensis
Millettia dura
Rainfall

Figure 3.4

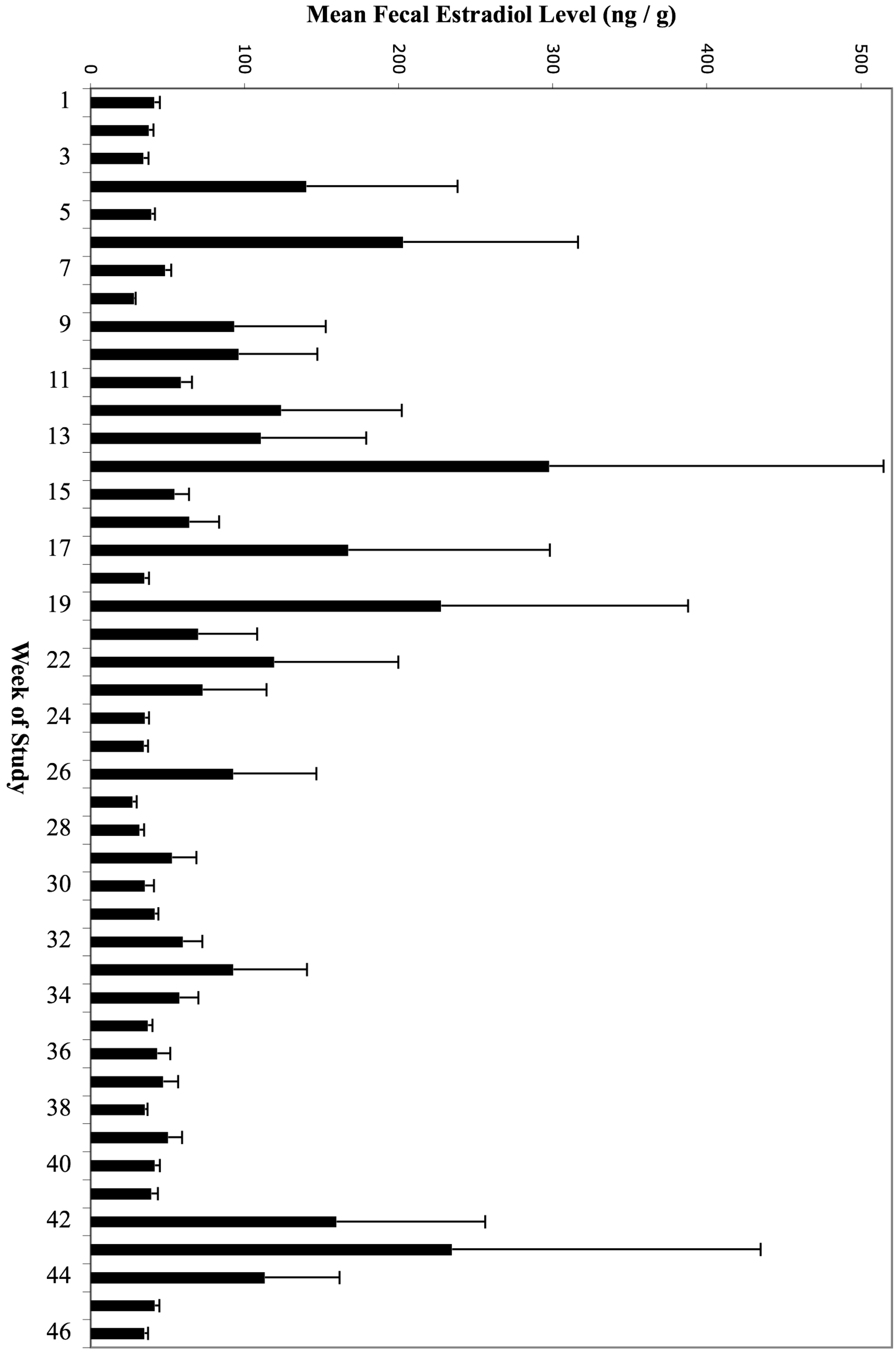
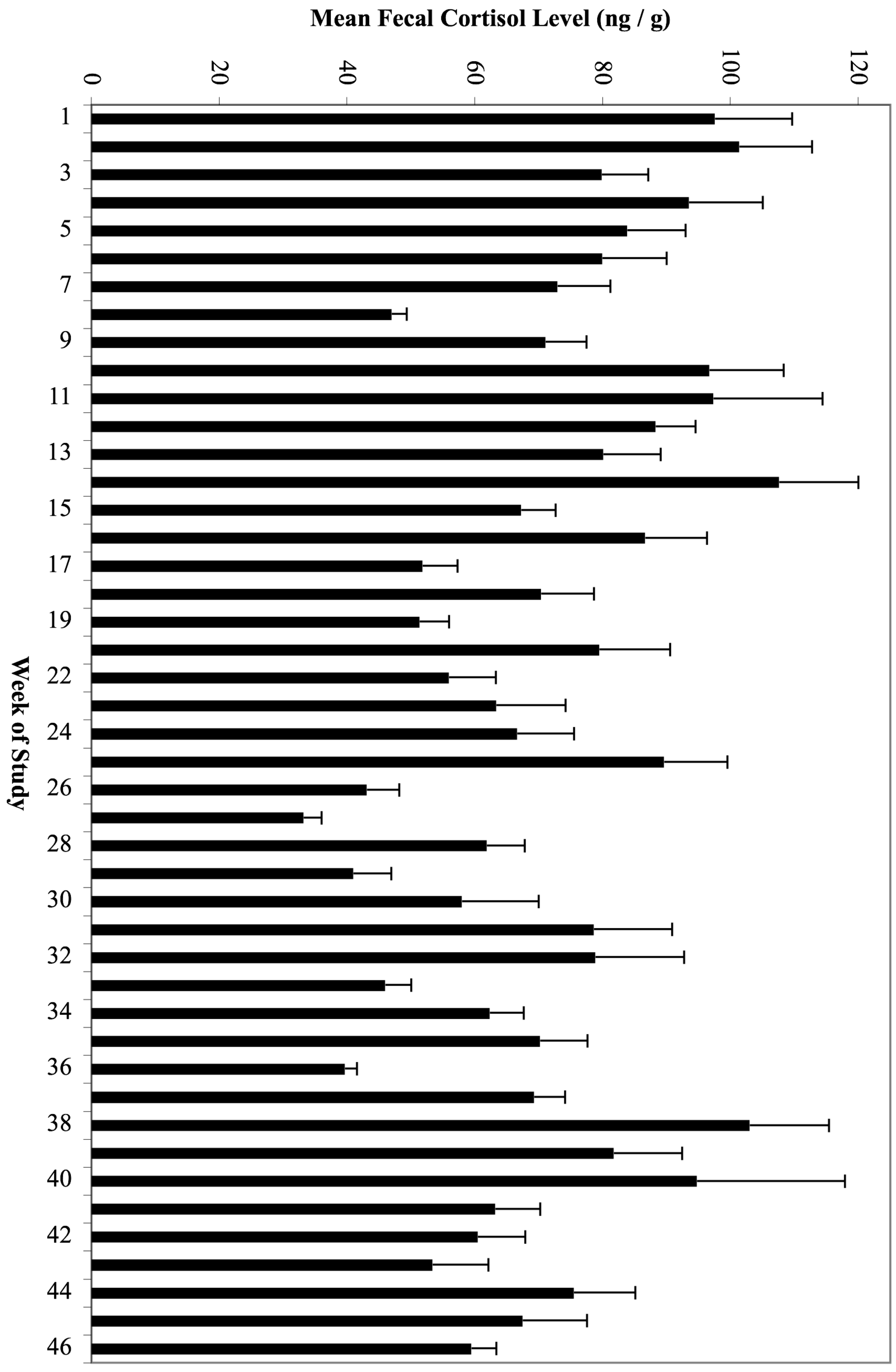

Mean Fecal Estradiol Level (ng / g)
0
100
200
300
400
500
Week of Study
1
3
5
7
9
11
13
15
17
19
22
24
26
28
30
32
34
36
38
40
42
44
46

Figure 3.5

Figure 3.6


Mean Fecal Cortisol Level (ng / g)
0
20
40
60
80
100
120
Week of Study
1
3
5
7
9
11
13
15
17
19
22
24
26
28
30
32
34
36
38
40
42
44
46

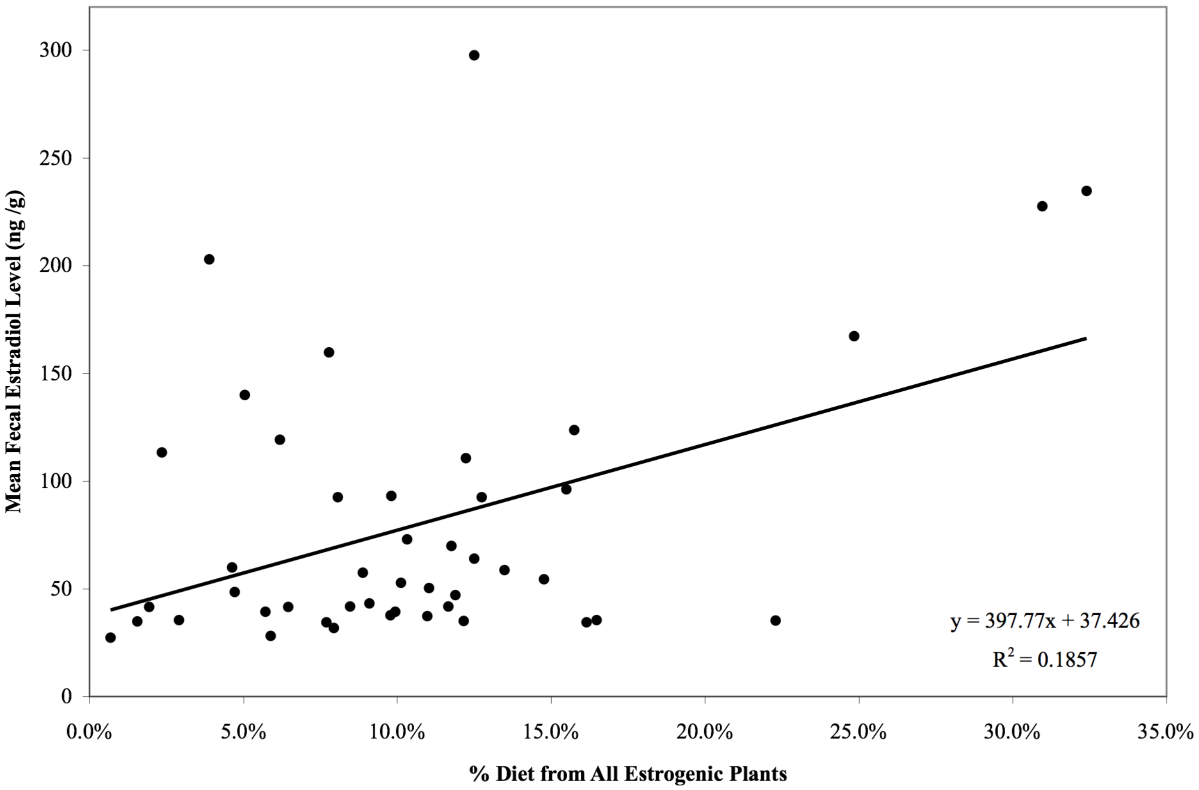

Mean Fecal Estradiol Level (ng /g)
0
50
100
150
200
250
300
0.0%
5.0%
10.0%
15.0%
20.0%
25.0%
30.0%
35.0%
% Diet from All Estrogenic Plants
y = 397.77x + 37.426
R2 = 0.1857

Figure 3.7

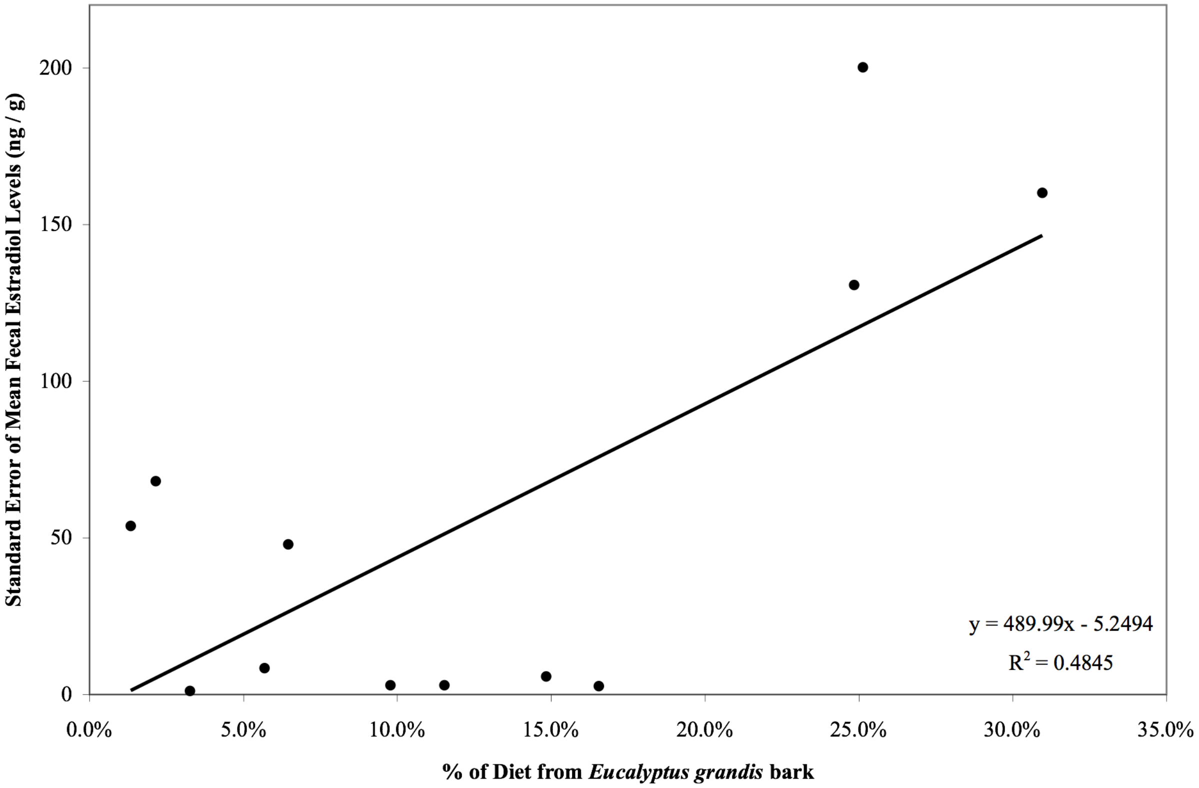

200
150
100
50
0
Standard Error of Mean Fecal Estradiol Levels (ng / g)
y = 489.99x - 5.2494
R2 = 0.4845
0.0%
5.0%
10.0%
15.0%
20.0%
25.0%
30.0%
35.0%
% of Diet from Eucalyptus grandis bark

Figure 3.8

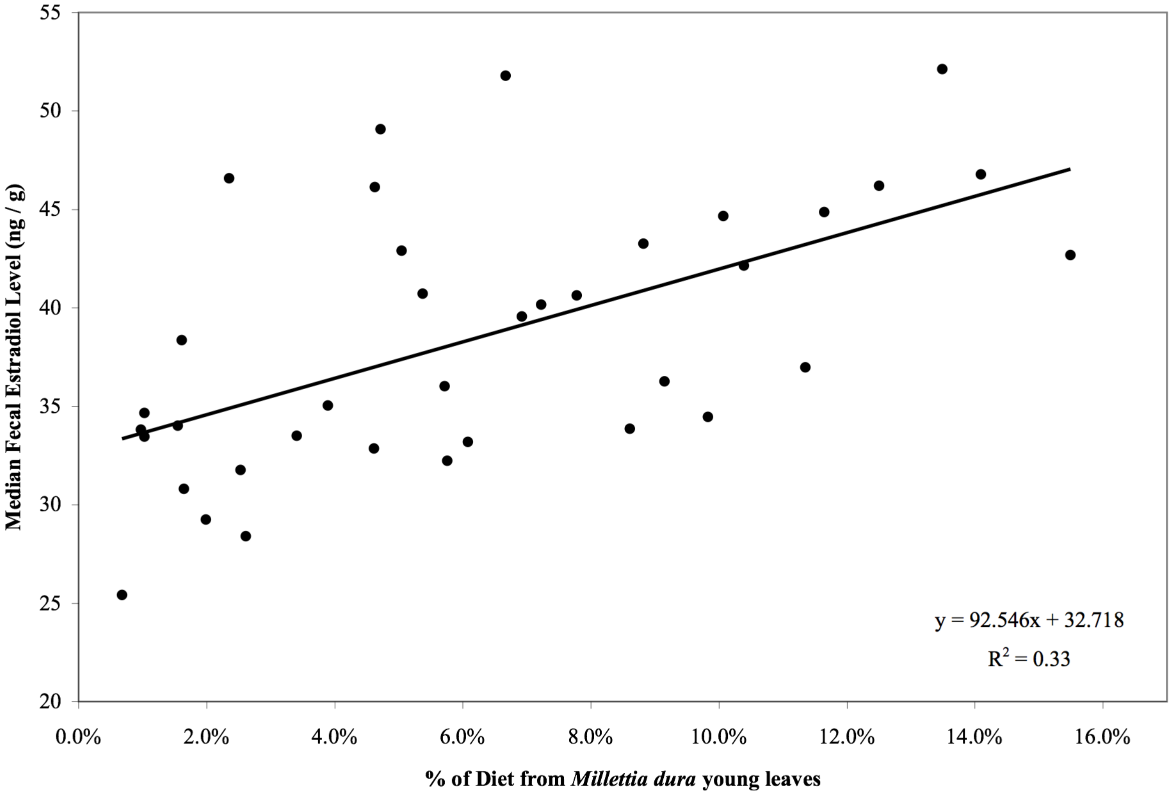

Median Fecal Estradiol Level (ng / g)
55
50
45
40
35
30
25
20
0.0%
2.0%
4.0%
6.0%
8.0%
10.0%
12.0%
14.0%
16.0%
% of Diet from *Millettia dura* young leaves
y = 92.546x + 32.718
$R^2 = 0.33$

Figure 3.9

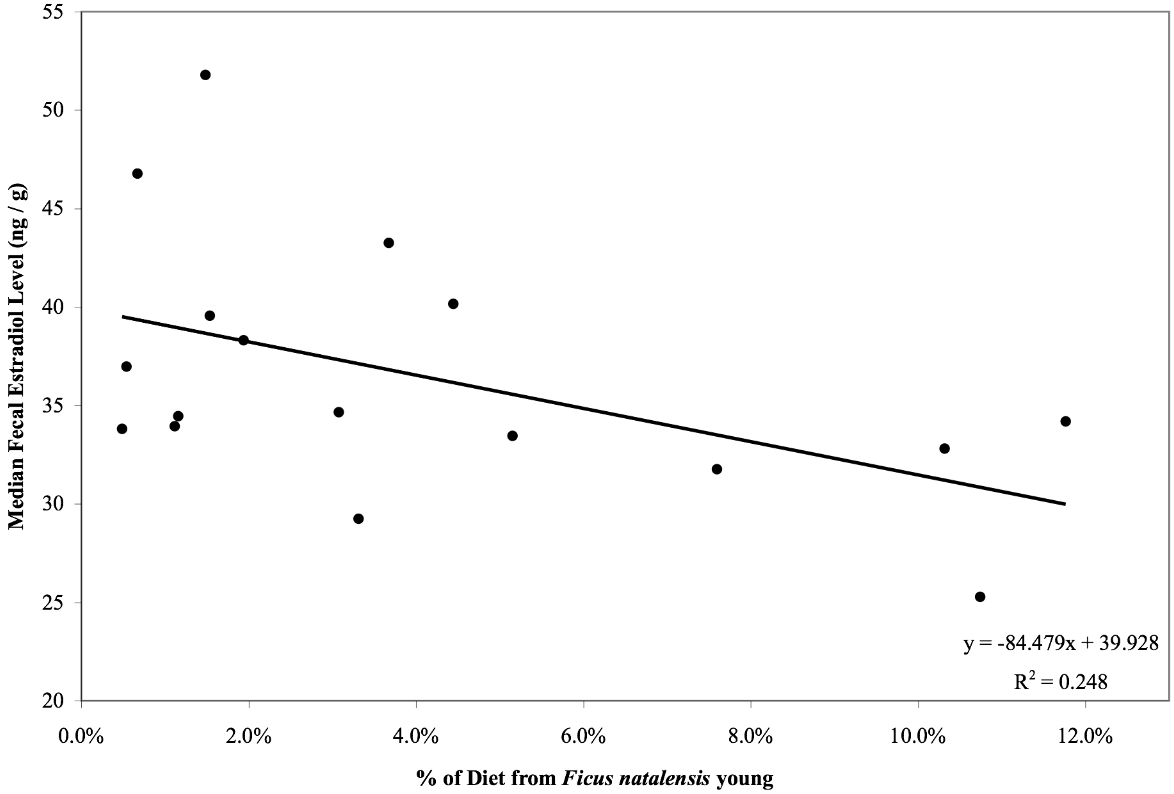

55
50
45
40
35
30
25
20
Median Fecal Estradiol Level (ng / g)
y = -84.479x + 39.928
$R^2 = 0.248$
0.0%
2.0%
4.0%
6.0%
8.0%
10.0%
12.0%
% of Diet from *Ficus natalensis* young

Figure 3.10

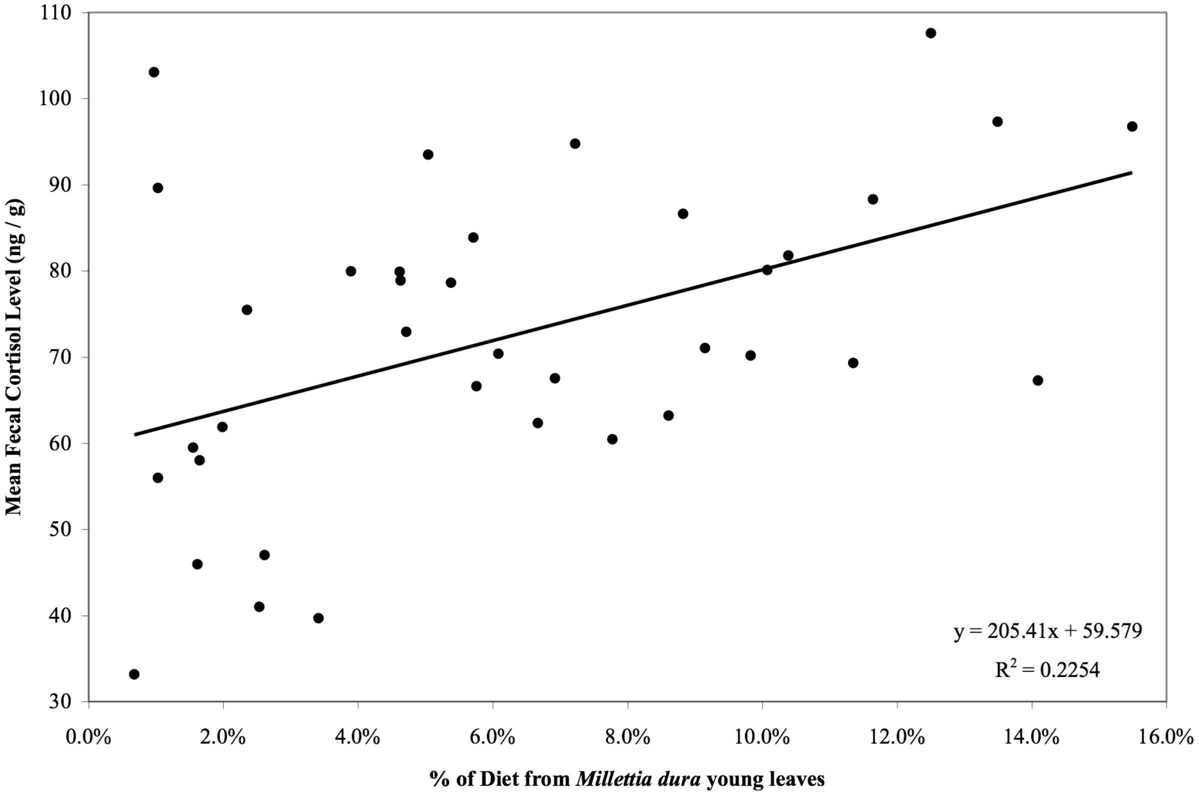

Mean Fecal Cortisol Level (ng / g)
110
100
90
80
70
60
50
40
30
y = 205.41x + 59.579
R² = 0.2254
0.0%
2.0%
4.0%
6.0%
8.0%
10.0%
12.0%
14.0%
16.0%
% of Diet from *Millettia dura* young leaves

Figure 3.11

Table 3.1. Correlation matrix for relationships between various indices of % of diet from estrogenic foods or climatic variables and fecal steroid hormone levels (estradiol and cortisol) of ten adult male red colobus monkeys for each week of the study (n = 45). The relationship is given for the mean, median, and standard error of the mean of the hormone levels to examine influence of estrogenic foods and climate on both the central tendency and variation of estradiol and cortisol.

	Estradiol Mean		Estradiol Median		Estradiol SEM		Cortisol Mean		Cortisol Median		Cortisol SEM	
Predictor	r	p	r	p	r	p	r	p	r	p	r	p
All Estrogenic Foods	**0.432**	**0.003**	-0.082	0.591	**0.495**	**0.001**	-0.148	0.332	-0.170	0.264	-0.045	0.770
Staple Estrogenic Foods	**0.428**	**0.003**	-0.056	0.717	**0.479**	**0.001**	-0.156	0.306	-0.182	0.232	-0.035	0.819
Rare Estrogenic Foods	0.119	0.437	-0.122	0.425	0.181	0.233	0.013	0.931	0.021	0.891	-0.034	0.825
Rare Estrogenic Foods*	0.615	0.104	0.003	0.995	0.628	0.095	-0.366	0.373	-0.330	0.425	-0.320	0.440
Millettia dura young leaves	0.034	0.826	**0.602**	**<0.001**	-0.026	0.863	**0.402**	**0.006**	**0.358**	**0.016**	0.191	0.208
Millettia dura young leaves*	0.272	0.104	**0.574**	**<0.001**	0.250	0.136	**0.475**	**0.003**	**0.461**	**0.004**	0.237	0.158
Ficus natalensis young leaves	-0.081	0.597	**-0.315**	**0.035**	-0.041	0.788	-0.165	0.278	-0.181	0.234	0.087	0.568
Ficus natalensis young leaves*	0.085	0.745	**-0.500**	**0.041**	0.114	0.664	-0.329	0.197	-0.37	0.144	0.059	0.822
Eucalyptus grandis Bark	**0.386**	**0.009**	*-0.280*	*0.063*	**0.450**	**0.002**	**-0.309**	**0.039**	**-0.298**	**0.047**	-0.179	0.241
Eucalyptus grandis Bark*	**0.697**	**0.012**	-0.028	0.931	**0.696**	**0.012**	-0.064	0.844	-0.111	0.730	0.174	0.589
Rainfall	**0.322**	**0.031**	**0.298**	**0.047**	**0.332**	**0.026**	0.157	0.302	0.114	0.454	0.061	0.689
Max Mean Temp	0.205	0.176	-0.114	0.456	0.214	0.159	-0.145	0.343	-0.111	0.469	-0.077	0.615

*Correlations excluding weeks the predictor variable was not fed on.

CHAPTER 4:

Conclusions and future research objectives

CONCLUSIONS

Chapter 1

In Chapter 1, I examined the potential roles of estrogenic plants in primate ecology and evolution by reviewing what is known about phytoestrogens from an ecological and evolutionary perspective, as well as the role of sex steroid hormones in vertebrate physiology and behavior and how phytoestrogens can interfere with this. Although numerous hypotheses regarding the phytoestrogen-primate relationship have been postulated (i.e., "plant defense hypothesis", "self-medication hypothesis", and "neutral hypothesis"), little data exist on their presence in the diets of wild primates, the physiological and behavioral effects of consuming phytoestrogens for wild primates, or the ecological and evolutionary relationships between estrogenic plants and primates. On the other hand, phytoestrogens in human plant-based foods are the focus of much research, including experimental studies that examine the physiological and behavioral effects of phytoestrogens for captive rodents and non-human primates. Based upon evidence from these studies, the conservative nature of the endocrine system across vertebrates, and the prevalence of phytoestrogens in leguminous plants, it is possible that hormonal interactions between plants and primates have played important roles in their ecological and evolutionary relationships. Therefore, I concluded this chapter by suggesting that if wild primates do consume phytoestrogens and significant inter- and intra-specific variation in the amount consumed exists in natural systems, then the potential physiological and behavioral effects seen in captive and laboratory studies likely promote differential survival and reproduction of individuals. Consequently, phytoestrogens would have an important, thus far neglected, role in primate ecology and evolution and may have important implications for primate conservation.

Chapter 2

In Chapter 2, I began building a comprehensive database on the presence of phytoestrogens in the diets of wild primates by investigating the prevalence of estrogenic plant species in the diets of red colobus monkeys (*Procolobus rufomitratus*) of Kibale National Park and mountain gorillas (*Gorilla beringei*) of Bwindi National Park, both in Uganda. To determine the composition of their diets, I collected 1327 hours of behavioral observations on one red colobus group and used published data on one gorilla group. To examine plant foods for estrogenic activity, I screened 44 plant items 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 bound to and activated ERβ, but not ERα. These results confirmed that estrogenic plant foods are routinely consumed by two highly folivorous African primate species. Phytoestrogens in the wild plant foods of these and many other wild primates may have important implications for better understanding primate reproductive ecology.

Chapter 3

In Chapter 3, I began examining the physiological effects of ingesting phytoestrogens for the red colobus monkey using my 11-month field study of their diet and fecal estradiol and cortisol levels. I found the overall percent of diet coming from these estrogenic plant foods averaged 10.7% (n = 45 weeks; range: 0.7% to 32.4%). Climatic factors were important for understanding variation in the proportion of diet coming from estrogenic plants, particularly for the consumption of *Millettia dura* young leaves. Although red colobus did not feed more heavily on *M. dura* young leaves when they were more available, they did feed more heavily on them during months of higher rainfall. In stepwise regressions examining the relationship of both climatic factors and estrogenic plant consumption with male red colobus hormone levels, the weekly median fecal estradiol level was best predicted by the percent of diet from estrogenic *M. dura* young leaves; the weekly median fecal cortisol level was also best predicted by the percent of diet from *M. dura* young leaves. Thus, it appeared that climatic factors influenced how much red colobus consumed estrogenic plant foods and the consumption of these estrogenic plant foods influenced the hormone levels of red colobus monkeys. Further, these results suggested that consumption of estrogenic plants by red colobus monkeys may have important implications for their health and fitness through interactions with the endocrine system. It is likely that the ecology and evolution of wild primates have been influenced by plants that produce phytoestrogens in ways not yet fully appreciated.

FUTURE RESEARCH OBJECTIVES

Further analyses using data collected during dissertation research

In this dissertation I have limited my scope to a review of what is currently known about the role of phytoestrogens in primate ecology and evolution, an examination of the prevalence of estrogenic plants in the diets of the red colobus monkey and mountain gorilla of western Uganda, and how the consumption of phytoestrogens relates to physiological changes in male red colobus. However, other types of data were also collected during this study. Using these data, I will test to see if the relationships found between the consumption of estrogenic plants and male red colobus hormone levels holds for adult female red colobus by examining how variation in estradiol, progesterone, and cortisol levels of 701 fecal samples from 14 adult females relates to the consumption of estrogenic plants. I will also use behavioral data to examine if the apparent hormonal response to phytoestrogen consumption found here translates into behavioral endpoints, particularly rates of mating, aggression, and anxiety-related behaviors (e.g., self-scratching).

Phytoestrogens in the primate diet: Understanding the evolutionary and ecological implications of estrogenic plants

The results of my dissertation raise a number of intriguing questions with relevance to both the dietary ecology of primates and the evolution of modern human biology: How prevalent are phytoestrogens in the diets of other primate species? Are there differences in exposure between frugivores and folivores? Are there differences between monkeys and apes or Latin American primates and African primates? What environmental factors influence phytoestrogen content and how does this affect primate feeding behavior? Could non-native estrogenic plants threaten the survival of endangered primates? Now that I have the necessary baseline information showing that phytoestrogens are consumed by wild primates and their consumption does relate to primate physiology, the next step in my research program is to use a threefold approach at understanding the evolutionary and ecological implications of estrogenic plants in the diets of wild primates.

First, I plan to examine the evolutionary implications of phytoestrogens by comparing the prevalence of estrogenic plants in the primate diet based upon dietary niche and phylogeny using numerous wild primate species. To examine the relative importance of dietary niche and phylogeny to phytoestrogen exposure, communities of sympatric primate species must be studied. Many closely related sympatric primate species are suggested to be able to coexist largely because of differences in their dietary niches (Terborgh 1983). Often, communities of primates include species classified as obligate frugivores, dependent on fruit even during times of fruit scarcity, and species classified as either opportunistic frugivores, able to use fibrous leaves during periods of fruit scarcity, or obligate folivores, dependent almost entirely on parts of plants other than ripe fruits throughout the year (Lambert 1998). Well-studied examples of such sympatry in primate communities include the folivorous howler monkey (*Alouatta*) and frugivorous spider monkey (*Ateles*) of Latin America (Milton 1981) and the frugivorous chimpanzee (*Pan*) and folivorous gorilla (*Gorilla*) of Africa (Stanford & Nkurunungi 2003). Although I found estrogenic plants in the diets of two highly folivorous Ugandan primate species, it is still unclear whether such compounds regularly occur in ripe fruits and are ingested to any notable extent by frugivorous primates. Therefore, I plan to expand upon my dissertation results by screening the diets of frugivores living sympatrically with the red colobus and mountain gorillas, as well as the diets of sympatric primate species living in the forests of Latin America, for estrogenic activity. Studying such sympatric species and using a comparative approach (e.g., Nunn & Barton 2001) will allow a test of the "plant defense hypothesis", while also providing important insights into the potential role of these compounds in human evolution, as pre-human ancestors were likely highly frugivorous.

Secondly, to provide a more detailed understanding of the ecological relationship between the red colobus of Kibale National Park and their estrogenic staple foods (i.e., *Millettia dura*, *Ficus natalensis*, and *Eucalyptus grandis*), variation in the phytoestrogen levels of these three plant species will be examined at the seasonal and intraspecific levels. It is possible that more phytoestrogens are produced during the time of year with more intense sunlight or for individual trees located in sunnier locations (Mazur & Adlercreutz 1998) or during periods of low (Leopold et al. 1976, Mazur & Adlercreutz 1998) or high rainfall (Rochester & Millam 2009). The influence of climate on phytoestrogen levels is likely very important for red colobus ecology, as the availability of their preferred dietary item, young leaves (Chapman et al. 2002), is lowest during the dry season for their preferred food species (Struhsaker 1997). Thus, if phytoestrogen levels are highest at this time of year due to light intensity and rainfall, and if estrogenic plants are used as a source of food during this "crunch" period, then this may be the time of year when phytoestrogen consumption exerts the most dramatic effects on the red colobus.

Finally, I plan to examine the physiological effects of feeding on *Eucalyptus grandis* for a number of primate species in both Uganda and Latin America, as this tree is a non-native species with the potential to act as an endocrine disruptor on many primates throughout the tropics. Black-and-white colobus monkeys (Harris & Chapman 2007), mountain gorillas (Rothman et al. 2006b), and howler monkeys (Serio Silva et al. 2006) are all known to consume *Eucalyptus*, likely as a source of sodium (Rode et al. 2003, Rothman et al. 2006b). Interestingly, a widespread female reproductive tract disease, similar to "clover disease" in sheep, has caused reduced fertility in koalas, which naturally depend on various species of *Eucalyptus* as a dietary source. It has been suggested that the consumption of phytoestrogens in eucalypt foliage was the cause of this reduction in fertility (Martin 1981), while the immediate cause is now known to be

Chlamydia psittaci infections (Canfield 1989). Since more recent studies have shown phytoestrogens to suppress inflammation and immunity in lab mice (Yellayi et al. 2002, 2003), such infections could result from the immunosuppressive actions of phytoestrogens. Further, studies on amphibians have shown immunosuppression caused by anthropogenic endocrine disruptors in pesticides, with a possible mechanism of action via increased stress hormone production (Hayes et al. 2006). In combination, these studies and results warrant a further, more detailed examination of the potential for *E. grandis* to act as an endocrine disruptor on wild primates.

Ultimate goal of research plan

To test my hypothesis that estrogenic plants play important roles in primate ecology and evolution through the effects of phytoestrogens on differential survival and reproduction, future studies should attempt to examine the relationship between phytoestrogen consumption and primate fitness. This can be done by studying a series of groups with varying levels of estrogenic plants in the diet and relating this to the reproductive output and survival rates of these groups. Considering the long life span of primates, such studies will take time. Nonetheless, they are well worth doing to fully understand the potential for estrogenic plants to influence the ecology and evolution of primates, as well as evaluate their importance in our own evolution and current health situation.

LITERATURE CITED

Adams, N. 1990. Permanent infertility in ewes exposed to plant oestrogens. Australian Veterinary Journal 67: 197-201.

Adams, N. 1995. Detection of the effects of phytoestrogens on sheep and cattle. Journal of Animal Science 73: 1509-1515.

Adlercreutz, H., P. Musey, T. Fotsis, C. Bannwart, K. Wähälä, T. Mäkelä, G. Brunow, and T. Hase. 1986. Identification of lignans and phytoestrogens in urine of chimpanzees. Clinica Chimica Acta 158: 147-154.

Adlercreutz, H., K. Höckerstedt, C. Bannwart, S. Bloigu, E. Hämäläinen, T. Fotsis, and A. Ollus. 1987. Effect of dietary components, including lignans and phytoestrogens, on enterohepatic circulation and liver metabolism of estrogens and on sex hormone binding globulin (SHBG). Journal of Steroid Biochemistry 27: 1135-1144.

Almstrup, K., M. Fernández, J. Petersen, N. Olea, N. Skakkebæk, and H. Leffers. 2002. Dual effects of phytoestrogens result in u-shaped dose-response curves. Environmental Health Perspectives 110: 743-748.

An, J., C. Tzagarakis-Foster, T. Scharschmidt, N. Lomri, and D. Leitman. 2001. Estrogen receptor β-selective transcriptional activity and recruitment of coregulators by phytoestrogens. The Journal of Biological Chemistry 276: 17808-17814.

Ball, E., M. Caniglia, J. Wilcox, K. Overton, M. Burr, B. Wolfe, B. Sanders, A. Wisniewski, and C. Wrenn. 2010. Effects of genistein in the maternal diet on reproductive development and spatial learning in male rats. Hormones and Behavior 57: 313-322.

Bauchop, T. and R. Martucci. 1968. Ruminant-like digestion of the langur monkey. Science 161: 698-700.

Beck, V., E. Unterrieder, L. Krenn, W. Kubelka, and A. Jungbauer. 2003. Comparison of hormonal activity (estrogen, androgen and progestin) of standardized plant extracts for large scale use in hormone replacement therapy. Journal of Steroid Biochemistry and Molecular Biology 84: 259-268.

Bennetts, H. 1946. Metaplasia in the sex organs of castrated male sheep maintained on early subterranean clover pastures. Australian Veterinary Journal 22: 70-78.

Bennetts, H. and E. Underwood. 1951. The oestrogenic effects of subterranean clover (*Trifolium subterraneum*): Uterine maintenance in the ovariectomised ewe on clover grazing. Australian Journal of Experimental Biology and Medical Science 29: 249-253.

Berger, P., E. Sanders, P. Gardner, and N. Negus. 1977. Phenolic plant compounds functioning as reproductive inhibitors in *Microtus montanus*. Science 195: 575-577.

Berger, P., N. Negus, E. Sanders, and P. Gardner. 1981. Chemical triggering of reproduction in *Microtus montanus*. Science 214: 69-70.

Burgess, M. and C. Chapman. 2005. Tree leaf chemical characters: selective pressures by folivorous primates and invertebrates. African Journal of Ecology 43: 242-250.

Caldwell, C., S. Britz, and R. Mirecki. 2005. Effect of temperature, elevated carbon dioxide, and drought during seed development on the isoflavone content of dwarf soybean [*Glycine max* (L.) Merrill] grown in controlled environments. Journal of Agricultural and Food Chemistry 53: 1125-1129.

Canfield, P. 1989. A survey of urinary tract disease in New South Wales koalas. Australian Veterinary Journal 66: 103-106.

Cederroth, C., J. Auger, C. Zimmermann, F. Eustache, and S. Nef . 2010a. Soy, phyto-oestrogens and male reproductive function: a review. International Journal of Andrology 33: 304-316.

Cederroth, C., C. Zimmermann, J. Beny, O. Schaad, C. Combepine, P. Descombes, D. Doerge, F. Pralong, J. Vassalli, and S. Nef. 2010b. Potential detrimental effects of a phytoestrogen-rich diet on male fertility in mice. Molecular and Cellular Endocrinology 321: 152-160.

Chamorro, S., V. Hernández, E. Monsalvez, J. Becerra, M. Mondaca, B. Piña, and G. Vidal. 2010. Detection of estrogenic activity from kraft mill effluents by the yeast estrogen screen. Bulletin of Environmental Contamination and Toxicology. 2010: 165-169.

Chapman, C. and J. Lambert. 2000. Habitat alteration and the conservation of African primates: Case study of Kibale National Park, Uganda. American Journal of Primatology 50: 169-185.

Chapman, C. and C. Peres. 2001. Primate conservation in the new millennium: The role of scientists. Evolutionary Anthropology 10:16-33.

Chapman, C., R. Wrangham, L. Chapman, D. Kennard, and A. Zanne. 1999. Fruit and flower phenology at two sites in Kibale National Park, Uganda. Journal of Tropical Ecology 15: 189-211.

Chapman, C., L. Chapman, K. Bjorndal, and D. Onderdonk. 2002. Application of protein-to-fiber ratios to predict colobine abundance on different spatial scales. International Journal of Primatology 23: 283-310.

Chapman, C., L. Chapman, K. Rode, E. Hauck, and L. McDowell. 2003. Variation in the nutritional value of primate foods: Among trees, time periods, and areas. International Journal of Primatology 24: 317-333.

Chapman, C., L. Chapman, T. Struhsaker, A. Zanne, C. Clark, and J. Poulsen. 2005. A long-term evaluation of fruiting phenology: importance of climate change. Journal of Tropical Ecology 21: 31-45.

Chapman, C., L. Chapman, A. Jacob, J. Rothman, P. Omeja, R. Reyna-Hurtado, J. Hartter, and M. Lawes. 2010. Tropical tree community shifts: Implications for wildlife conservation. Biological Conservation 143: 366-374.

Cline, J. and C. Wood. 2009. Estrogen/isoflavone interactions in cynomolgus macaques (*Macaca fascicularis*). American Journal of Primatology 71: 722-731.

Colborn. T., F. vom Saal, and A. Soto. 1993. Developmental effects of endocrine-disrupting chemicals in wildlife and humans. Environmental Health Perspectives 101: 378-384.

Coley, P. and J. Barone. 1996. Herbivory and plant defenses in tropical forests. Annual Review of Ecology and Systematics 27: 305-335.

Cornwell T, Cohick W, and Raskin I. 2004. Dietary phytoestrogens and health. Phytochemistry 65: 995-1016.

Cvoro, A., S. Paruthiyil, J. Jones, C. Tzagarakis-Foster, N. Clegg, D. Tatomer, R. Medina, M. Tagliaferri, F. Schaufele, T. Scanlan, M. Diamond, I. Cohen, and D. Leitman. 2007. Selective activation of estrogen receptor-β transcriptional pathways by an herbal extract. Endocrinology 148: 538-547.

DeGabriel, J., B. Moore, W. Foley, and C. Johnson. 2009. The effects of plant defensive chemistry on nutrient availability predict reproductive success in a mammal. Ecology 90: 711-719.

Dixon, R. 2004. Phytoestrogens. Annual Review of Plant Biology 55: 225-261.

Emery Thompson, M., M. Wilson, G. Gobbo, M. Muller, and A. Pusey. 2008. Hyperprogesteronemia in response to *Vitex fischeri* consumption in wild chimpanzees (*Pan troglodytes schweinfurthii*). American Journal of Primatology 70: 1064-1071.

Fidler, A., S. Lawrence, and K. McNatty. 2008. An hypothesis to explain the linkage between kakapo (*Strigops habroptilus*) breeding and the mast fruiting of their food trees. Wildlife Research 35: 1-7.

Forbey, J., A. Harvey, M. Huffman, F. Provenza, R. Sullivan, and D. Tasdemir. 2009. Exploitation of secondary metabolites by animals: A response to homeostatic challenges. Integrative and Comparative Biology 49: 314-328.

Fox, J., M. Starcevic, P. Jones, M. Burow, and J. McLachlan. 2004. Phytoestrogen signaling and symbiotic gene activation are disrupted by endocrine-disrupting chemicals. Environmental Health Perspectives 112: 672-677.

Freeland, W. and D. Janzen. 1974. Strategies in herbivory by mammals: Role of plant secondary compounds. American Naturalist 108: 269-289.

Garey, J. 1993. Dietary estrogens as a cue for primate breeding seasons. American Journal of Physical Anthropology S16: 92 (*meeting abstract*).

Glander, K. 1979. Howling monkey feeding behavior and plant secondary compounds: A study of strategies. In: The Ecology of Arboreal Folivores. G.G. Montgomery (ed.). Smithsonian Institution Press: Washington, D.C.

Glander, K. 1980. Reproduction and population growth in free-ranging mantled howling monkeys. American Journal of Physical Anthropology 53: 25-36.

Greaves, M. 2007. Darwinian medicine: a case for cancer. Nature Reviews Cancer 7: 213-221.

Guillette, L. 2000. Contaminant-induced endocrine disruption in wildlife. Growth Hormone & IGF Research Supplement B: S45-S50.

Gultekin E, and Yildiz F. 2006. Introduction to phytoestrogens. In: Phytoestrogens in Functional Foods. F. Yildiz (ed.). CRC Press: Boca Raton, Florida.

Guschanski, K., L. Vigilant, A. McNeilage, M. Gray, E. Kagoda, and M. Robbins. 2009. Counting elusive animals: Comparing field and genetic census of the entire mountain gorilla population of Bwindi Impenetrable National Park, Uganda. Biological Conservation 142: 290-300.

Hadley, M. 2000. Endocrinology (5th edition). Prentice Hall: Upper Saddle River, New Jersey.

Harborne, J. 1993. Introduction to Ecological Biochemistry (4th edition). Elsevier Academic Press: San Francisco, California.

Harris, T. and C. Chapman. 2007. Variation in diet and ranging of black and white colobus monkeys in Kibale National Park, Uganda. Primates 48: 208-221.

Hartley, D., J. Edwards, C. Spiller, N. Alom, S. Tucci, P. Seth, M. Forsling, and S. File. 2003. The soya isoflavone content of rat diet can increase anxiety and stress hormone release in the male rat. Psychopharmacology 167: 46-53.

Hayes, T. 2005. Welcome to the revolution: Integrative biology and assessing the impact of endocrine disruptors on environmental and public health. Integrative and Comparative Biology 45: 321-329.

Hayes, T., A. Collins, M. Lee, M. Mendoza, N. Noriega, A. Stuart, and A. Vonk. 2002. Hermaphroditic, demasculinized frogs after exposure to the herbicide atrazine at low ecologically relevant doses. Proceedings of the National Academy of Sciences 99: 5476-5480.

Hayes, T., P. Case, S. Chui, D. Chung, C. Haeffele, K. Haston, M. Lee, V. Mai, Y. Marjuoa, J.

Parker, and M. Tsui. 2006. Pesticide mixtures, endocrine disruption, and amphibian declines: Are we underestimating the impact? Environmental Health Perspectives 114: 40-50.

Heftmann, E. 1975. Functions of steroids in plants. Phytochemistry 14: 891-901.

Heistermann, M., S. Tari, and J. Hodges. 1993. Measurement of faecal steroids for monitoring ovarian function in New World primates, Callitrichidae. Journal of Reproduction and Fertility 99: 243-251.

Heldring, N., A. Pike, S. Andersson, J. Matthews, G. Cheng, J. Hartman, M. Tujague, A. Ström, E. Treuter, M. Warner, and J. Gustafsson. 2007. Estrogen receptors: how do they signal and what are their targets. Physiological Reviews 87: 905-931.

Henry, L. and D. Witt. 2002. Resveratrol: Phytoestrogen effects on reproductive physiology and behavior in female rats. Hormones and Behavior 41: 220-228.

Higham, J., C. Ross, Y. Warren, M. Heistermann, and A. MacLarnon. 2007. Reduced reproductive function in wild baboons (*Papio hamadryas anubis*) related to natural consumption of the African black plum (*Vitex doniana*). Hormones and Behavior 52: 384-390.

Howard, P., T. Davenport, F. Kigenyi, P. Viskanic, M. Baltzer, C. Dickinson, J. Lwanga, R. Matthews, and E. Mupada. 2000. Protected area planning in the tropics: Uganda's national system of forest nature reserves. Conservation Biology 14: 858-875.

Huffman, M. 1997. Current evidence for self- medication in primates: a multidisciplinary perspective. Yearbook of Physical Anthropology 40: 171–200.

Huffman, M. 2001. Self-medicative behavior in the African great apes: An evolutionary perspective into the origins of human traditional medicine. Bioscience 51: 651-661.

Hughes, C. 1988. Phytochemical mimicry of reproductive hormones and modulation of herbivore fertility by phytoestrogens. Environmental Health Perspectives 78: 171-175.

Iino, M., T. Nomura, Y. Tamaki, Y.Yamada, K. Yoneyama, Y. Takeuchi, M. Mori, T. Asami, T. Nakano, and T. Yokota. 2007. Progesterone: Its occurrence in plants and involvement in plant growth. Phytochemistry 68: 1664-1673.

Janeczko, A. and A. Skoczowski. 2005. Mammalian sex hormones in plants. Folia Histochemica Et Cytobiologica 43: 71-79.

Jondhale, N., U. Shanker, S. Mehrotra, S. Agarwal, S. Singh, G. Das, and S. Deori. 2009. Effect of *Aegle marmelos* and *Ficus religiosa* leaves extracts on the ovarian function in rats. Indian Veterinary Journal 86: 1141-1144.

Karangi, M. 2008. Revisiting the roots of Gikuyu culture through the sacred Mugumo tree. Journal of African Cultural Studies 20: 117-132.

Ketcha Wanda, G., D. Njamen, E. Yankep, M. Tagatsing Fotsing, Z. Tanee Fomum, J. Wober, S. Starcke, O. Zierau, and G. Vollmer. 2006. Estrogenic properties of isoflavones derived from *Millettia griffoniana*. Phytomedicine 13: 139-145.

Kouki, T., M. Kishitake, M. Okamoto, I. Oosuka, M. Takebe, and K. Yamanouchi. 2003. Effects of neonatal treatment with phytoestrogens, genistein and daidzein, on sex difference in female rat brain function: estrous cycle and lordosis. Hormones and Behavior 44: 140-145.

Krishnan, A., P. Stathis, S. Permuth, L. Tokes, and D. Feldman. 1993. Bisphenol-A: An estrogenic substance is released from polycarbonate flasks during autoclaving. Endocrinology 132: 2279-2286.

Kubo, I., J. Klocke, and S. Asano. 1983. Effects of ingested phytoecdysteroids on the growth and development of two lepidopterous larvae. Journal of Insect Physiology 29: 307-316.

Kuiper, G., J. Lemmen J, B. Carlsson, J. Corton, S. Safe, P. van der Saag, B. van der Burg, and J. Gustafsson. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β. Endocrinology 139: 4252-4263.

Kurzer, M. and X. Xu. 1997. Dietary phytoestrogens. Annual Review of Nutrition 17: 353-381.

Lambert, J. 1998. Primate digestion: interactions among anatomy, physiology, and feeding ecology. Evolutionary Anthropology 7: 8-20.

Leitman, D., S. Paruthiyil, O. Vivar, E. Saunier, C. Herber, I. Cohen, M. Tagliaferri, and T. Speed. 2010. Regulation of specific target genes and biological responses by estrogen receptor subtype agonists. Current Opinion in Pharmacology 10: 629-636.

Leopold, A., M. Erwin, J. Oh, and B. Browning. 1976. Phytoestrogens: Adverse effects on reproduction in California quail. Science 191: 98-100.

Lu, A., J. Beehner, N. Czekala, A. Koenig, E. Larney, C. Borries. 2010. Phytochemicals and reproductive function in wild female Phayre's leaf monkeys (*Trachypithecus phayrei crepusculus*). Hormones and Behavior 59: 28-36.

Martin, R. 1981. Age-specific fertility in three populations of the koala, *Phascolarctos cinereus* (Goldfuss), in Victoria. Australian Wildlife Research 8: 275-283.

Mazur, W. 1998. Phytoestrogen content in foods. Baillière's Clinical Endocrinology and Metabolism 12: 729-742.

Mazur, W. and H. Adlercreutz. 1998. Naturally occurring oestrogens in food. Pure and Applied Chemistry 70: 1759-1776.

McKey, D., J. Gartlan, P. Waterman, and G. Choo. 1981. Food selection by black colobus monkeys (*Colobus satanas*) in relation to plant chemistry. Biological Journal of the Linnean Society 16: 115-146.

McNeilage, A., A. Plumptre, A. Brock-Doyle, and A. Vedder. 2001. Bwindi Impenetrable National Park, Uganda: gorilla census 1997. Oryx 35: 39-47.

Messina, M. 2010. A brief historical overview of the past two decades of soy and isoflavone research. The Journal of Nutrition 140: 1350S-1354S.

Milnes, M., D. Bermudez, T. Bryan, T. Edwards, M. Gunderson, I. Larkin, B. Moore, and L. Guillette. 2006. Contaminant-induced feminization and demasculinization of nonmammalian vertebrate males in aquatic environments. Environmental Research 100: 3-17.

Milton, K. 1979. Factors influencing leaf choice by howler monkeys: a test of some hypotheses of food selection by generalist herbivores. American Naturalist 114: 362-378.

Milton, K. 1980. The Foraging Strategy of Howler Monkeys: A Study in Primate Economics. Columbia University Press: New York.

Milton, K. 1981. Food choice and digestive strategies of two sympatric primate species. American Naturalist 117: 496-505.

Milton, K. 1991. Leaf change and fruit production in six neotropical Moraceae species. Journal of Ecology 79: 1-26.

Milton, K. 1998. Physiological ecology of howlers (*Alouatta*): Energetic and digestive considerations and comparison with the Colobinae. International Journal of Primatology 19: 513-548.

Milton, K. 1999. Nutritional characteristics of wild primate foods: Do the diets of our closest living relatives have lessons for us? Nutrition 15: 488-498.

Milton, K. 2000. Back to basics: Why foods of wild primates have relevance for modern human health. Nutrition 16: 480-483.

Morrison, M., E. Cober, M. Saleem, N. McLaughlin, J. Frégeau-Reid, B. Ma, and L. Woodrow. 2010. Seasonal changes in temperature and precipitation influence isoflavone concentration in short-season soybean. Field Crops Research 117: 113-121.

Musey, P., H. Adlercreutz, K. Gould, D. Collins, T. Fotsis, C. Bannwart, T. Mäkelä, K. Wähälä, G. Brunow, and T. Hase. 1995. Effect of lignans and isoflavonoid phytoestrogens in chimpanzees. Life Sciences 57: 655-664.

Nash, L. and P. Whitten. 1989. Preliminary observations on the role of *Acacia* gum chemistry in *Acacia* utilization by *Galago senegalensis* in Kenya. American Journal of Primatology 17: 27-39.

Nunn, C. and R. Barton. 2001. Comparative methods for studying primate adaptation and allometry. Evolutionary Anthropology 10: 81-98.

Oates, J., G. Whitesides, A. Davies, P. Waterman, S. Green, G. Dasilva, and S. Mole. 1990. Determinants of variation in tropical forest primate biomass: new evidence from West Africa. Ecology 71: 328-343.

Olukoya, D., N. Idika, and T. Odugbemi. 1993. Antibacterial activity of some medicinal plants from Nigeria. Journal of Ethnopharmacology 39: 69-72.

Ososki, A. and E. Kennelly. 2003. Phytoestrogens: A review of the present state of research. Phytotherapy Research 17: 845-869.

Patisaul, H. and H. Bateman. 2008. Neonatal exposure to endocrine active compounds or an ERβ agonist increases adult anxiety and aggression in gonadally intact male rats. Hormones and Behavior 53: 580-588.

Patisaul, H., J. Luskin, and M. Wilson. 2004. A soy supplement and tamoxifen inhibit sexual behavior in female rats. Hormones and Behavior 45: 270-277.

Patisaul, H., K. Burke, R. Hinkle, H. Adewale, and D. Shea. 2009. Systemic administration of diarylpropionitrile (DPN) or phytoestrogens does not affect anxiety-related behaviors in gonadally intact male rats. Hormones and Behavior 55: 319-328.

Propper, C. 2005. The study of endocrine-disrupting compounds: Past approaches and new directions. Integrative and Comparative Biology 45: 194-200.

Ray, B., and A. Pal. 1966. Estrogenic activity of tree leaves as animal feed. Indian Journal of Physiology and Allied Sciences 20: 6-10.

Remis, M. 2000. Initial studies on the contributions of body size and gastrointestinal passage rates to dietary flexibility among gorillas. American Journal of Physical Anthropology 112: 171-180.

Reynaud, J., D. Guilet, R. Terreux, M. Lussignol, and N. Walchshofer. 2005. Isoflavonoids in nonleguminous families: An update. Natural Product Reports 22: 504-515.

Rochester, J. and J. Millam. 2009. Phytoestrogens and avian reproduction: Exploring the evolution and function of phytoestrogens and possible role of plant compounds in the breeding ecology of wild birds. Comparative Biochemistry and Physiology, Part A 154: 279-288.

Rode, K., C. Chapman, L. Chapman, and L. McDowell. 2003. Mineral resource availability and consumption by colobus in Kibale National Park, Uganda. International Journal of Primatology 24: 541-573.

Rothman, J., E. Dierenfeld, D. Molina, A. Shaw, H. Hintz, and A. Pell. 2006a. Nutritional chemistry of foods eaten by gorillas in Bwindi Impenetrable National Park, Uganda.

American Journal of Primatology 68: 675-691.

Rothman, J., P. Van Soest, and A. Pell. 2006b. Decaying wood is a sodium source for mountain gorillas. Biology Letters 2: 321-324.

Rothman, J., A. Plumptre, E. Dierenfeld, and A. Pell. 2007. Nutritional composition of the diet of the gorilla (*Gorilla beringei*): a comparison between two montane habitats. Journal of Tropical Ecology 23: 673-682.

Rothman, J., E. Dierenfeld, H. Hintz, and A. Pell. 2008. Nutritional quality of gorilla diets: consequences of age, sex, and season. Oecologia 155: 111-122.

Sapolsky, R. 2005. The influence of social hierarchy on primate health. Science 308: 648-652.

Serio-Silva, J., G. Pozo-Montuy, and Y. Bonilla-Sanchez. 2006. Black howler monkeys (*Alouatta pigra*) inhabiting eucalyptus (*Eucalyptus grandis*) plantations in Tabasco, Mexico: Habitat use and questions to answer. American Journal of Primatology 68: S10 (*meeting abstract*).

Setchell, K. and A. Cassidy. 1999. Dietary isoflavones: Biological effects and relevance to human health. The Journal of Nutrition 129: 758-766S.

Setchell, K. and C. Clerici. 2010. Equol: History, chemistry, and formation. The Journal of Nutrition 140:1355-1362S.

Simon, N., J. Kaplan, S. Hu, T. Register, and M. Adams. 2004. Increased aggressive behavior and decreased affiliative behavior in adult male monkeys after long-term consumption of diets rich in soy protein and isoflavones. Hormones and Behavior 45: 278-284.

Sindambiwe, J., M. Calomme, P. Cos, J. Totté, L. Pieters, A. Vlietinck, and D. Vanden Berghe. 1999. Screening of seven selected Rwandan medicinal plants for antimicrobial and antiviral activities. Journal of Ethnopharmacology 65: 71-77.

Snaith, T., C. Chapman, J. Rothman, and M. Wasserman. 2008. Bigger groups have fewer parasites and similar cortisol levels: A multi-group analysis in red colobus monkeys. American Journal of Primatology 70: 1072-1080.

Sousa, M. and T. Ziegler. 1998. Diurnal variation on the excretion patterns of fecal steroids in common marmoset (*Callithrix jacchus*) females. American Journal of Primatology 46: 105-117.

Stanford, C. and J. Nkurunungi. 2003. Behavioral ecology of sympatric chimpanzees and gorillas in Bwindi Impenetrable National Park, Uganda: Diet. International Journal of Primatology 24: 901-918.

Strier, K. 1993. Menu for a monkey. Natural History 102: 34-43.

Struhsaker, T. 1997. Ecology of an African Rain Forest: Logging in Kibale and the Conflict between Conservation and Exploitation. University Press of Florida: Gainesville, Florida.

Struhsaker, T. 2005. Conservation of red colobus and their habitats. International Journal of Primatology 26: 525-538.

Terborgh, J. 1983. Five New World Primates: A Study in Comparative Ecology. Princeton University Press: Princeton, New Jersey.

Thornton, J. 2001. Evolution of vertebrate steroid receptors from an ancestral estrogen receptor by ligand exploitation and serial genome expansions. Proceedings of the National Academy of Sciences 98: 5671-5676.

Thornton, J., E. Need, and D. Crews. 2003. Resurrecting the ancestral steroid receptor: ancient origin of estrogen signaling. Science 301: 1714-1717.

Touma, C. and R. Palme. 2005. Measuring fecal glucocorticoid metabolites in mammals and birds: The importance of validation. Annals of the New York Academy of Sciences 1046:

54-74.

Trisomboon, H., S. Malaivijitnond, W. Cherdshewasart, G. Watanabe, and K. Taya. 2007. Assessment of urinary gonadotropin and steroid hormone profiles of female cynomolgus monkeys after treatment with *Pueraria mirifica*. Journal of Reproduction and Development 53: 395-403.

Vivar, O., E. Saunier, D. Leitman, G. Firestone, and L. Bjeldanes. 2010. Selective activation of estrogen receptor-β target genes by 3,3'-diindolylmethane. Endocrinology 151: 1662-1667.

Wallace, P., E. Marfo, and W. Plahar. 1998. Nutritional quality and antinutritional composition of four non-conventional leafy vegetables. Food Chemistry 61: 287-291.

Wallis, J. 1997. A survey of reproductive parameters in the free-ranging chimpanzees of Gombe National Park. Journal of Reproduction and Fertility 109: 297-307.

Wasser, S., L. Risler, and R. Steiner. 1988. Excreted steroids in primate feces over the menstrual cycle and pregnancy. Biology of Reproduction 39: 862-872.

Wasser, S., R. Thomas, P. Nair, C. Guidry, J. Southers, J. Lucas, D. Wildt, and S. Monfort. 1993. Effects of dietary fibre on faecal steroid measurements in baboons (*Papio cynocephalus cynocephalus*). Journal of reproduction and fertility 97: 569-574.

Wasserman, M. and C. Chapman. 2003. Determinants of colobine monkey abundance: the importance of food energy, protein and fibre content. Journal of Animal Ecology 72: 650-659.

Watcho, P., E. Ngadjui, N. Alango, N. Benoît, and A. Kamanyi. 2009. Reproductive effects of *Ficus asperifolia* (Moraceae) in female rats. African Health Sciences 9: 49-53.

Weingrill, T., D. Gray, L. Barrett, and S. Henzi. 2004. Fecal cortisol levels in free-ranging female chacma baboons: relationship to dominance, reproductive state and environmental factors. Hormones and Behavior 45: 259-269.

Weiser, M. and R. Handa. 2009. Estrogen impairs glucocorticoid dependent negative feedback on the hypothalamic-pituitary-adrenal axis via estrogen receptor alpha within the hypothalamus. Neuroscience 159: 883-895.

Whitten, P. 1983. Flowers, fertility, and females. American Journal of Physical Anthropology 60: 269-270 (*meeting abstract*).

Whitten, P., D. Brockman, and R. Stavisky. 1998. Recent advances in noninvasive techniques to monitor hormone-behavior interactions. Yearbook of Physical Anthropology 41: 1-23

Whitten, P. and H. Patisaul. 2001. Cross-species and interassay comparisons of phytoestrogen action. Environmental Health Perspectives 109: 5-20.

Wingfield, J. 2005. The concept of allostasis: Coping with a capricious environment. Journal of Mammalogy 86: 248-254.

Wingfield, J., M. Moore, and D. Farner. 1983. Endocrine responses to inclement weather in naturally breeding populations of white-crowned sparrows (*Zonotrichia leucophrys pugetensis*). The Auk 100: 56-62.

Wingfield, J., and R. Sapolsky. 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology 15: 711-724.

Wynne-Edwards, K. 2001. Evolutionary biology of plant defenses against herbivory and their predictive implications for endocrine disruptor susceptibility in vertebrates. Environmental Health Perspectives 109: 443-448.

Yellayi, S., A. Naaz, M. Szewczykowski, T. Sato, J. Woods, J. Chang, M. Segre, C. Allred, W. Helferich, and P. Cooke. 2002. The phytoestrogen genistein induces thymic and immune changes: A human health concern? Proceedings of the National Academy of Sciences 99: 7616-7621.

Yellayi, S., M. Zakroczymski, V. Selvaraj, V. Valli, V. Ghanta, W. Helferich, and P. Cooke. 2003. The phytoestrogen genistein suppresses cell-mediated immunity in mice. Journal of Endocrinology 176: 267-274.

Yenesew, A., J. Midiwo, and P. Waterman. 1996. Four isoflavones from seed pods of *Millettia dura*. Phytochemistry 41: 951-955.

Ziegler, T. and D. Wittwer. 2005. Fecal steroid research in the field and laboratory: Improved methods for storage, transport, processing, and analysis. American Journal of Primatology 67: 159-174.