Understanding female social dominance: comparative behavioral endocrinology in the Genus *Eulemur*

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
of Philosophy in the University Program
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ABSTRACT

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Abstract

Female social dominance over males is unusual in mammals, yet characterizes most Malagasy lemurs, which represent almost 30% of all primates. Despite its prevalence in this suborder, both the evolutionary trajectory and proximate mechanism of female dominance remain unclear. Potentially associated with female dominance is a suite of behavioral, physiological and morphological traits in females that implicates 'masculinization' via androgen exposure; however, relative to conspecific males, female lemurs curiously show little evidence of raised androgen concentrations. In order to illuminate the proximate mechanisms underlying female dominance in lemurs, I observed mixed-sex pairs of related *Eulemur* species, and identified two key study groups -- one comprised of species expressing female dominance and, the other comprised of species (from a recently evolved clade) showing equal status between the sexes (hereafter 'egalitarian'). Comparing females from these two groups, to test the hypothesis that female dominance is an expression of an overall masculinization of the female, I 1) characterize the expression of female dominance, aggression, affiliation, and olfactory communication in *Eulemur*; 2) provide novel information about the hormonal and neuroendocrine correlates associated with the expression of female dominance; 3) investigate the activational role of the sex-steroid hormones in adult female *Eulemur* using seasonal correlates of hormonal and behavioral change; and 4) examine the

specific role of estrogen in the regulation and expression of sex-reversed female behavior in these species. In doing so I highlight significant behavioral and physiological differences between female-dominant and egalitarian *Eulemur* and show that female dominance is associated with a more masculine behavioral and hormonal profile. I also suggest that these behavioral and hormonal differences may be the result of fundamental differences in the biosynthetic pathway associated with estrogen production. Moreover, I assert that these putative physiological differences could provide a parsimonious proximate mechanism explaining the evolution of female dominance and its subsequent relaxation in egalitarian *Eulemur* species.

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1. General Introduction

In his 1963 treatise on the science of ethology, Nikolaas Tinbergen distilled the study of animal behavior to the simple question: "Why do these animals behave as they do?" (Tinbergen, 1963). Expanding upon earlier work by Julian Huxley and Konrad Lorenz, in his 1963 paper Tinbergen famously provides four clear aims that must be addressed to fully answer this question: the causation, survival value, ontogeny, and evolution of a behavior. These aims, restated within the modern paradigm, are the proximate mechanisms, function or adaptive value, development, and phylogeny of a behavior. Although it has been over 50 years since Tinbergen presented these four problems they are as applicable today as they were then. The question "Why do these animals behave as they do?" becomes particularly interesting for species that behave in ways that deviate from what is perceived as the phylogenetic "norm".

In most of the approximately 5000 species of mammals, males are larger and more aggressive than females and thus tend to be dominant socially (Darwin, 1871, Beach, 1975, French et al., 2013). The standard explanation for the presence of these sexually dimorphic traits, that facilitate intra-sexual competition, is sexual selection acting on males (Darwin, 1871, Trivers, 1972., French et al., 2013). In many species, this explanation can account for male-biased traits, like increased size, aggression, and weaponry, as well as for other male-biased behavior, like territoriality and scent marking. There are, however, several remarkable species of mammal in which some of

the typical sex differences between males and females, particularly in aggression and social dominance, are absent or even reversed. Notable species expressing increased female aggression and dominance relative to males include the spotted hyena, *Crocuta crocuta* (Glickman et al., 1992a, Frank et al., 1991, Goymann et al., 2001, Dloniak et al., 2006, Holekamp, 2006), the marmosets and tamarins or Callitrichid primates (Abbott and Hearn, 1978, Epple, 1982, Abbott, 1984, Birnie et al., 2010, Ross and French, 2011), the naked mole rat, *Heterochepalus glaber* (Margulis et al., 1995, Clarke and Faulkes, 1997), the Syrian (or Golden) hamster, *Mesocricetus auratus* (Vandenbergh, 1971, Payne and Swanson, 1972, Payne, 1976), the California mouse, *Peromyscus californicus* (Barkley and Goldman, 1977, Perché, 2001, Davis and Marler, 2003), the rock hyrax, *Procavia capensis* (Koren et al., 2006, Koren and Geffen, 2009), and various lemurs or Strepsirrhine primates (Jolly, 1966, Richard, 1987, Pereira et al., 1990, Curtis and Zaramody, 1999, Radespiel and Zimmermann, 2001, Ostner et al., 2003, Drea, 2009, Drea, 2011). Of this list, the lemurs stand out as the largest and most varied group of species expressing female dominance.

Today, it is generally agreed that fully addressing Tinbergen's four problems requires knowledge of the physiological mechanisms that underlie a given behavior (Adkins-Regan, 2005). Often playing a critical role in the physiological mechanisms underlying behavior are hormones. Hormonal studies can shed light directly onto two of the four aims, the proximate mechanisms and the development of behavior, while

helping to integrate all four aims (Adkins-Regan, 2005). To add to our knowledge of the physiological mechanisms underlying female dominance in lemurs - in order to ultimately help answer the question "Why do lemurs behave as they do?"- I conducted novel comparative research on the behavior and hormones of several closely related species within the genus *Eulemur*. The *Eulemur* clade is unique among Strepsirrhines in containing the only known lemur species reported not to show female dominance (Roeder and Fornasieri, 1995, Kaufman, 1996). Members of this clade thus make interesting subjects for comparatively exploring the proximate mechanisms underlying female dominance. I had three main objectives for this research: 1) To characterize the expression of female dominance, aggression, affiliation, and olfactory communication in *Eulemur*; 2) To provide novel information about the hormonal and neuroendocrine mechanisms that underlie the expression of female dominance; 3) To investigate the activational role of the sex-steroid hormones in adult female *Eulemur* using seasonal correlates of hormonal and behavioral change; and 4) To examine the specific role of estrogen in the regulation and expression of sex-reversed female behavior in these species.

Previous research on some of the species in which females dominate males has revealed that these species often show reductions or reversals of sex differences in other traits that also typically discriminate males from females. The reductions and reversals of these sex differences are driven by traits expressed by the female that include an

elongated, pendulous clitoris that is fully (e.g. hyena) or partially (e.g. some lemurs) traversed by the urethra (Glickman et al., 2006, Drea and Weil, 2008), lack of bimaturation relative to the male (Kappeler, 1990a, Drea and Frank, 2003, Drea, 2009), increased rates of rough-and-tumble play (Gould, 1990, Drea and Frank, 2003), increased role in territorial defense (French and Inglett, 1991, Jolly et al., 1993, Lazaro-Perea, 2001, Drea and Frank, 2003), and increased rates of scent marking (Albers and Prishkolnik, 1992, Drea and Frank, 2003, Scordato and Drea, 2007, Drea and Scordato, 2008). Female aggression, however, is often the focus of studies investigating sex-reversed behavior (e.g. Frank et al., 1991, Kappeler, 1990b, Goymann et al., 2001, Dloniak et al., 2006, Digby and Stevens, 2007, Drea, 2007, Koren and Geffen, 2009, French et al., 2013). Likewise, because of the importance of androgenic hormones in the development of sex-typical behavior in males (Phoenix et al., 1959, Jost, 1972, Goy, 1980, Jost, 1983, Wallen, 2005) and their role in the proximate expression of aggression in males (Bouissou, 1983, Harding et al., 1988, Soma, 2006, Soma et al., 2008) and females (Albert et al., 1991, Beehner et al., 2005, Gill et al., 2007), the assessment of androgenic hormones has also featured prominently in this research (see French et al., 2013 for a review).

Androgens are steroid hormones, as are estrogens, progestins, glucocorticoids, and mineralocorticoids. These five types of steroid hormones are related by having a common biosynthetic precursor, cholesterol, and similar enzymatic, biosynthetic pathways by which they are produced. The primary sources of circulating steroid

hormones are the endocrine tissues of the adrenal cortex and the gonads (ovaries and testis). Recently, researchers have found that circulating steroid hormones are often converted to different and active types in the peripheral tissues (Toran-Allerand, 1984, Trainor et al., 2006, Pradhan et al., 2010). Peripheral conversion seems to be particularly true for androgens, including testosterone and androstenedione, which are often converted to dihydrotestosterone and estradiol, respectively, in the target tissue before exerting an effect (Jost, 1983, Toran-Allerand, 1984, Fitch and Denenberg, 1998, Wallen, 2005, Soma et al., 2008, Pradhan et al., 2010). Alternately, it now also appears that many hormones can be created *de novo* from cholesterol in many target tissues, particularly the brain (Hau, 2007, Schmidt et al., 2008, Pradhan et al., 2010).

Steroid hormones have a wide variety of physiological functions. They coordinate the maintenance of homeostasis and water balance, the stress response, the regulation of metabolism, and have effects on development, cognition, and behavior. The sex-steroid hormones, including the androgens, estrogens, and progestins, regulate gametogenesis, sexual differentiation during development, and reproductive behavior - which can include social behavior (Adkins-Regan, 2005). Biochemically, steroids work in two distinct fashions. Traditionally, steroid hormones were thought to act only on intracellular receptors. As non-polar lipids, steroids pass easily through the cell membrane into the cytosol where they bind to specific receptors. Upon receptor binding, the steroid hormones trigger a cascade of cellular signals, which results in the

modification of gene transcription. As transcriptional regulators, hormones have long been known to exert effects that occur over a period of hours or days. More recently, it has become clear that steroid hormones can also bind to receptors found on the cell surface (membrane bound receptors) and exert non-genomic effects over a much shorter time scale, from seconds to minutes (Losel and Wehling, 2003, Schmidt et al., 2008), allowing hormones to regulate physiology and behavior more immediately in response to dynamic stimuli, such as ongoing social interactions.

The regulation of steroid hormone synthesis occurs via a complex feedback process that includes the hypothalamus in the brain and, located just below the hypothalamus, the anterior pituitary gland. Two parallel systems, the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis, allow for the integration of physiological and environmental stimuli, including social and behavioral events, into the hormonal response of the organisms (Harris, 1955, Raisman, 1997, Adkins-Regan, 2005). Regulation of the HPA and HPG axes can be considered three tiered. The hypothalamus, responding to external and internal stimuli, produces peptide releasing factors from neurosecretory cells, which enter the hypothalamic-pituitary portal system. This portal system is a large bundle of capillaries that connect the anterior pituitary to the hypothalamus via blood flow. In response to specific releasing factors from the hypothalamus, the anterior pituitary, in turn, releases a specific set of peptide hormones. These peptide hormones enter the circulatory system

and act on the peripheral endocrine glands (the adrenals for the HPA, or the gonads for the HPG) and stimulate or inhibit their function and hormone release (Becker and Breedlove, 2002). For the HPG axis, the hypothalamic-releasing hormone is gonadotropic-releasing-hormone (GnRH). GnRH stimulates the anterior pituitary to release the peptide hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate the testes or ovaries to produce androgens and estrogens.

A consideration of the sex-steroid hormones in research on the proximate mechanisms underlying sex-reversed, female behavior in lemurs, or any other female-dominant species, is important due to the critical role of sex-steroid hormones in the expression and development of sexually differentiated traits. A conceptual and heuristic framework for the study of hormones and sexual differentiation that is still largely valid and widely applied today, stems from pioneering work by Phoenix, Goy, Gerald and Young, who, in 1959, injected testosterone propionate into pregnant guinea pigs and observed the subsequent mating behavior of the treated guinea pig's offspring (Phoenix et al., 1959). This work, together with earlier work by Lillie (1916) and Jost (1947), established the "organizational hypothesis" of hormone action (Wallen, 2005, Arnold, 2009). Within this framework, hormones are considered to exert their effect on sexually differentiated traits in two primary ways, as outlined below:

1. Organizational: Hormones, acting during critical, finite, and specific periods of development, cause permanent and irreversible changes in the developing individual, which determine the trajectory of sexual differentiation. These actions of hormones have been termed "organizing" actions or effects (Phoenix et al., 1959, Young et al., 1964b, Jost, 1972, Toran-Allerand, 1984, Fitch and Denenberg, 1998, Breedlove and Hampson, 2002, McCarthy and Becker, 2002, Wallen, 2005). Historically, the organizing actions of hormones were thought to occur only during very early development, pre- or perinatal, and were typified by the role of androgens in the morphological and behavioral differentiation of the male fetus. More recently, evidence from both birds and rodents have shown that post-pubertal hormone manipulation can also result in long term changes in neural anatomy (Arnold and Breedlove, 1985, Fitch and Denenberg, 1998). Thus, as scientific techniques allow greater insight into cellular biology and physiology, particularly in the brain, the strict definition constraining organizing, hormonal effects to early development has been broadened, by some, to include any permanent change whenever they occur in life (for a discussion see Arnold and Breedlove, 1985, Fitch and Denenberg, 1998).

2. Activational: Hormones acting during adulthood regulate, coordinate, permit, or stimulate changes in physiology or behavior that are temporary and reversible.

These actions of hormones have been termed "activating" actions or effects (Young et al., 1964b, Breedlove and Hampson, 2002, McCarthy and Becker, 2002, Wallen, 2005). In rodents, for example, the activational effects of hormones are seen in the lordosis reflex of female rats. Lordosis in female rats normally occurs only during a specific period of her estrous cycle and in response to a mounting male. Ovariectomy eliminates this response (along with the whole estrous cycle). The lordosis response in ovariectomized female rats can be reinstated, however, with exogenous treatment of estradiol followed approximately 48 hours later by treatment with progesterone. The hormone treatment is said to have activated the lordotic response.

It is important to note that the activational effects of hormones are typically constrained by the organizational effects. This is because the organizational actions of steroid hormones change the substrates (which when applied to behavior implicate regions of the brain) upon which the activational hormones act. For instance, given the same estrogen/progesterone treatments that activate lordosis in ovariectomied females, the normal male rat, castrated in adulthood, does not display the lordosis reflex in response to being mounted (Breedlove and Hampson, 2002). Thus, in this context, the organizational effects of hormones, particularly owing to the presence or absence of testosterone pre- or perinatally, influences the ***process*** of sexual differentiation, whereas the activational effects of hormones, on the other hand, influence or modify the

expression of sexual differentiation. This is not to say that all sexually dimorphic behavior is activated by hormones nor that all masculine or feminine behavior is constrained by the organizational actions of hormones during development. In some cases, as opposed to the generally accepted idea that hormonal changes influence behavior, social cues can activate sexually dimorphic behavior, which then causes changes in circulating hormones. For instance, in male song sparrows, territorial intrusions by foreigners during the breeding season illicit very aggressive responses by residents and result in a subsequent increase in the residents' circulating testosterone concentrations (Soma, 2006, Pradhan et al., 2010). Alternately, some sexually biased behavior can be activated by hormones in both sexes, as exemplified by yawning in rhesus macaques (Graves and Wallen, 2006). Yawning is an androgen-dependent, male-biased behavior in rhesus monkeys. Male rhesus monkeys yawn more frequently than do females, due to their naturally greater circulating concentrations of testosterone. Moreover, male yawning frequencies are reduced following castration. Yawning frequencies in females can be brought up to intact male levels with the administration of exogenous testosterone and this effect can be reversed by treatment with the androgen receptor inhibitor flutamide. This research, by Graves and Wallen (2006), reveals that, in some cases, androgens can regulate behavior similarly in the two sexes. The sex differences in expression of behavior in these cases is due to sex differences in such

things as circulating hormone concentrations or receptor number and sensitivity, rather than with the direct mechanisms regulating the behavior.

The process of mammalian anatomical and behavioral sexual differentiation regulated by the organizational action of hormones begins, of course, with the sex chromosomes (Wallen, 2005, Bocklandt and Vilain, 2007, Drea, 2009). The process occurs as follows: In males, sexual differentiation begins when the presence of the Y chromosome initiates a cascade of developmental events via a gene known as the sex determining region of the Y (*Sry*). The presence of the *Sry* gene ultimately causes the indifferent fetal gonads to develop into testes. The testes in turn, with the activation of the HPG axis, produce testicular hormones, including androgens, primarily testosterone, and the peptide hormone anti-mullerian hormone. These two hormones, respectively, drive the development of masculine characteristics, a process known as 'masculinization', and suppress the development of feminine characteristics, a process known as 'defeminization' (Jost, 1972, MacLusky and Naftolin, 1981, Fitch and Denenberg, 1998, Wallen, 2005). In the absence of the Y chromosome and the *Sry* gene, the fetal gonads develop as ovaries and in the continued absence of either masculinizing or defeminizing hormones, the fetus develops and differentiates toward female endpoints. The terminology used in describing the process of sexual differentiation has sometimes been taken to suggest that 'default' female development is a passive process. On the contrary, feminization, or the process of developing female characteristics, also

requires a variety of active physiological processes, which include the action of sex-steroid hormones (Toran-Allerand, 1984, Fitch and Denenberg, 1998, Wallen, 2005).

Importantly, for the purposes of considering naturally masculinized females, numerous experiments investigating the process of sexual differentiation have shown that both the male and the female fetus are responsive to the masculinizing effects of testosterone (Phoenix et al., 1959, Young et al., 1964a, Harris and Levine, 1965, Goy, 1970, Beach, 1975, Beach et al., 1982, Wallen and Hassett, 2009). Furthermore, masculinization and defeminization are independent and thus can occur separately (Phoenix et al., 1959, Goy, 1970, Beach, 1975, MacLusky and Naftolin, 1981, Wallen, 2005, Drea, 2009, Arnold, 2009), allowing for the possibility of masculinization without defeminization under certain developmental conditions.

Experimentally exposing the female mammalian fetus to exogenous testosterone *in utero* produces a 'pseudohermaphrodite' (Goy, 1970, Pomerantz et al., 1986). These experimentally masculinized females express phenotypic characteristics of both sexes, but do not actually have both male and female reproductive organs as do true hermaphrodites (such as many annelids and mollusks). Pseudohermaphroditic females have been generated experimentally in a number of species, including guinea pigs (Phoenix et al., 1959), dogs (Beach et al., 1982, Beach et al., 1983), and rhesus monkeys (Goy, 1981, Pomerantz et al., 1986, Pomerantz et al., 1988, Goy et al., 1988, Wallen, 1996). The extent of experimental morphological and behavioral masculinization of the female

pseudohermaphrodite depends on the timing and duration of treatment with exogenous testosterone during gestation (Wallen, 1996, Drea, 2009, Drea, 2011). These experimentally manipulated females can show masculinized external genitalia, in the form of a hypertrophied clitoris and fused or partially fused labial folds (Goy, 1981, Wallen, 1996), masculine behavioral traits (Goy et al., 1988, Wallen, 1996), or, if treatment is extensive enough, both masculinized genitalia and masculinized behavior (Wallen, 1996). In rhesus monkeys, pseudohemaphroditic females masculinized at the appropriate prenatal time, express male-like frequencies of rough-and-tumble play and play mounting as juveniles, and increased frequencies of male-typical sexual behavior as adults (Pomerantz et al., 1986, Goy et al., 1988).

A similar masculinized phenotype is found in human females with the clinical condition known as congenital adrenal hyperplasia (CAH) (Ehrhardt and Meyer-Bahlburg, 1981, Hampson, 2002, Mathews et al., 2009). CAH is a result of a deficiency of one or more of the enzymes needed to convert cholesterol to cortisol, leading to significantly reduced cortisol production. Because the normal physiology of the HPA axis regulating hormone homeostasis still function normally in these patients, the reduction in cortisol results in an increase in precursor steroid production by the adrenal cortex via feedback mechanisms. The excess precursor steroids, unable to progress down the biosynthetic pathway towards cortisol because of the enzyme deficiency, progress instead down the androgenic pathway, resulting in an excess of androgens. The excess

androgens produced via this process act on the female fetus in the same way as exogenous androgens do in the experimental cases. Like experimentally produced pseudohermaphroditic animals, human females with CAH show masculinized morphological and behavioral traits. Behaviorally, relative to their non-CAH peers, girls with CAH are reported to engage in more male-like frequencies of rough play, identify as 'tom-boys,' and engage in higher frequencies of aggressive behavior (Ehrhardt and Meyer-Bahlburg, 1981, Hampson, 2002, Mathews et al., 2009).

Based on the phenotypic similarity between females from certain female-dominant species and these experimentally and clinically generated female pseudohermaphrodites, the organizational role of steroid hormones during gestation becomes a likely candidate as a mechanism for natural female masculinization (Drea 2009). Despite this promising hypothesis, investigating its veracity remains difficult. To fully and effectively answer questions about the organizational effects of hormones on masculine female development requires a captive breeding population of animals that can be hormonally sampled and manipulated. If the animals selected are long lived, hormonal manipulation raises logistical and ethical issues concerning the long-term care of experimentally 'abnormal' individuals (such as pseudohermaphroditic rhesus monkeys). Furthermore, the mammals that are characterized by female dominance are not typically found in large numbers in captivity, and many are rare or even endangered, raising more logistical and ethical issues related to their study.

Nevertheless, the organizational role of hormones has been investigated in at least two female-dominant species, the ring-tailed lemur and the spotted hyena.

Of the two female-dominant species used to investigate the organizational role of hormones in the expression of their particular phenotypes, only the spotted hyena has been the subject of experimental manipulation, in the form of anti-androgen treatment during pregnancy (Drea et al., 1998, Drea et al., 2002). Like many lemurs, the spotted hyena is characterized by complete female dominance over males. The female spotted hyena is larger and more aggressive than the male, with a female dominance hierarchy based on maternal rank 'inheritance' (Smale et al., 1993, Frank, 1996). Unique even among female-dominant species (Drea et al., 1998, Drea, 2007), female spotted hyenas are also characterized by extremely masculinized external genitalia, with a hypertrophied clitoris that fully encompasses the urethra and reproductive tract (Kruuk, 1972, Frank et al., 1990). Female spotted hyenas also show evidence of hormonal masculinization: Relative to males, adult female spotted hyenas have high circulating concentrations of the androgen androstenedione (A_4), a precursor to both testosterone (T) and estradiol (E_2) (Glickman et al., 1987, Glickman et al., 1992b). Produced by the ovaries, A_4 concentrations rise even further during spotted hyena pregnancy. This A_4 is converted by the placenta to T, which then bathes the fetus at high levels during gestation (Yalcinkaya et al., 1993, Licht et al., 1998). Interestingly, the developmental exposure of the fetus to T cannot completely account for the extreme morphology of the

female genitalia, as 'phallic' development begins prior to the differentiation of the fetal gonad (Licht et al., 1998, Cunha et al., 2005) and anti-androgen treatment during gestation does not prevent the development of the peniform clitoris in female offspring (Drea et al., 1998). Nevertheless, anti-androgen treatment does modify some morphological characteristics of both the male and female phallus, causing them to be generally shorter and thicker, and the meatus (or opening) wider and more elastic, confirming at least a partial role for androgens in hyena genital development (Drea et al., 1998).

Prenatal hormone exposure in spotted hyenas also appears to organize aspects of their postnatal behavior. For instance, the frequencies of mounting and aggressive behavior in both male and female juveniles are correlated with androgen concentrations measured in the mother while pregnant (Dloniak et al., 2006). Also correlated with prenatal androgen exposure are the rates of intersexual aggression produced by females as adults (Van Meter, 2009). Furthermore, the offspring of mothers treated with anti-androgens during pregnancy show reduced A_4 concentrations (Drea, 2007) and a reduction of the postnatal aggression typically directed between siblings of this species (Drea, 2007, Van Meter, 2009). Hormones also appear to play a role in activating the expression of inter-sexual aggression in spotted hyenas, with ovariectomy effectively reducing rates of inter-sexual aggression in captivity (Baker, 1990). Thus it appears that

androgens act both organizationally and activationally in the expression of 'male-like' behavior in female spotted hyenas.

The role of specific hormones in the organization and activation of 'male-like' traits in female ring-tailed lemurs is perhaps less clear (Drea, 2007). Female ring-tailed lemurs are unequivocally dominant over males (Jolly, 1966, Kappeler, 1990b, Pereira et al., 1990, Pereira and Kappeler, 1997, Drea, 2007) and are characterized by several male-like traits, including size monomorphism with males (Kappeler, 1990a, Drea and Weil, 2008), more prominent territorial defense than males (Jolly et al., 1993), and male-like rates of scent marking (Scordato et al., 2007, Drea and Scordato, 2008). Morphologically, female ring-tailed lemurs possess an elongated, pendulous clitoris that is partially traversed by the urethra, as well as prominent anogenital glands (Hill, 1953, Drea and Weil, 2008, Drea, 2009) showing a degree of morphological masculinization reminiscent of the spotted hyena (Drea and Weil, 2008, Drea, 2009). Hormonally, female ring-tailed lemurs show slightly elevated A_4 concentrations relative to other female mammals, but these concentrations are still lower than those of conspecific males (Drea, 2007). Like the spotted hyena, female ring-tailed lemurs also show significantly lower circulating concentrations of T than their male counterparts (Drea, 2007). The pattern of hormonal fluctuation during ring-tailed lemur pregnancy is similar, but less extreme, than that seen in the spotted hyena (Drea, 2011). During pregnancy, female ring-tailed lemurs show increases in circulating concentrations of A_4, T, and E_2, with the early timing of

hormone increase pointing to a maternal ovarian source of androgens (Drea, 2011). Relative to pre- and post-gestational levels, measurable increases in all three hormones are evident in singleton female pregnancies, particularly during developmental periods consistent with the differentiation of the external genitalia and the brain (Drea, 2011). Increases in gestational A_4, T and E_2, however, are most pronounced in females carrying male fetuses (Drea, 2011). Thus, despite some consistency with an organizational hypothesis of female masculinization, correlative data alone cannot be used to draw a definitive connection between gestational hormone concentrations and the expression of male-like traits in female ring-tailed lemurs (Drea, 2011).

It is more than likely that hormones are acting both organizationally and activationally to shape at least some of the unusual aspects of lemur morphology and behavior. The question, however, of which hormones are doing what remains. The endangered status of lemurs makes performing the experiments necessary to definitively answer this question logistically difficult (Drea, 2011). One way to address this challenge is to focus on the activational effects of hormones and the correlations between adult hormones and behavior (e.g. Cavigelli et al., 2003, Ostner and Heistermann, 2003, Drea, 2007). In ring-tailed lemurs, such studies show that seasonal increases in A_4 and E_2 correlate with increases in adult, female aggression (Drea, 2007), consistent with an activational effect of hormones, but these studies still leave the question of the primary hormonal mechanisms underlying female masculinization open.

1.1 Dissertation Overview

In this dissertation, I aim to increase our understanding of which hormones are acting to influence the expression of female lemur behavior. I do so through a new set of studies that explore adult lemur behavior, including aggression, scent marking, and affiliation, as well as products of the endocrine system, including the sex-steroid hormones, A_4, T, and E_2, and the monoamine serotonin. Using the data generated by these studies, performed on animals of both sexes from multiple species, I test the hypothesis that female dominance in lemurs is an expression of overall masculinization of the female. In this research, I take advantage of the variation in the expression of female dominance in the species of the genus *Eulemur* to provide a comparative perspective lacking in earlier work. I provide insight into the proximate mechanisms underlying female dominance in lemurs with the hope that this will help integrate Tinbergen's four aims in pursuit of the answer to why lemurs behave as they do.

In the next section (Chapter 2), I begin by comparatively examining the expression of behavior in female and male *Eulemur*, from both female-dominant and egalitarian species. I test the hypothesis of female behavioral masculinization by measuring the relative rates of several behaviors, which typically show sex differences in expression. These behaviors are supplants (a measure of dominant behavior), aggression, scent marking, and allogrooming. I predicted that female-dominant species would show reduced or reversed sex differences for these behaviors, and that, relative to

egalitarian females, female-dominant females would show more 'male-like' rates of behavioral expression.

In Chapter 3, I test the hypothesis that female-dominant female lemurs are hormonally masculinized. I do so by comparing the relative circulating concentrations of the sex-steroid hormones, A_4, T, and E_2, and novelly, the monoamine serotonin in female-dominant and egalitarian females and males. I predicted that sex differences in female-dominant species would be smaller, relative to those found within egalitarian species. More importantly, I also predicted that female-dominant females would show a more masculine endocrine profile, with greater circulating concentrations of androgens, and lower circulating concentrations of serotonin, than egalitarian females.

In Chapter 4, I explore the activational role of the sex-steroid hormones on *Eulemur* behavior by examining the correlations between seasonal changes of behavior and hormones, within both female-dominant and egalitarian species. I predicted that, if hormones are activating masculine behavior in female-dominant female lemurs, seasonal changes in hormone concentrations, particularly androgens, would positively correlations with changes in the expression of masculine behaviors, like aggression and scent marking. I also predicted that these correlations between hormones and behavior would differ between female-dominant and egalitarian females, with egalitarian females showing weaker correlations between androgens and behavior.

Lastly, in Chapter 5, I present the results of an experimental hormonal manipulation, using the aromatase letrozole, on the behavior of female-dominant and egalitarian lemur. Letrozole is a potent inhibitor of aromatase, which catalyzes the final and rate-limiting step in the biosynthesis of estrogen from its androgenic precursors (Bhatnagar, 2007). The effect of letrozole treatment should be seen as a significant decrease in circulating E_2 concentrations (Geisler et al., 2002, Pepe et al., 2003, Bhatnagar, 2007), and possibly as a concurrent increase in circulating androgens (Kumru et al., 2007, Gallicchio et al., 2011). If estrogens or androgens are activating the expression of any of the behaviors measured, which include supplants, aggression, scent marking, and sniffing, then the rates of these behaviors should be altered upon treatment.

I also conducted a series of behavioral bioassays on each subject, presenting them with conspecific male and female odors while untreated, and again during letrozole treatment, to further explore the role of hormones on the response to conspecific odors. Olfactory communication plays an important role in lemur ecology, and both signal deposition (i.e. scent marking) and signal reception and response are important components. Ring-tailed lemurs, in particular, possess an extremely complex system of olfactory communication (Scordato et al., 2007, Scordato and Drea, 2007, Drea and Scordato, 2008). They express up to over 300 volatile, chemical compounds in their genital secretions (Scordato et al., 2007, Boulet et al., 2009), which signal information about sex, reproductive status, individual identity, and genetic diversity (Charpentier et

al., 2008, Drea and Scordato, 2008, Boulet et al., 2009, Boulet et al., 2010). Moreover, sex, reproductive state, and social status affects both the deposition of, and the response to, these odor signals (Drea and Scordato, 2008). *Eulemur* too, rely heavily on olfactory communication (Colquhoun, 2011, delBarco-Trillo et al., 2012), producing hundreds of chemical compounds in their odor secretions, which also signal information about the signaler's sex, identity, and reproductive state. Based on differences in chemical complexity between males and females, in both female-dominant and egalitarian species (Scordato and Drea, 2007, delBarco-Trillo et al., 2012), as well as results from previous experiments in *L. catta* (Scordato and Drea, 2007), in the current experiment, I predict a preference for female odors over male odors by female-dominant subjects, and a reduction or reversal of this preference for egalitarian females. Because the sex and hormonal state of an individual impact their odorants and the responses these odorants generate in conspecifics (Dorries et al., 1997, Lundstrom et al., 2006, Renfro and Hoffmann, 2013, Martinec Nováková et al., 2014), conspecific response can be indicative of different hormonal influences on olfactory cues. Therefore, I predicted that letrozole treatment would, at the least, alter the response of subjects to conspecific odors. If the hormonal mechanisms regulating olfactory behavior differ between female-dominant and egalitarian females, then treatment should result in different patterns of change between the two groups.

These investigations provide strong support for the masculinization of female-dominant female lemurs, and for an activational role of both androgens and estrogens on the expression of social behavior in *Eulemur.* The results also raise the possibility that the differences in behavior and hormones found between female-dominant and egalitarian species, are the result of differences in the expression or function of the enzymes responsible for the production of the sex-steroid hormones, particularly estrogen. I therefore suggest that future effort into answering the question of "why lemurs behave as they do" would be well served by investigating the molecular biology and genetics of estrogen synthesis, and the aromatase enzyme in particular, within these unique and endangered species.

2. Female-dominant female *Eulemur* are behaviorally masculinized

2.1 Introduction

Lemurs make up a diverse, monophyletic radiation of primates (Yoder et al., 1996) that have been evolving separately from other primates since the Eocene (Martin, 1990). As a result of this separate, but parallel evolution, interest in lemur social structure and behavior, as a comparator to anthropoid primates, emerged in the mid 1960's (e.g. Jolly, 1966) and has been ongoing ever since. During early field studies it became apparent that, despite some similarity in social structures, lemurs differed significantly from anthropoid primates in the social relationships between males and females (Jolly, 1966, Jolly, 1984). Since then, female social dominance (FSD) in lemurs has remained an interesting evolutionary puzzle (Kappeler, 1996, Pereira et al., 1999, von Engelhardt et al., 2000, Drea, 2007, Dunham, 2008).

Several evolutionary explanations have been put forward for FSD in lemurs. One of the most widely accepted, and debated, hypotheses proposes that FSD is an adaptation to high reproductive costs in a harshly seasonal environment (Hrdy, 1981, Jolly, 1998, Pereira et al., 1999). FSD thus provides priority of access to resources for females, allowing them to avoid excess reproductive stress, which would otherwise be imposed by the seasonal Malagasy environment (Pereira et al., 1999). An alternate, but not necessarily mutually exclusive, hypothesis proposed by van Schaik and Kappeler (1996) suggests that the recent ecological changes brought about by the arrival of man to

the island of Madagascar have resulted in an evolutionary disequilibrium. They suggest the behavioral and social traits that distinguish diurnal and social lemurs from anthropoid primates are adaptations, not to their current environmental situation, but to environmental conditions present before the Holocene. The premise of this latter hypothesis is that the ancestral lemur condition was nocturnal, monogamous, pair-living. Given the typical adaptations to monogamy, including size monomorphism, this condition provides the opportunity for the development of FSD, especially given high female need (vanSchaik and Kappeler, 1996). While both these hypotheses have their champions and detractors, they both unquestionably focus on the ultimate (evolutionary) reasons underlying female dominance in lemurs. Following Tinbergen's paradigm (Tinbergen, 1963), an understanding of the proximate mechanisms is of equal importance. A better understanding of the proximate mechanisms will help reconstruct the evolutionary forces that may have influenced the development of FSD by providing insight into the variation of trait expression, mechanisms of inheritance, and correlating social and environmental variables (Drea, 2007).

One hypothesis put forth explaining the proximate mechanisms underlying the expression of FSD is female masculinization (Drea, 2007, Drea, 2009, Drea, 2011, Petty and Drea, 2015). At least two lines of evidence exist that are consistent with female masculinization in lemurs. First, ring-tailed lemurs (Hill, 1953, Drea and Weil, 2008, Petty and Drea, 2015) and members of the *Eulemur* clade are characterized

morphologically by masculinized genitalia in the form of an elongated, pendulous clitoris that is partially traversed by the urethra, and by prominent anogenital glands that are often more elaborate than those of males (Hill, 1953, delBarco-Trillo et al., 2012, Petty and Drea, 2015; Fig. 1). Second, in both ring-tailed lemurs (Scordato et al., 2007, Boulet et al., 2010) and female-dominant species of *Eulemur* (delBarco-Trillo et al., 2012, Petty and Drea, 2015*)*, females produce olfactory signals that are more chemically complex than those produced by conspecific males. Physiological evidence of masculinization, however, particularly evidence of hormonal masculinization, remains equivocal (von Engelhardt et al., 2000, Drea, 2007, Drea, 2011). For this reason, some studies that focus on the proximate mechanisms underlying FSD in lemurs discount or ignore the possible masculinization of females as a mechanism (e.g. Engelhardt et al., 2000, Dunham, 2008). Nevertheless, female masculinization (Drea, 2007, Drea, 2009, Drea, 2011, Petty and Drea, 2015) remains a very parsimonious proximate explanation for this trait.

The hypothesis that FSD in lemurs is an expression of female masculinization generates a number of testable predictions. In most mammals, there are measurable sex differences in the expression of dominance, aggression (Darwin, 1871, Beach, 1975, French et al., 2013), scent marking (Kimura and Hagiwara, 1985, Rozenfeld et al., 1987, Hurst, 1990, , Albers and Prishkolnik, 1992, Gosling et al., 1996, Allen, 1999, Gosling and Roberts, 2001), and affiliation (Kaufman, 1967, Bernstein, 1970, Smuts, 1985, Gould,

1996). Dominance, aggression, and scent marking are typically considered male-biased traits and affiliative behavior (i.e. grooming) is usually found to be female biased. Thus, if female lemurs are masculinized, FSD should co-vary with other male-like behavior in females, such as aggression, scent marking, and affiliation. Specifically, in female-dominant species one would expect to see reductions or reversals in the sex differences for these behaviors, with females expressing greater rates of aggression and scent marking and lower rates of affiliative behavior relative to males. Moreover, these reductions and reversals should not be evident in closely related species that lack FSD. In order to test these predictions, a comparative approach is necessary. The requisite trait variation to test these predictions is uniquely present in the genus *Eulemur* (Table 1). This phylogenetic group contains closely related species (Fig. 2) that both do (Jolly, 1998, Curtis and Zaramody, 1999, Digby and Stevens, 2007, Marolf et al., 2007) and do not (Roeder and Fornasieri, 1995, Kaufman, 1996, Pereira and McGlynn, 1997) express FSD.

Using variation in the expression of dominance-subordination behavior, I first validated the categorizations of species as female dominant or egalitarian. I then comparatively tested the following prediction: relative to egalitarian species (*Efc* and *Efr*), female-dominant species (*Er, Emf, Ec,* and *Em)* should show greater reductions, or outright reversals, of the sex differences in aggression, scent marking, and affiliation that are typical for these behaviors. Moreover, relative to egalitarian females,

female-dominant females should show: 1) more overt aggression over conspecific males, 2) more time engaged in scent marking, and 3) less female bias in initiating or time spent allogrooming.

Figure 1. Across *Eulemur* species, females appear to be equally 'masculinized' in their morphological features, in that all females have an elongated, pendulous clitoris that is partially traversed by the urethra and their peri-anal glands are more elaborate than are those of conspecific males. Pictured are the anogenital regions (in cephalocaudal orientation from left to right) of representative, adult female (left column) and male (right column) members of *E. rubriventer* (top row), a species characterized by female social dominance, and *E. f. collaris* (bottom row), a species characterized by egalitarianism.

Table 1. Socio-demographic variables in the six *Eulemur* study species.

Species (and abbreviation) in phylogenetic context (following Horvath et al., 2008)	**Social organization**	**Group size**	**FSD / Egalitarian**	**Intersexual aggression**	**Male care**
E. rubriventer (*Er*)	pair bonded	2-10	FSD	low	yes
E. m. flavifrons (*Emf*)	MM/MF	2-15	FSD	high	no
E. coronatus (*Ec*)	MM/MF	5-15	FSD	high	no
E. mongoz (*Em*)	pair bonded	2-6	FSD	medium	yes
E. f. rufus (*Efr*)	MM/MF	4-17	CD	very low	no
E. f. collaris (*Efc*)	MM/MF	4-17	CD	very low	no

Note. Abbreviations are as follows: Female social dominance (FSD); co-dominance/egalitarian (CD); multi-male/multi-female (MM/MF)

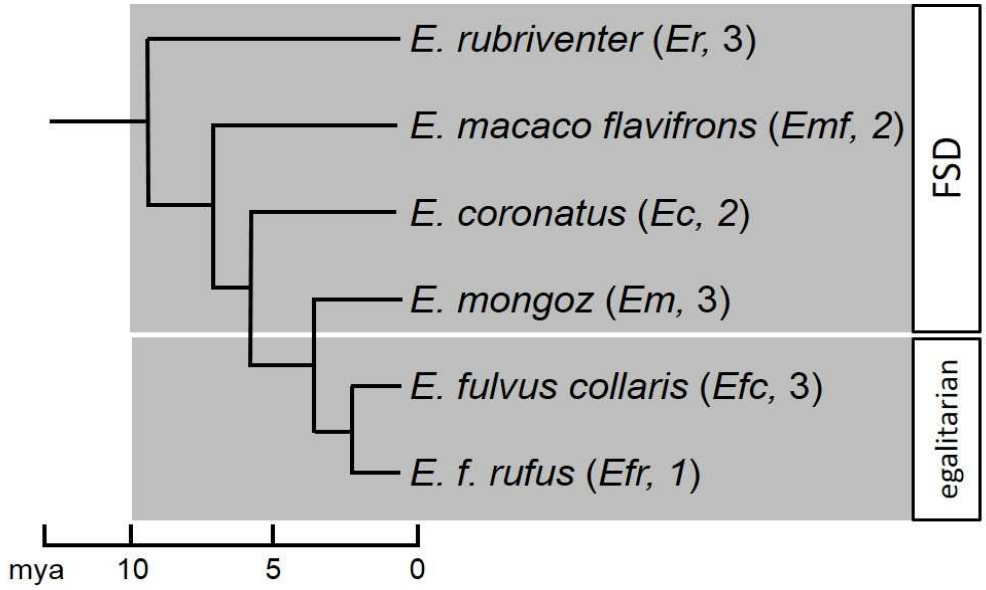

Figure 2. The phylogeny, adapted from Horvath *et al.*. (2008), and estimated divergence times of the *Eulemur* species that served as subjects in the present study. The study groups of species that show female social dominance ('FSD') or sexual 'co-dominance' (egalitarianism) are shown on the far right; species abbreviation and sample size of mixed-sex dyads for each species are shown in parentheses following species names.

2.2 Materials and Methods

2.2.1 Subjects and housing

Our subjects were 28 reproductively intact, adult animals (14 males, 14 females), aged 9–29 (mean + S.E.M.: 20.32 + 0.98) years, representing six species of *Eulemur* (Table 1, Fig. 2). The animals of each species were similarly maintained in 14 established, mixed-sex pairs at the Duke Lemur Center (DLC) in Durham, NC, USA. They included 10 pairs from four species characterized by FSD (*Er,* n = 3; *Emf*, n = 2; *Ec,* n = 2; and *Em*, n = 3) and 4 pairs from two species characterized by egalitarianism (*Efc,* n = 3, and *Efr*, n = 1; Table 1 and Fig. 2). The sample sizes for individual species were unavoidably small, owing to the rarity of the species being studied.

The animals were housed in large indoor/outdoor enclosures (23.2–951.3 m^2) and were exposed to natural daylight and the local photoperiod. During the warmer months, some of the animals had access to larger, forested enclosures (1.5–27.2 acres), often with several species occupying the same habitat. *Eulemur mongoz* were fed folivore chow (Leaf-Eater Primate Diet, Mazuri, Land O' Lakes Purina Feed, St. Paul, MN, USA), whereas the other *Eulemur* species were fed Old World monkey chow (Monkey Diet, LabDiet, St. Louis, MO, USA). The diets of all of the animals were supplemented with a mixture of fruits and vegetables. Those animals that semi-free-ranged could additionally supplement their normal diet with local vegetation and with insects foraged from the forest. The housing has been described previously (delBarco-Trillo et al., 2012).

Like female *Lemur catta,* females of *Eulemur* species exhibit strictly seasonal estrous cycles, with seasons in the Northern Hemisphere being shifted by six months from those in Madagascar (Van Horn, 1975, Drea, 2007). *Eulemur* breeding at the DLC generally begins in October, with the peak in births occurring in March and weaning being completed by the end of May. The data presented in this chapter were recorded during a 3-month period in the NBS, from June to September 2010. All protocols were performed in accordance with USDA guidelines and were approved by the DLC research committee and the Institutional Animal Care and Use Committee of Duke University (protocols: #MO-4-10-2, A102-10-04).

2.2.2 Data collection

Because each dyad was housed separately and confined to a defined area, I used continuous focal sampling of both dyad members, concurrently, focusing on didactic interactions. A comprehensive ethogram (Table 2) included dominance behavior (e.g. supplant), aggression (e.g. lunge, bite, chase, cuff), affiliation (e.g. proximity, body contact, allogroom), and scent marking (e.g. deposit, sniff, overmark). Data were entered, with a time stamp, directly into hand-held, portable computers (Psion 'Workabout', Noldus Information Technology, Inc., Leesburg, VA, USA) in an 'actor-behavior-recipient' format, with the actor and recipient being identified by their sex. This approach allowed me to record frequency, directionality, and duration of interactions between males and females, concurrently. Each dyad was observed for 1

hour twice per week in the mornings between 8:30 h and 12:30 h, with the distribution of observation periods being randomized across dyads (for a total of 24 hrs per dyad). I collected data with the help of three undergraduate volunteers. I trained all volunteers prior to data collection and tested their scoring against mine, routinely, for inter-observer reliability. I tested inter-observer reliability approximately once per month by having all observers, including myself, collect data on the same subjects at the same time. Agreement on actor, behavior, recipient, duration, and frequency was assessed using the Observer software (the Observer 3.0, Noldus Information Technology, Inc., Leesburg, VA, USA), which can be used to calculate an overall percent agreement between data sets. Agreement was high and mean reliability +/- S.D. across the study period equaled 88.7 +/- 0.1%. To further minimize the effect of variation in the data potentially owing to multiple observers, each dyad was observed an equal number of times by each observer over the course of the study.

2.2.3 Statistical analysis

To determine behavioral frequencies for each individual, I tallied the occurrences and directions of each behavior for each focal dyad during every observation period, using the actor as the focal individual. I then calculated, in acts/hour, an average frequency for each individual for each behavior. For the case of initiation of grooming, I recorded the individual from each dyad that was the first to groom in each observation period (female, male, both, or no grooming) and, for each case, calculated a proportion

of the number of total observations. Using these values, I tested for sex effects within each study group or type of dominance structure (e.g. egalitarian females vs. egalitarian males), and for effects of dominance structure within female sex (i.e. female-dominant females vs. egalitarian females). I also calculated a sex difference (sdiff) each, for female-dominant and egalitarian species, by subtracting the male frequency of behavior from the female frequency of behavior, to visualize the direction and magnitude of the sex differences for each behavior. More positive values of sdiff indicate a greater female bias and more negative values indicate a greater male bias in behavior.

Although large sample sizes are always preferred, the unpaired-t test can be applied to small sample sizes ($N \leq 5$), particularly if effect size is predicted to be large (de Winter, 2013). Here, I used the two-tailed Student's *t*-test, with or without Welch's correction for unequal variances, as needed, for all comparisons. I also calculated the effect size, d (Cohen, 1988, Coe, 2002), of the mean differences observed. Effect size can greatly aid in interpreting mean differences, independent of statistical significance, because it is equivalent to a 'Z-score' of a standard normal distribution. In other words, an effect size of 1.0 is equivalent to one standard deviation between two means. By convention, $d = 0.2$ is often interpreted as a "small" effect, $d = 0.5$ as a "medium" effect, and $d = 0.8$ as a "large" effect (Cohen, 1988, Coe, 2002). I used Graphpad Prizm v.6.0 (Graphpad Software, LaJolla, Ca) to calculate population means, standard deviations, standard errors, t-statistics and p-values. I calculated the effects sizes by hand.

Table 2. Ethogram used to collect *Eulemur* behavioral data.

Behavior	State or event	Coding sequence	Description
break contact	state	actor/recipient	actor moves out of physical contact, but stays within an arm's-length distance of recipient
withdraw	state	actor/recipient	actor that was within one arm's length moves away to within 1-m distance of recipient
move away	state	actor/recipient	actor that was between arm's length and 1 m moves away beyond 1-m distance of recipient
move toward	state	actor/recipient	actor moves to within 1 m, but more than an arm's lengths away of recipient
approach	state	actor/recipient	actor moves to within an arm's length, but not into physical contact of its partner
follow	state	actor/recipient	within 5 seconds after dyadic partner moves, actor moves in the same direction/path as dyadic partner
huddle	state	actor/recipient	actor is in physical contact with the other animal
groom	state	actor/recipient	actor runs tooth comb through fur of recipient
supplant	event	actor/recipient	actor takes the spot occupied by the recipient forcing recipient to move away
cuff	event	actor/recipient	actor uses hand and arm to aggressively swipe at recipient
lunge	event	actor/recipient	actor lurches toward opponent as if to attack
chase	state	actor/recipient	actor aggressively pursues partner
bite	event	actor/recipient	actor places mouth and teeth on partner, with aggressive force
fight bout	state	actor/recipient	multiple, fast, aggressive interactions between both individuals, with the actor being the initiator
scent mark	event	actor/substrate	deposition of genital scent mark on either substrate or partner
over mark	event	actor	actor scent marks directly over recipient's recent scent mark, within 5 secs of deposition
sniff mark	event	actor	actor sniffs other individual's recent scent mark within 5 secs of its deposition
out of sight	state		one or both subjects move out of visual range

2.3 Results

2.3.1 Dominance

The results for dominance behavior, measured by the frequency of supplants, are shown in Figure 3. Female-dominant females supplanted their dyadic, male partner over 17 times (d = 1.16) more frequently than the reverse (Unpaired Student's *t* = 2.602, df = 18, P = 0.018 two-tailed; Table 3), and 52 times (d = 1.0) more often than egalitarian females supplanted their dyadic male partner (Unpaired Student's *t* = 2.739, df =9.062, P = 0.023 two-tailed; Table 4). By contrast, egalitarian females supplanted their dyadic male partners almost 9 times less frequently (d = -2.81) than the reverse (Unpaired Student's *t* = 3.989, df = 6, P = 0.007 two-tailed; Table 5). The patterns and effects sizes of the sex differences in these rates of behavior (Fig. 3 insert) showed a strong female bias for supplants in female-dominant species (FSD; d = 1.16) and a strong male bias (d = -2.81) for supplants in egalitarian species.

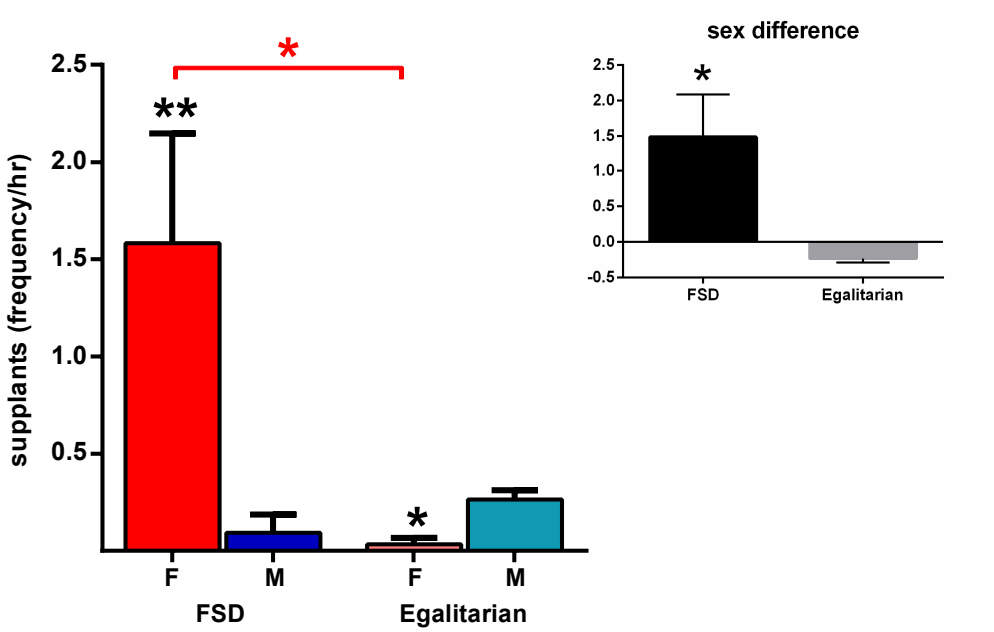

Figure 3. Rates of dominant behavior. Female *Eulemur* characterized by female social dominance (FSD-F, red) expressed significantly higher rates of dominance behavior relative to both conspecific males (FSD-M, blue) and egalitarian females (Egalitarian-F, pink), whereas egalitarian females expressed significantly lower rates of dominance behavior relative to conspecific males (Egalitarian-M, light blue). Data shown: mean + S.E.M.; Between-sex comparisons (no brackets); between-female comparisons (red brackets): **P < 0.01 and *P < 0.05. Inset: Sex difference in behavioral rates within each group (female rate – male rate). Female-dominant species showed a female bias in rates of dominance behavior, as opposed to egalitarian species that showed a male bias.

Table 3. Average behavioral rates comparing females and males from female-dominant *Eulemur*. P-values < 0.05 and effects sizes d > 0.6 in bold.

Behavior	Female mean	Male mean	p-value	effects size d
Supplants	1.58 acts/hr	0.09 acts/hr	**0.018**	**1.16**
Aggression	0.51 acts/hr	0.1 acts/hr	**0.02**	**1.13**
Scent Marking	23.8 acts/hr	19.26 acts/hr	0.59	0.25
Grooming Rate	2.23 acts/hr	2.33 acts/hr	0.92	-0.04
Groom Initiation	0.17 acts/ob	0.21 acts/ob	0.67	-0.21

Table 4. Average behavioral rates comparing *Eulemur* females from species expressing female social dominance (FSD) and egalitarianism. P-values < 0.05 and effects sizes d > 0.6 in bold.

Behavior	FSD female mean	Egalitarian female mean	p-value	effects size d
Supplants	1.58 acts/hr	0.03 acts/hr	**0.023**	**1**
Aggression	0.51 acts/hr	0.21 acts/hr	0.26	**0.69**
Scent Marking	23.8 acts/hr	2.26 acts/hr	**0.019**	**1.04**
Grooming Rate	2.23 acts/hr	6.36 acts/hr	**0.042**	**-1.34**
Groom Initiation	0.17 acts/ob	0.33 acts/ob	0.26	**-0.68**

Table 5. Average behavioral rates comparing females and males from egalitarian *Eulemur*. P-values < 0.05 and effects sizes d > 0.6 in bold.

Behavior	Female mean	Male mean	p-value	effects size d
Supplants	0.03 acts/hr	0.26 acts/hr	**0.007**	**-2.81**
Aggression	0.21 acts/hr	0.37 acts/hr	0.44	-0.59
Scent Marking	2.26 acts/hr	5.1 acts/hr	0.22	**-0.96**
Grooming Rate	6.36 acts/hr	6.76 acts/hr	0.92	-0.07
Groom Initiation	0.33 acts/ob	0.14 acts/ob	0.36	**0.69**

2.3.2 Aggression

Consistent with the data on dominance behavior, female-dominant females showed the greatest frequency of aggression directed toward their respective male partners (Figure 4), which was, on average, 5 times greater (d = 1.13) than the reverse (Welch's corrected Unpaired Student's t = 2.536, df = 10.45, P = 0.0032 two-tailed; Table 3). Likewise, egalitarian females showed rates of aggression that were 1.76 times less (d = -0.59) than rates observed by their egalitarian males (Unpaired Student's t = 0.828, df = 6,

P = 0.44 two-tailed; Table 5), and 2.5 times less (d = - 0.69) than those rates for female-dominant females (Unpaired Student's *t* = 0.2615, df =12, P = 0.26 two-tailed; Table 4). The patterns (Fig. 4 inset) and effects sizes of the sex differences in aggression showed a strong female bias for aggressive behavior in female-dominant species (d = 1.13; p = 0.02; Table 3) and a more moderate male bias for aggressive behavior in egalitarian species (d = - 0.59), the latter likely owing to chance (p = 0.44; table 5).

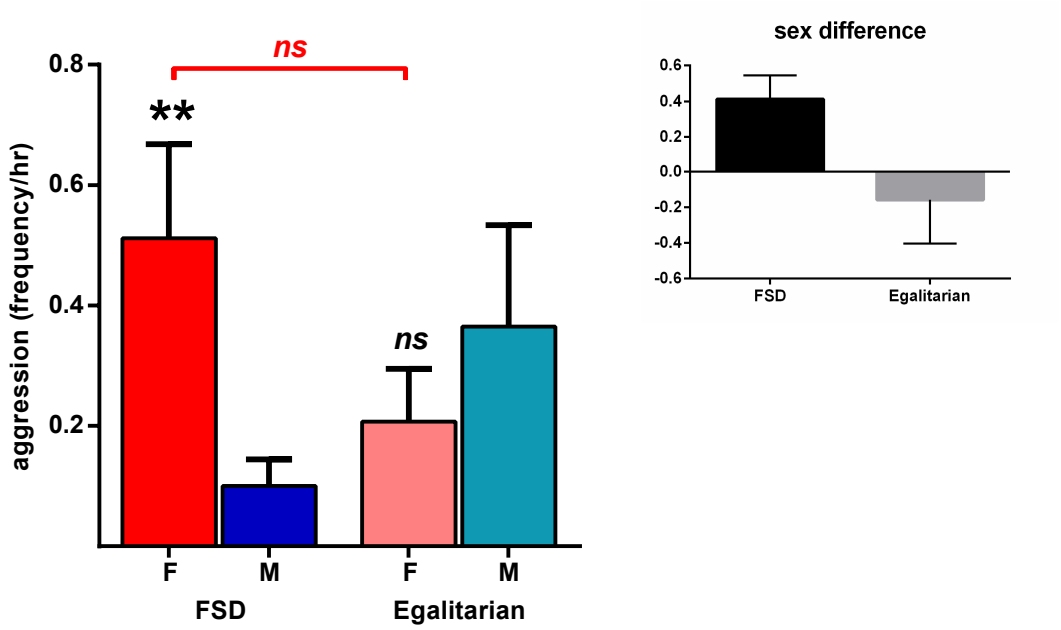

Figure 4. Rates of aggressive beahvior. Female *Eulemur* characterized by female social dominance (FSD-F, red) were significantly more aggressive than their conspecific males (FSD-M, blue) during the non-breeding season. Females from egalitarian species (Egalitarian-F, pink), on the other hand, did not differ in their rates of aggression relative to their male counterparts (Egalitarian, light blue). Data shown mean + S.E.M.; Between-sex comparisons (no brackets); between-female comparisons (red brackets): *P < 0.05, ns = non-significant. Inset: Sex difference in behavioral rates within each group (female rate – male rate). Female-dominant species showed a female bias in rates of aggressive behavior as opposed to egalitarian species that showed a male bias.

2.3.3 Scent Marking

Female-dominant females had the highest mean rate of scent marking of any group (Figure 5), although this value was not statistically different from the mean rate observed for conspecific males (Welch's corrected Unpaired Student's $t = 0.5563$, df = 11.75, p = 0.59; Table 3). Female-dominant females scent marked 10.5 times (d = 1.04) more frequently than did females from egalitarian species (Welch's corrected Unpaired Student's $t = 2.839$, df = 9.269, P = 0.019 two-tailed; Table 4). For egalitarian species, the mean rate of male scent marking (μ ± S.E. = 5.1 ± 1.9 acts/hour) was 2.25 times greater (d = -0.96) than the mean rate of female scent marking (μ ± S.E. = 2.26 ± 0.93 acts/hour; Unpaired Student's $t = 1.361$, df = 6, p = 0.22; Table 5). In female-dominant species, the patterns (Fig. 5 inset) and effects sizes of the sex differences for scent marking revealed a small female bias (d = 0.25), likely due to chance (p = 0.59; Table 3), indicating that both sexes scent marked at roughly equivalent rates. In egalitarian species, on the other hand, the mean difference, measured by effect size, in scent-marking rates was large (d = -0.96) and male biased, and less likely owed to chance (p = 0.22; Table 5), suggesting that these males scent marked more often than females.

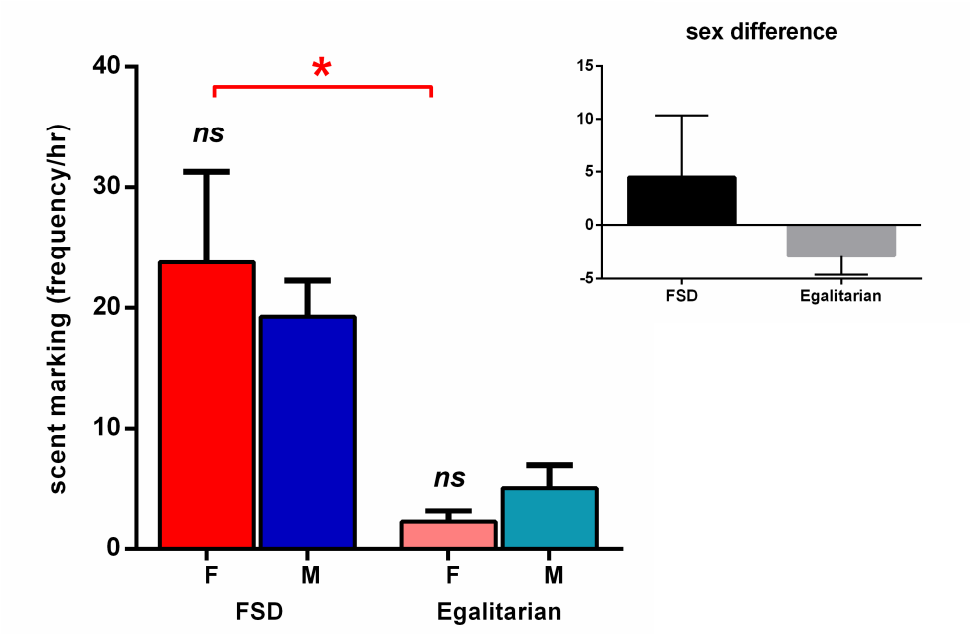

Figure 5. Rates of scent marking. Female *Eulemur* characterized by female social dominance (FSD-F, red) and female *Eulemur* characterized by egalitarianism (Egalitarian-F, pink) scent mark at equal rates relative to their con-specific male partners (FSD-M, blue; Eaglitarian-M, light blue). FSD females, however, engage in significantly higher rates of scent marking relative to egalitarian females despite similar glandular morphology. Between-sex comparisons (no brackets); between-female comparisons (red brackets): **P < 0.02, *ns* = non-significant. Inset: Sex difference in behavioral rates within each group (female rate – male rate). Female-dominant species showed a female bias in rates of scent-marking as opposed to egalitarian species which showed a male bias.

2.3.4 Grooming

Figure 6 shows the results for allogrooming frequency. Within both female-dominant and egalitarian species, males and females groomed one another at roughly equal rates, showing trivial mean differences (d = -0.04 and -0.07 respectively; Table 3 and 5). Female-dominant females, however, groomed their male partners 2.8 times less frequently (d = -1.34) than egalitarian females groomed their male partners (t = 2.267, df = 12, p = 0.042; Table 4).

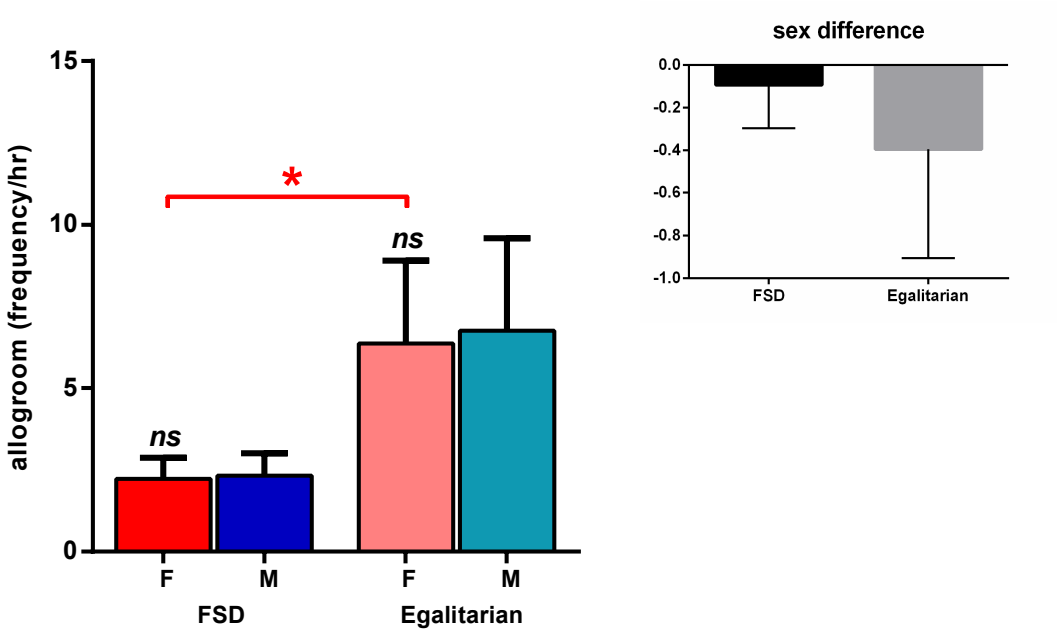

Figure 6. Rates of allogrooming. Female *Eulemur* characterized by female social dominance (FSD-F, red) and female *Eulemur* characterized by egalitarianism (Egalitarian-F, pink) groomed at rates similar to those of their conspecific male partners (FSD-M, blue; Egalitarian males, light blue). FSD females, however, engage in significantly lower rates of allogrooming relative to egalitarian females. Between-sex comparisons (no brackets); between-female comparisons (red brackets): *P < 0.05, ns = non-significant. Insert: Sex difference in behavioral rates within each group (female rate – male rate). Female-dominant species and egalitarian species both showed a slight male bias in grooming frequency.

The results for grooming initiation are shown in Figure 7. As expected, female-dominant females were slightly less likely to initiate grooming than were conspecific males (d = -0.21), but not significantly so (Unpaired Student's t = 0.436, df = 18, p = 0.67; Table 3). Female-dominant females were also moderately less likely to initiate grooming of their male partners (d = -0.68, p = 0.26; Table 4) compared to the initiation rates of egalitarian females. Egalitarian males initiated grooming the least often (μ ± S.E. = 0.14 ± 0.11 initiations/observation), based on absolute mean frequencies, followed by female-

dominant females (μ ± S.E. = 0.17 ± 0.06 initiations/observation). Although there is a reasonable probability that the sex differences for both female-dominant and egalitarian species were due to chance (p = 0.67 and p = 0.36; Tables 3 and 5 respectively), the proportion of grooming initiations revealed a small male bias (d = -0.21; Table 3) in female-dominant species and a moderate female bias (d = 0.69; Table 5) in egalitarian species (Fig 7 inset).

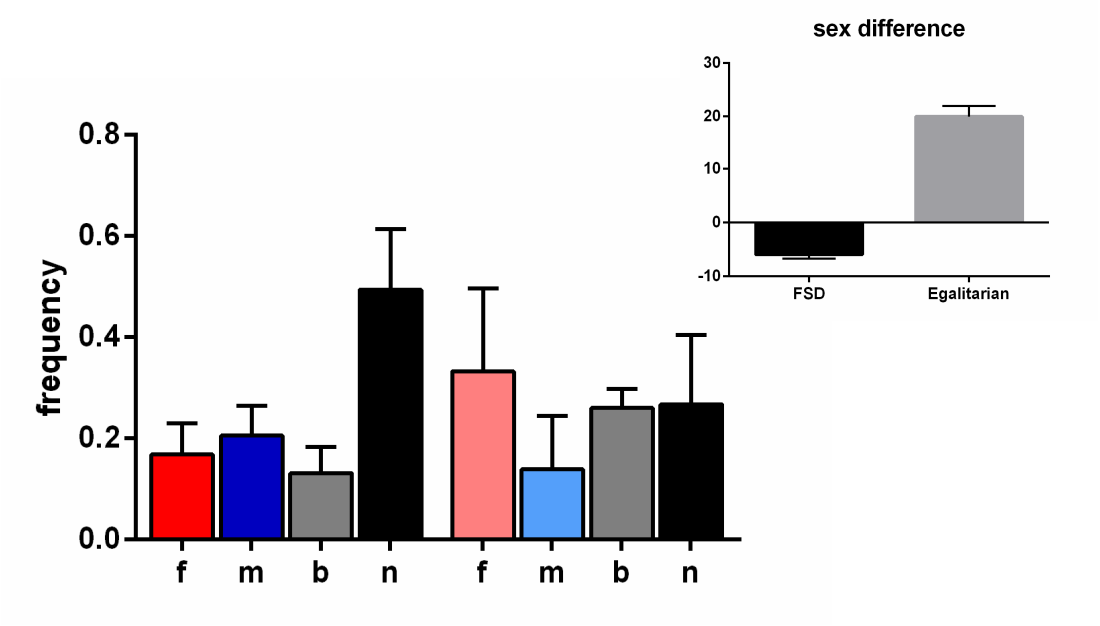

Figure 7. Frequency of grooming initiation. Females from female-dominant *Eulemur* species initiated grooming during observation periods less frequently (17% of observations) than did conspecific males (21% of observations). Females from egalitarian species, on the other hand, initiated grooming during observation periods more than twice as frequently (33% of observations) than did conspecific males (14% of observations). f = proportion of observations with female initiation of grooming, m = proportion of observations with male initiation, b = proportion of observations with mutual (both) initiation, n = proportion of observations with no grooming observed. Inset: Sex difference (female – male) in the proportion of observations in which one sex initiated grooming within each group. For grooming initiation, female-dominant species showed a slight male bias, whereas egalitarian species showed a female bias.

2.4 Discussion

There is substantial variation in the social organization of species within *Eulemur*, ranging from small, apparently monogamous, family units, to larger, promiscuous, multi-male/multi-female groups. It has been known for some time, however, that the social relationships in *E. fulvus* species differ relative to other lemur (Pereira and McGlynn, 1997). For instance, relative to other lemur species, female *E. fulvus* seem to show weaker social bonds with related females; individual females tend instead, to form a close, 'special relationship' with a male partner within their larger multi-male/multi-female groups (Pereira and McGlynn, 1997). Additionally, *E. fulvus* species seem to lack the clear female dominance structure (i.e. FSD) that is prevalent in other lemurs (Roeder and Fornasieri, 1995, Kaufman, 1996, Pereira and McGlynn, 1997). Although some of the social and ecological factors potentially influencing these differences have been considered previously (Overdorff, 1996, Pereira and McGlynn, 1997), the differences in dominance structure between *E. fulvus* species and other *Eulemur* species have not been comparatively assessed in the context of female masculinization. I have addressed this gap in the present study, comparatively examining *Eulemur* behavior, looking specifically for evidence of female behavioral masculinization in female-dominant species relative to egalitarian species.

Even under consistent social and environmental conditions, my data clearly indicate that *E. rubriventer*, *E. macaco flavifrons*, *E., coronatus* and *E. mongoz* expressed

FSD, and that *E. fulvus* species did not. Thus, I rigorously validated prior categorizations of *Eulemur* species into female-dominant and egalitarian groups (Pereira et al., 1990, Roeder and Fornasieri, 1995, Kaufman, 1996, Overdorff, 1996, Pereira and McGlynn, 1997, Jolly, 1998, Curtis and Zaramody, 1999, Digby and Stevens, 2007, Marolf et al., 2007). Under the paradigm of female masculinization, FSD should then co-vary in these species with other male-like behavior in females. Because in most mammals there are measurable sex-differences in the expression of aggression (Darwin, 1871, Beach, 1975, French et al., 2013), scent marking (Rozenfeld et al., 1987, Hurst, 1990, Albers and Prishkolnik, 1992, Gosling et al., 1996, Allen, 1999, Gosling and Roberts, 2001) and affiliation (Kaufman, 1967, Bernstein, 1970, Smuts, 1985, Gould, 1996), these are the behaviors on which I focused.

Aggression is often considered a characteristic hallmark of male behavior in mammals (Darwin, 1871, Beach, 1975, French et al., 2013), and thus is a logical behavioral focus for investigating masculinization in females. As in other female-dominant species, like the spotted hyena (Frank et al., 1991, Glickman et al., 1992a, Holekamp et al., 1996, Goymann et al., 2001, Dloniak et al., 2006,Drea, 2009) and the ring-tailed lemur (Jolly, 1966, Pereira et al., 1990, Kappeler, 1993, Drea, 2007, Drea, 2009, Drea, 2011), females from female-dominant *Eulemur* species are more aggressive towards conspecific males than vice versa. Female-dominant females are also more aggressive towards their male partners than are egalitarian females towards their

partners. It is worth noting that female mammals are capable of substantial levels of aggression without being masculinized. For instance, Beehner et al. (2005) reported rates of aggression for female Ethiopian baboons that are similar to the rates I report here. These female rates of aggression, however, are still lower than those expressed by male baboons (Beehner et al., 2006). Thus, although suggestive, high rates of female aggression do not necessarily require female masculinization to become manifest. It is therefore important to examine other sexually dimorphic behavior, such as scent marking and affiliation.

The patterns evident in my analysis of scent marking behavior also fit well with the hypothesis of female masculinization. Scent marking is often considered a form of male intrasexual competition (Gosling and Roberts, 2001), and there is often a strong correlation between scent marking frequency and social status. Notably, resource holders – typically the territorial or dominant males - mark more than do non-resource holders (Rozenfeld et al., 1987, Hurst, 1990, Gosling et al., 1996, Allen, 1999, Gosling and Roberts, 2001). The chemical complexity of scent secretions is, likewise, thought to be sexually dimorphic in many species, with males showing greater chemical complexity than females (see delBarco-Trillo et al., 2012). In egalitarian species of *Eulemur*, males have greater chemical complexity than do conspecific females, consistent with the pattern of typical mammals (delBarco-Trillo et al., 2012). In female-dominant species of *Eulemur*, on the other hand, females express more complex scent secretions than do

males (delBarco-Trillo et al., 2012), which may be consistent with female masculinization (Scordato et al., 2007, delBarco-Trillo et al., 2012). Supporting these previously reported patterns of chemical complexity for female-dominant and egalitarian *Eulemur*, and consistent with the hypothesis of female masculinization, I found that female-dominant females scent mark at similar frequencies as conspecific males. They also scent mark at much greater rates (10x more) than do egalitarian females, consistent with the evidence I report for aggression.

Affiliative behavior, in the form of grooming, has also been reported to be sexually dimorphic in expression; however, with an opposite female bias. In species in which male-female dyadic relationships form and have been examined, females usually initiate grooming and groom their male partners more frequently than the reverse (Kaufman, 1967, Bernstein, 1970, Smuts, 1985, Gould, 1996). In the female-dominant spotted hyena, however, this pattern is reversed, with males typically initiating affiliative interactions with females (Szykman et al., 2001). Although the variance in my grooming data set was high across all groups, with standard deviations in each case approaching the mean, I found that female-dominant lemurs groomed far less often than did egalitarian lemurs. Within groups, however, males and females tended to groom at nearly identical rates and durations (data not shown) regardless of their social system. These findings revealed a 'male-like' pattern of reduced sex differences in affiliation in both female-dominant and egalitarian species. Nonetheless, like spotted hyenas, the two *Eulemur*

groups were differentiated in that female-dominant females initiated grooming less frequently, on average, than did either conspecific males or egalitarian females.

Female lemurs from female-dominant species express aggressive and scent making behavior more frequently, and groom, and initiate grooming of, their partners less frequently than do egalitarian females. While not all of these direct female-female comparisons showed statistically significant differences between means, the preponderance of evidence based on the patterns of differences across the totality of the behavioral data, as well as on the relatively large effect sizes (all $d > 0.65$), suggest that female-dominant, female lemurs behave more like the males of most 'traditional' species than do egalitarian females. Additionally, the sex differences evident for each of these behaviors within each group are also supportive of a fundamental difference between female-dominant and egalitarian species, consistent with the hypothesis of female masculinization in species with FSD. Thus, I suggest behavioral masculinization should be added to the morphological (e.g. Hill, 1953; Drea and Weil, 2008) and chemical evidence (Scordato and Drea, 2007; Boulet et al., 2010; delBarco-Trillo et al., 2012), supporting the hypothesis that female masculinization is the underlying proximate explanation for the expression of FSD in lemurs.

3. Female-dominant female *Eulemur* are hormonally masculinized

3.1 Introduction

Females from female-dominant lemur species are characterized by a number of 'male-like' traits. Morphologically, female lemurs grow to be as large as males (Kappeler, 1990a, Drea, 2009), they have exaggerated external genitalia, characterized by an elongated, pendulous, clitoris that is partially traversed by the urethra (Hill, 1953; Drea and Weil, 2008), and they have prominent anogenital glands that are often more elaborate than those of males (Hill, 1953, delBarco-Trillo et al., 2012). Physiologically, unlike females from egalitarian species, females from female-dominant lemur species produce scent secretions that are chemically more complex than those of conspecific males (Scordato and Drea, 2007, Boulet et al., 2010; delBarco-Trillo et al., 2012). Behaviorally, relative to females from more 'traditional' species, females from female-dominant lemur species show increased rates of rough-and-tumble play (Gould, 1990) and territorial defense (Jolly et al., 1993). Female-dominant females are also more aggressive than conspecific males (Drea, 2007; also see Section 2), and scent mark and groom as often as do conspecific males (see Section 2). Based on our understanding of the processes of sexual differentiation, including the organizational and activational actions of the sex-steroid hormones in the development and expression of 'male-like' behavior in mammals (Phoenix et al., 1959, Goy, 1970, Jost, 1972, Jost, 1983, Wallen, 2005), it would be logical to suspect a hormonal mechanism underlying these traits in female-dominant lemurs.

In mammals, the development and expression of 'male-like' behavior in males relies on the actions of both androgens and estrogens (Jost, 1972, Whalen and Debold, 1974, Naftolin and Ryan, 1975, Goy, 1980, MacLusky and Naftolin, 1981, Bakker et al., 2004a, Bakker et al., 2004b, Wallen, 2005, Zuloaga et al., 2008, Wu et al., 2009). The androgens testosterone (T) and dihydrotestosterone (DHT) act to organize male morphology during development (Phoenix *et al.* 1959, Jost, 1970, Goy, 1980, Goy, 1985, Wallen 2005). T and its metabolite DHT, as well as its precursor androstenedione (A_4), also have been shown to activate male behavior, including aggression and sexual behavior, in adults (Whalen and Debold, 1974). The development and expression of male behavior is not, however, simply due to circulating concentrations of androgens. Hormones can exert their effects via changes in production, receptor sensitivity and availability, or metabolism and synthesis (Adkins-Regan, 2005, Zuloaga et al., 2008). Androgen binding of the androgen receptor for instance, can alter both aromatase and estrogen receptor expression and thus influence estrogen activity (Zuloaga et al., 2008). Some of the actions of the aromatizable androgens T and A_4, particularly in the brain, are actually due to their local conversion to estradiol (E_2) by the enzyme aromatase (Naftolin and Ryan, 1975, MacLusky and Naftolin, 1981, Bakker et al., 2004a, Bakker et al., 2004b, Wu et al., 2009). Aggressive behavior in rodents, in particular, appears to be reliant on the role of estrogenic metabolites of T (Scordalakes and Rissman, 2004, Zuloaga et al., 2008). Complicating the issue somewhat, the specific roles of androgens or estrogens in the development and expression of masculine traits may differ between species. For instance, masculinization of the brain (and presumably behavior) in

primates, seems to be less reliant on the aromatization of androgens to E_2 than it is in rodents (MacLusky and Naftolin, 1981, Wallen, 2005, Zuloaga et al., 2008).

Importantly, the masculinizing effects of androgens and estrogens can act in females as well as males (Phoenix et al., 1959, Young et al., 1964a, Harris and Levine, 1965, Goy, 1970, Beach, 1975, MacLusky and Naftolin, 1981, Beach et al., 1982, Graves and Wallen, 2005, Wallen, 2006, Wallen and Hassett, 2009). Developmentally, females experimentally exposed to androgens (Phoenix et al., 1959, Young et al., 1964a, Harris and Levine, 1965, Goy, 1970, Beach, 1975, Beach et al., 1982, Wallen and Hassett, 2009) or estrogens (MacLusky and Naftolin, 1981) as fetuses can develop male-like morphological and behavioral traits. Androgens and estrogens also regulate many of the same behaviors in adult females as they do in males. In males, T often mediates aggression (Wingfield et al., 1990, Wallen, 2005, Hau, 2007, Soma et al., 2008, Sperry et al., 2010) and correlates (often positively) with dominance rank (Bonson et al., 1994, Setchell et al., 2008). Similarly in females, T mediates aggression in some birds (Gill et al., 2007) and rodents (Barkley and Goldman, 1977), as well as in human women (Cashdan, 1995, Udry et al., 1995, Grant and France, 2001). Likewise, social rank is positively correlated with T in female baboons (Beehner et al., 2005). In the rock hyrax, females uniquely express more circulating T than conspecific males and dominate males socially (Koren et al., 2006, Koren and Geffen, 2009). T has also been shown to mediate female scent-marking behavior, in some species (Owen and Thiessen, 1973, Brown, 1978), much as it does for males (Blum and Thiessen, 1970). In both rats and gerbils, gonadectomy abolishes scent marking behavior in males and females. Scent marking is

reinstated in both sexes only by administration of exogenous T (Owen and Thiessen, 1973, Brown, 1978). Like T, E_2 also mediates aggression in various taxa. Increased E_2 in males can act to stimulate aggressive territorial defense (Trainor et al., 2006b) and inter-male aggression (Cologer-Clifford et al., 1999). In females, E2 positively mediates aggression in reptiles (Woodley and Moore, 1999), birds (Parn et al., 2008), rodents (Lonstein and Gammie, 2002), and primates (Michael and Zumpe, 1993).

Because of their obvious role in mediating behavior and 'male-like' traits in particular, it is no surprise that androgens and estrogens have been investigated in the context of the 'male-like' behavior exhibited by females from species characterized by female social dominance (FSD). For instance, androgens are implicated in the masculinization of female spotted hyenas (Glickman et al., 1992a, Glickman et al., 1992b, Licht et al., 1992, Yalcinkaya et al., 1993, Drea et al., 1998, Dloniak et al., 2006, Van Meter, 2009). Pregnant female hyenas produce high quantities of A_4, which is converted by the placenta to T. Exposure of the developing fetus to this T then organizes some aspects of the unique masculine physiology and behavior found in female spotted hyenas (Licht et al., 1992, Yalcinkaya et al., 1993, Drea et al., 1998). Activationally, circulating A_4 concentrations positively correlate with rates of aggression in adult female hyenas (Dloniak et al., 2006, Van Meter, 2009). A_4, as well as E_2, have also been implicated as mediators of aggression in female lemurs. In ring-tailed lemurs (Drea, 2007), both A_4 and E_2 positively correlate with seasonal aggression. Likewise, in ruffed lemurs (Shideler et al., 1983), which also express FSD, E_2 positively correlates with aggression, but

unfortunately, as is the case for many hormonal studies in females, androgens were not measured in that study.

Despite the evidence supporting a role for androgens in the expression of 'male-like' behavior in adult females, measurements of circulating concentrations of androgens continue to vex investigators studying the role of hormones in the context of FSD (von Engelhardt et al., 2000, Goymann et al., 2001). Although there is strong evidence showing that female spotted hyenas have greater circulating concentrations of the androgen A_4 than do males (Glickman et al., 1987, Glickman et al., 2006, Drea, 2009), relative to males, female spotted hyenas still show significantly lower circulating concentrations of the more biologically active T (Glickman et al., 1987, Drea, 2009). Similarly, in the ring-tailed lemur, both A_4 and T circulate in females at lower concentrations than they do in males (Drea, 2007, Drea, 2009). This difference in androgen concentrations between the sexes has led some investigators to doubt that a hormonal mechanism underlies the expression and evolution of FSD in these species (von Engelhardt et al., 2000, Goymann et al., 2001). Previous studies on both sides of this argument have compared hormones in females to those in conspecific males. Critically, however, females may be more sensitive to the actions of androgens than are males (Sherwin, 1988, Staub and De Beer, 1997a). Thus, male-female comparisons may be an inappropriate and misleading analysis of androgen action in females.

Given that intersexual comparisons may not be entirely informative, in this study I make use of the trait variation present in *Eulemur* (as discussed in the prior chapter), to conduct both male-female and female-female hormonal comparisons. As noted earlier,

the *Eulemur* clade uniquely contains species that both show or lack FSD (Table 1). Using the data generated from this study, I will test the hypothesis that hormonal mechanisms underlie female masculinization in female-dominant lemurs. Following the traditional focus on reproductive hormones, I measure circulating adult concentrations of the androgens, A_4 and T, and the estrogen, E_2. Importantly, I measure each of these hormones in both males and females from both female-dominant and egalitarian *Eulemur*. The few endocrine studies that currently exist for *Eulemur* have not examined 'heterologous' hormones (i.e. androgens in females or estrogens in males). Moreover, these studies have been focused only on egalitarian *E. fulvus* species (Ostner and Heistermann, 2003, Ostner et al., 2003, Ostner et al., 2008) leaving gaps in our understanding about the comparative hormonal mechanisms at work in this behaviorally diverse group.

Because, as noted above, hormones can exert their effects via changes in production, receptor sensitivity and availability, or metabolism and synthesis, assessing relative hormone concentration ratios, in addition to absolute, hormone concentrations may provide greater insight into underlying hormonal mechanisms. Indeed, even when comparing individuals from the same species, absolute concentrations of hormones can vary widely (Adkins-Regan, 2005) and do not always explain differences in behavior (Grunt and Young, 1953, Adkins-Regan, 2005). Steroid hormone ratios have been used as a diagnostic tool for the prenatal detection of CAH (Lucas-Herald et al., 2015), and for determining the potential role of sex hormones in coronary atherosclerosis in both men and postmenopausal women (He et al., 2007). Thus, in addition to measuring absolute

hormone concentrations, I also compared the relative hormone concentration ratios of T to A_4, E_2 to A_4, and T to E_2, between female-dominant and egalitarian females to aid in interpreting my results.

Additionally, because of the various mechanisms by which hormones can act, a consideration of a more integrated neuroendocrine network may be beneficial to our understanding of the endocrine regulation of behavior associated with FSD. One potential, candidate for study in lemurs is the monoamine neurotransmitter serotonin (5-HT). 5-HT is measurable in cerebral spinal fluid (CSF) and in serum, with concentrations being positively correlated between the two (Yan et al., 1993). In other species, 5-HT concentrations, in both CSF and serum, have been linked mechanistically to both dominance (Raleigh et al., 1985, Winberg et al., 1997, Howell et al., 2007, Miller-Butterworth et al., 2007, Riddick et al., 2009) and aggression (Reisner et al., 1996, Birger et al., 2003b, Howell et al., 2007, Miller-Butterworth et al., 2007, Rosado et al., 2009). Specifically, increased expression of 5-HT predicts decreased rates of aggression and lower social rank in several species, including primates and humans (Korzan and Summers, 2004, Summers et al., 2005, Howell et al., 2007, Miller-Butterworth et al., 2007). Also, 5-HT concentrations have been shown to be sexually dimorphic in several species, with females traditionally showing increased expression and activity over males (humans: Rubinow et al., 1998, Weiss et al., 2005; rats: Carlsson et al., 1985, Carlsson and Carlsson, 1988, Rubinow et al., 1998; fish: Telgkamp et al., 2007). Lastly, 5-HT concentrations and activity are also closely tied to the regulation and activity both of androgens (Simon et al., 1998, Cologer-Clifford et al., 1999, Birger et al., 2003a, Clark and

Henderson, 2003) and of estrogens (Rubinow et al., 1998, Cologer-Clifford et al., 1999, Bethea et al., 2002). In rodents and primates, both T and E2 have been shown to inhibit the function of the serotonergic system, thereby increasing aggression (Bonson et al., 1994, Cologer-Clifford et al., 1999, Birger et al., 2003a). Although research on the role of 5-HT in the expression of female behavior in female-dominant species is currently ongoing in hyenas (Jones et al., 2015), 5-HT has not been measured previously in lemurs. Thus, in addition to the traditional sex-steroid hormones mentioned above, I also measure circulating adult concentration of serum 5-HT in both males and females, to provide a more integrated perspective on the neuroendocrine mechanisms that may be mediating female dominance in *Eulemur*.

If differences in the endocrine system underlie the expression of masculine characteristics in female *Eulemur*, as I hypothesize, then evidence of this difference should be apparent in the relative comparisons of absolute hormone concentrations between groups. Specifically, female-dominant species should have reduced or reversed sex-differences in all hormone concentrations relative to egalitarian species. Relative to egalitarian females, female-dominant females should also show lower absolute concentrations of 5-HT, and increased absolute concentrations of A_4, and T. In other words, female-dominant females should show a more 'male-like' androgenic profile compared to egalitarian females. If the differences in the expression of 'male-like' traits in female-dominant *Eulemur* owe to differences in absolute concentrations of circulating hormones, and not to specific differences between androgenic or estrogenic metabolism, I would also expect female-dominant females to show increased E_2 concentrations,

relative to egalitarian females, and expect no differences in the comparisons of the ratios of hormone concentrations between female-dominant and egalitarian groups.

3.2 Material and Methods

3.2.1 Subjects and housing

The subjects and housing conditions were identical to those of the behavioral study outlined in Section 2.2.1.

3.2.2 Sampling procedures.

With the assistance of DLC veterinary personnel, we obtained blood samples from each subject once monthly during a 3-month study period in the NBS, from June to September 2010 (for a total of n = 42 male and n = 42 female samples). On blood-draw days, animals that previously had been corralled into their indoor enclosures were netted and processed individually, to minimize the time delay between capture and blood draw (mean ± S.E.M. = 5.00 ± 0.68 min). Handling occurred primarily in the morning (between 9:00 and 12:30 h, mean ± S.E.M. = 10:12 ± 0:07 h). Using a 23-gauge needle and syringe, we drew blood samples (3 cc) from the femoral vessels of awake, manually restrained animals, all of which were habituated to these procedures. We immediately transferred the blood samples to serum separator tubes (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ 07417, USA), allowed them to clot at ambient temperature, centrifuged them at 1500 × g for 20 min, and stored the decanted serum at −80 °C until analysis.

3.2.3 Hormone assays

Concentrations of 5-HT, A_4, T and E_2 were determined for each serum sample collected using commercial competitive enzyme immunoassay (EIA) kits (ALPCO diagnostics, Salem NH 03079, USA). I validated the assays both for 1) analyte recovery - by spiking a known amount of analyte into a pooled serum sample and comparing the observed and expected results, and 2) linearity - by running a serial dilution of the pooled serum and comparing the slopes against the standard curves. For all assays, recovery ranged from 85% to 105% and each dilution curve was parallel to the appropriate assay standard curve. The 5-HT assay had a sensitivity of 5 ng/ml using a 25-ul dose, with an intra- and inter-assay coefficient of variation (CV) of 5.4% and 6%, respectively. The A_4 assay had a sensitivity of 0.04 ng/ml using a 25-ul dose, with an intra- and inter-assay CV of 5.23% and 8.7%, respectively. The T assay had a sensitivity of 0.02 ng/ml using a 50-ul dose, with an intra- and inter-assay CV of 7.9% and 7.3%, respectively. The E_2 assay had a sensitivity of 10 pg/ml using a 50-ul dose, with an intra- and inter-assay CV of 7.7% and 8.7%, respectively. I performed hormone assays in duplicate. A coefficient of variance (CV = standard deviation / mean) was derived for each sample duplicate. If the CV for any sample duplicate from any EIA plate was more than 10%, the sample was re-analyzed on a subsequent plate. Following these procedures for each individual, I generated three sample per hormone. Using these absolute hormone concentrations, I also generated three relative hormone ratio values, T/A_4, E_2/A_4, and T/E_2, for each individual.

3.2.4 Statistical analyses.

The analyses of the hormonal data were conducted similarly to the analyses of behavior presented in Section 2. Briefly, I averaged each individual's data points across the study to generate data sets both of absolute hormone concentrations and of relative hormone ratios for each group. In the event that an individual's assay result was below the level of detectability, I used the minimum sensitivity value for that assay in the calculations. The data were log transformed, and mean differences were assessed using the two-tailed Student's *t*-test and the effect size, d, (Cohen, 1988, Coe, 2002). I used Graphpad Prizm v.6.0 (Graphpad Software, LaJolla, Ca) to calculate population means, standard deviations, standard errors, t-statistics and p-values. The effects sizes were calculated by hand. I tested for sex differences in absolute hormone concentrations within each study group or type of dominance structure (e.g. egalitarian females vs. egalitarian males), and for differences between females from each group (i.e. female-dominant females vs. egalitarian females). I tested the hormone ratio data for differences between female-dominant and egalitarian females only.

3.3 Results

3.3.1 Serotonin

Average male and female concentrations of circulating 5-HT did not differ significantly in either female-dominant or egalitarian *Eulemur* (Tables 6 and 7). As predicted, female-dominant females did show, on average, lower absolute concentrations of circulating 5-HT compared to conspecific males (FSD-F, 5-HT = 1302 ng/mL < FSD-M, 5-HT = 1404 ng/mL), but the effect size for this difference was small (d =

-0.21; Table 6). Likewise, egalitarian females showed greater average concentrations of circulating 5-HT relative to egalitarian males (Egal-F, 5-HT = 1755 ng/mL > Egal-M, 5-HT = 1604 ng/mL; Table 7), but with a similarly small effect size (d = 0.32). Comparing females to females (Table 8), female-dominant females had predictably lower average concentrations of circulating 5-HT relative to egalitarian females (Unpaired Student's t = 1.712, d.f. = 12, p = 0.11, d = 1.05), but despite a relatively large effect size (d > 0.8), this difference failed to reach statistical significance at the $p < 0.05$ level (Table 8; Figure 8A).

3.3.2 Androstenedione

In both female-dominant and egalitarian species, males and females showed similar average circulating concentrations of A_4 (Tables 6 and 7, respectively). Female-dominant *Eulemur*, however, show a smaller sex difference in mean A_4 concentrations than do egalitarian *Eulemur* (FSD d = -0.56 < Egalitarian d = -0.79). A_4 circulated in significantly greater absolute concentrations in female-dominant females than in egalitarian females (Unpaired Student's t = 3.06, d.f. = 12, p = 0.0099; effect size d = 1.22; Table 8, Fig. 8B).

Table 6. Mean hormone concentrations of females and males from female-dominant *Eulemur*. P-values < 0.05 and effect sizes d > 0.6 are shown in bold.

Hormone	Mean concentration		p-value	effect size
	Female	Male		(d)
5-HT	1302 ng/mL	1404 ng/mL	0.72	-0.21
A_4	0.49 ng/mL	1.10 ng/mL	0.24	-0.59
T	0.28 ng/mL	5.19 ng/mL	**0.0005**	**-0.87**
E_2	119.3 pg/mL	90.4 pg/mL	0.15	0.48

Table 7. Mean hormone concentrations of females and males from egalitarian *Eulemur*. P-values < 0.05 and effects sizes d > 0.6 are shown in bold.

Hormone	Mean concentration		p-value	effect size
	Female	Male		(d)
5-HT	1755 ng/mL	1604 ng/mL	0.61	0.32
A_4	0.18 ng/mL	0.39 ng/mL	0.38	**-0.79**
T	0.06 ng/mL	2.81 ng/mL	**0.005**	**-1.16**
E_2	73.1 pg/mL	47.78 pg/mL	0.26	**0.76**

Table 8. Mean hormone concentrations of females from *Eulemur* species with female social dominance (FSD) and egalitarianism. P-values < 0.1 and effect sizes d > 0.6 are shown in bold.

Hormone	Mean concentration		p-value	effect size
	FSD	Egalitarian		(d)
5-HT	1302 ng/mL	1755 ng/mL	0.11	**1.05**
A_4	0.49 ng/mL	0.18 ng/mL	**0.0099**	**1.22**
T	0.28 ng/mL	0.06 ng/mL	**0.0014**	**1.48**
E_2	119.3 pg/mL	73.1 pg/mL	**0.099**	**0.86**

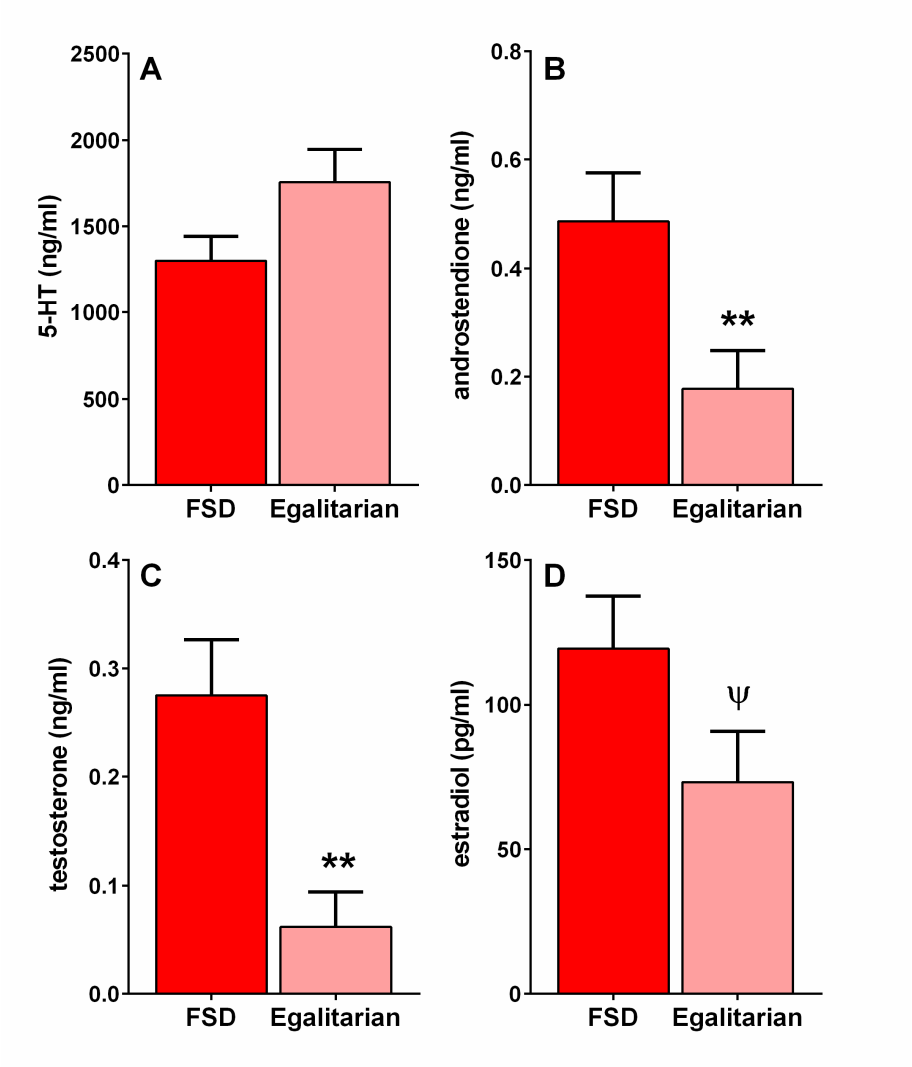

Figure 8. Female-female absolute sex steroid concentrations. Female-dominant female lemurs (FSD, red bars) showed decreased circulating concentrations of (A) serotonin (5-HT), and increased circulating concentrations of (B) androstenedione, (C) testosterone and (D) estradiol relative to egalitarian females (pink bars). $^{\Psi}P < 0.1$ and $^{**}P < 0.01$.

3.3.3 Testosterone

As expected, in both female-dominant and egalitarian *Eulemur* species, circulating T concentrations were much greater in males than in females in (Tables 6 and 7, respectively). The sex differences between absolute mean concentrations of T are smaller in in female-dominant *Eulemur* than in egalitarian *Eulemur* (FSD d = 0.87 < Egalitarian d

= 1.16). T concentrations were much greater in female-dominant females than in egalitarian females (Unpaired Student's t = 4.136, d.f. = 12, p = 0.0014; effect size d = 1.48; Table 8, Fig. 8C).

3.3.4 Estradiol

In both female-dominant and egalitarian *Eulemur*, males and females showed similar average circulating concentrations of E_2 (Tables 6 and 7, respectively). Effect sizes indicate that the sex difference in absolute mean circulating E_2 concentrations was smaller in female-dominant *Eulemur* (d = 0.48) than in egalitarian *Eulemur* (d = 0.76). The difference in circulating E_2 between female-dominant and egalitarian females is relatively large (d = 0.86) and approached statistical significance (p = 0.099), with female-dominant females expressing greater absolute mean circulating concentrations of E_2 relative to egalitarian females (Table 8, Fig 8D).

3.3.5 Hormone Ratios

Female-dominant females had approximately 34% more circulating T relative to A_4 than did egalitarian females (Figure 9A; effect size d= 0.74), but this difference was not statistically significant (Unpaired Student's *t* = 1.45, d.f. = 12, p = 0.173). Contrasting the pattern shown by the T/A_4 ratio, egalitarian females *Eulemur* showed 155% more circulating E_2 relative to circulating A4 compared to female-dominant females (Unpaired Student's *t* = 2.69, d.f. = 12, p = 0.019; effect size d = 1.59; Figure 9B). Directly comparing T to E2 revealed 227% more T relative to E_2 circulating in female-dominant females compared to egalitarian females (Unpaired Student's *t* = 3.99, d.f. = 12, p = 0.0018: effect size d = 2.36; Figure 9C).

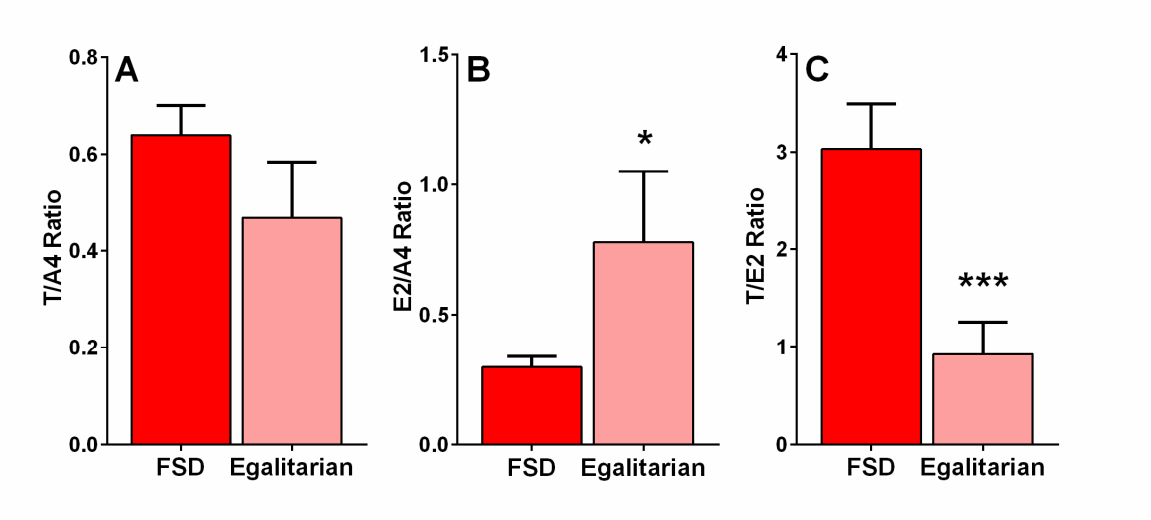

Figure 9. Female hormone ratios. (A) Female-dominant females (FSD, red bars) and egalitarian females (pink bars) show statistically similar T/A_4 ratios, suggesting T metabolism is similar in the two groups. (B) Egalitarian females show significantly increased E_2/A_4 ratios compared to FSD females, suggesting different E_2 metabolism between the two groups. (C) FSD females show T/E_2 ratios 3 times greater than those measured in egalitarian females, confirming an androgenic bias in circulating sex hormones. *$P < 0.05$ and ***$P < 0.005$.

3.4 Discussion

The expectation that female masculinization in female-dominant species is dependent on the diverse actions of the endocrine system, in general, and on the action of androgens, in particular, is based on a strong theoretical foundation. Our understanding of sexual differentiation (Jost, 1970, Jost, 1972, MacLusky and Naftolin, 1981, Fitch and Denenberg, 1998, Wallen, 2005), together with evidence from experimentally masculinized animals (Phoenix et al., 1959, Young et al., 1964a, Harris and Levine, 1965, Goy, 1970, Beach, 1975, Beach et al., 1982, Wallen and Hassett, 2009) and the pathophysiology of disorders like CAH, which result in naturally masculinized human women (Ehrhardt and Meyer-Bahlburg, 1981, Hampson, 2002, Mathews et al.,

2009), all support this supposition. Data from naturally masculinized females from female-dominant species, like the spotted hyena and the ring-tailed lemur, however, do not always clearly align with the predictions generated from the experimental and clinical research conducted in more 'traditional' species. For instance, despite elevated A_4 concentrations in female spotted hyenas relative to conspecific males (Glickman et al., 1987), and evidence of an organizational role of androgens during fetal gestation in female hyenas (Yalcinkaya et al., 1993, Drea et al., 1998, Licht et al., 1998, Dloniak et al., 2006, Van Meter, 2009), the more potent androgen T still circulates in lower concentrations in female hyenas than it does in males (Glickman et al., 1987, Drea, 2009). Moreover, androgen exposure cannot completely explain the morphological masculinization of the female spotted hyena (Drea et al., 1998, Licht et al., 1998, Cunha et al., 2005). Likewise in ring-tailed lemurs, although hormonal fluctuations during the timing of differentiation of the external genitalia and the brain in singleton female pregnancies are consitent with hormonal masculinization (Drea, 2011), hormonal correlates of masculinization are more pronounced in females carrying male fetuses (Drea, 2011). Both A_4 and E_2 correlate with aggression in adult female ring-tailed lemurs (Drea, 2007), and female ring-tailed lemurs also show lower circulating concentrations of both A_4 and T relative to males (Drea, 2007, Drea, 2009). This lack of alignment with the predictions based on our understanding of sexual differentiation, and work in more traditional species, leaves the role of hormonal (i.e., androgenic) mechanisms in the expression of female dominance an open question (Drea, 2011, see also von Engelhardt

et al., 2000, Goymann et al., 2001). The present study, by comparing females to females, sheds some light onto this question.

As I show in the previous chapter, female-dominant *Eulemur* females are behaviorally masculinized relative to egalitarian *Eulemur* females. Female-dominant female *Eulemur* are more aggressive, scent mark more frequently, and groom their partners less often, than do egalitarian females. Supporting an endocrine component in the masculinization of these females, the data presented here show that females from species characterized as female-dominant also have a significantly more 'masculine' neuroendocrine profile than do egalitarian females. This profile includes lower circulating 5-HT concentrations, and greater circulating androgen concentrations, in the form of both A_4 and T, in female-dominant females.

In mammals, males typically show greater circulating concentrations of A4 and T relative to females (Drea, 2007, French et al., 2013), and females usually show greater concentrations of E2 (although see Thompson et al., 1978). Likewise, in most species for which researchers have measured 5-HT, males show decreased concentrations of 5-HT relative to females (Carlsson et al., 1985, Carlsson and Carlsson, 1988, Rubinow et al., 1998, Weiss et al., 2005, Telgkamp et al., 2007). With the exception of T, the sex differences typically found for these compounds were absent in female-dominant and egalitarian *Eulemur*. Nevertheless, female-dominant *Eulemur* showed smaller sex differences in all compounds relative to differences measured in egalitarian *Eulemur,* supporting the hypothesis of endocrine mediation of female-masculinization in female-dominant species. Critically, female-dominant females also showed significantly

greater circulating concentrations of A_4 and T relative to egalitarian females, confirming an androgenic component in the female-masculinization of lemurs.

An important component of my study is its comparative framework. The lack of any statistical sex difference in mean concentrations of 5-HT, A_4 and E_2 among *Eulemur* is quite remarkable, because most mammals show strong sex differences in these hormones. Females typically have androgen concentrations well below those of males and estrogen concentrations well above. However, as these results are evident in both egalitarian *Eulemur* and female-dominant *Eulemur*, male-female comparisons of hormone concentrations alone, fail to support a role of the endocrine system in the behavioral masculinization of female-dominant species. It is only when females from the two groups are compared that the prediction that female masculinization of FSD females is due to endogenous hormones, particularly androgens, is supported.

Compared to their sex-specific female-dominant counterparts, both male and female members of egalitarian *Eulemur* species express lower absolute circulating concentrations of each sex hormone. The result is that the HPG axis of egalitarian *Eulemur* seems toned down, or less activated, than the endocrine systems of female-dominant *Eulemur* or ring-tailed lemurs (e.g. in Drea, 2007). The relative concentrations of circulating steroid hormones and the ratios of A_4 to T and E_2 suggest that it may be valuable to investigate differences in steroid biosynthesis between the two groups. While the absolutely greater concentrations of A_4 and T, as well as the larger T/E_2 ratio, leave little doubt that female-dominant female *Eulemur* maintain a more androgenic endocrine profile relative to egalitarian females, similar T/A_4 ratios between female-

dominant and egalitarian females suggest little difference in the physiology of their respective T metabolisms. The E_2/A_4 ratios from each group, however, differ significantly. Based on the differences in E_2/A_4 values between female-dominant and egalitarian lemurs, differences in E_2 biology may be a possible mechanism driving the masculinization of the endocrine system in female-dominant female lemurs.

Estrogen in vertebrates is synthesized via the actions of the aromatase enzyme. In humans, aromatase expression is regulated, genetically, by a number of tissue-specific, regulatory promotors (Sebastian and Bulun, 2001). These promoters independently regulate aromatase expression, in their specific tissues, in response to different hormones or cytokines and, therefore, also independently regulate local estrogen concentrations (Sebastian and Bulun, 2001). The function of these promotors changes over time in humans, altering estrogen production at the monthly scale of the menstrual cycle and pregnancy, as well as over longer time periods, as a function of age (Hemsell et al., 1974). Occasionally, gain-of-function mutations in any of these promotor regions can result in pathological estrogen production, which can result in conditions like prepubertal gynecomastia or even some cancers (Shozu et al., 2003). A similar promotor mutation is responsible for the 'henny-feathering' trait in chickens, which results in a female feather pattern in roosters (Matsumine et al., 1991, Shozu et al., 2003). In chickens, aromatase is typically only expressed in the hypothalamus in males, and in the ovary and hypothalamus in females (Matsumine et al., 1991). In henny-feathering, the tissue specificity of aromatase expression is lost in both males and female chickens due to a mutation in the promotor. The result is aromatase expression in a variety of

tissues, including the skin and connective tissue, resulting in a female feather pattern in roosters (Matsumine et al., 1991). Theoretically, a similar mutation affecting aromatase expression could explain the significantly increased E_2/A_4 ratio in egalitarian lemurs. Consistent with this hypothesis, a similar pattern in E_2/A_4 ratio is found in comparisons of female-dominant and egalitarian males (egalitarian males have 123% more E_2 relative to circulating A_4 compared to FSD males; Student's $t = 2.389$, d.f. = 12 $p = 0.03$; effect size d= 1.4), suggesting that the mechanism underlying the differences in E_2 biology are evident in both sexes, much as is the mutation in henny-feathering in chickens.

Given that evolution typically acts on the mechanisms of actions of a hormone, like enzymes, promotors, or receptors (Adkins-Regan, 2005), a mutation to one of the genes involved in E_2 biosynthesis, like an aromatase promotor for instance, provides a potential mechanism for the evolution of female-dominance, via female masculinization, in lemurs. Changes in aromatase expression in various tissues can result in measurable changes in plasma concentrations of both estrogens, and its androgenic precursors A_4 and T (Hemsell et al., 1974). Differences in aromatase promotor function in humans have been shown to result in changes in the circulating concentrations of T, as well as of the gonadotropins, LH and FSH (Shozu et al., 2003). Much like humans with CAH, where an enzyme mutation dramatically alters the relative synthesis of a whole class of steroid hormones (Breedlove and Hampson, 2002), potential differences in E_2 synthesis in female-dominant *Eulemur*, relative to other 'typical' species, could thus serve as a mechanism driving the higher A_4 and T they express. A similar evolutionary change in primitive *E. fulvus* species, altering the efficiency or expression of aromatase or estrogen,

might have had a cascade effect via feedback, and effectively altered their entire steroid endocrine milieu. This potentially serves as a parsimonious mechanism for both the evolutionary expression of female dominance in lemurs, and the relaxation of female dominance and female masculinization in egalitarian *Eulemur* species. Thus, future studies focusing on the genetic and functional differences in steroid biosynthesis, particularly related to estrogen and aromatase, may provide valuable insight into the mechanisms underlying female masculinization in female-dominant lemurs.

4. Testosterone and estrogen activate behavior differently in female-dominant and egalitarian female *Eulemur*

4.1 Introduction

In seasonal environments, reproduction and its associated behavioral and physiological components need to be coordinated with the relatively predictable annual cycles favorable to giving birth and raising young (Dawson et al., 2001, Goldman, 2001, Adkins-Regan, 2005). Thus, behavioral and hormonal changes, particularly in females, are often tied to photoperiod or other environmental factors, such as cyclical rainfall (Thompson et al., 1978, Perret and Schilling, 1995, Strier et al., 1999, Adkins-Regan, 2005). In males, although environmental cues (such as photoperiod or rainfall) can trigger the hypothalamic-pituitary-gonadal (HPG) axis to regulate reproductive physiology and behavior via the steroid hormones, social cues also have a profound effect on male hormonal and behavioral change (Vandenbergh and Drickamer, 1974, Wingfield et al., 1990, Wingfield et al., 1997, Wingfield et al., 2001). For instance, exposure to receptive females (Wingfield et al., 2001, Muller and Wrangham 2004) and competition with other males (Wingfield et al., 1990) can cause male testosterone (T) levels to increase significantly, even when already elevated (due to seasonal cues) above non-breeding baseline levels (Wingfield et al., 1990, Wingfield et al., 2001, Goymann et al., 2007).

Variation in the patterns of hormonal regulation of male behavior, particularly aggression during the breeding season in birds, has given rise to the "challenge hypothesis" (Wingfield et al., 1990). The challenge hypothesis explains seasonal patterns

of circulating androgen concentrations in seasonally breeding birds as a function of mating system, paternal care, and male-male aggression (Wingfield et al., 1990). Under the premises of the challenge hypothesis, species must balance the competitive benefits of increased T with the "costs" of T (Wingfield et al., 1997, Wingfield et al., 2001, Goymann et al., 2006). The costs of T can include energetic costs (due to direct metabolic effects or indirect behavioral effects), increased predation risk (due to increases in conspicuous behavior like courtship displays or territorial defense), increased risk of injury (due to increased escalated aggression and fighting), decreased parental care (in birds, increased T can negatively influence rates of offspring care), conflicts with pair formation or courtship (due to misdirected aggression towards females), and immunosuppression (Wingfield et al., 1990, Wingfield et al. 1997, Wingfield et al., 2001). Thus, the challenge hypothesis predicts that, for a given species, the seasonal change in male T will be related to the degree of social challenge that those males experience. In birds, non-territorial and long term pair-bonded species that experience little to no intrasexual competition typically show little to no seasonal increase in circulating androgens. Seasonally breeding species, with a high degree of male-male competition, show positive correlations between circulating androgen concentrations and periods of social instability and/or when females are receptive. Polygamous, territorial species, with little to no male parental care, show the greatest increases in, and the longest seasonal periods of elevated T, due to intense prolonged male-male competition and the regular availability of receptive females. These latter males often show little change in T in response to social cues (Wingfield et al., 1990, Wingfield et al., 2001, Goyman et al.,

2007). The challenge hypothesis has also been used to describe correlation between circulating T and various seasonal and social cues in males from several non-avian vertebrates, including lizards (Klukowski, et al., 1998), some fish (Hirschenhauser, et al., 2004, Desjardins et al., 2006), and chimpanzees (Muller and Wrangham, 2003, Sobolewski, et al., 2013). It has rarely, however, been applied to females (see Desjardins, et al., 2005 for an exception and a brief review).

Hormones, including androgens, regulate many of the same behaviors in females as they do in males (Barkley and Goldman, 1977, Udry et al., 1995, Cashdan, 1995, Grant and France, 2001, Zysling et al., 2005, Gill et al., 2007) and often incure similar costs (Zysling et al., 2005). For example, exogenous T given to female dark-eyed juncos increases intrasexual aggression (Zysling et al., 2005), and aggression in female dark-eyed juncos postively correlates with increased reproductive success (Cain and Kettering, 2012). However, increased T in female juncos also impairs immune functions and the stress response (Zysling et al., 2005). Thus, although the challenge hypothesis focuses primarily on males (Wingfield et al., 1990), it should also be applicable to the actions of hormones, particularly androgens, in females (Desjardins, et al., 2005). This may be particularly true in female-dominant lemurs, where, as I have shown, females express greater baseline levels of androgens than do egalitarian females. Following this line of thought, if female masculinization in lemurs is an evolutionary response to increased female competition for limited resources in a highly seasonal environment, as has been suggested (Hrdy, 1981, Jolly, 1998, Pereira et al., 1999), then according to the challenge hypothesis, there should be correlations between hormonal and behavioral

change during the breeding season when competive pressures are at their highest. These correltions should be evident in the form of correlated seasonal increases in androgens and aggression in female-dominant females, but not in egalitarian females. A test, in lemurs, of the challenge hypothesis provides the additional opportunity to establish correlative and comparative evidence for the activational role of the sex-steroid hormones in the expression of masculinized behavior in female-dominant species.

To test the predictions derived from the challenge hypothesis in female lemurs, and to establish evidence for an activational role of the sex-steroid hormones in the expression of masculinized behavior in female-dominant species, I examined the hormonal correlates of seasonal changes in behavior in female *Eulemur* from both female-dominant and egalitarian species. Several lines of evidence suggest that seasonal changes in behavior and hormones in *Eulemur* will provide appropriate data to accomplish my goal. Correlational studies examining the role of hormones in regulating adult behavior have been conducted in a number of primates, including muriquis (Strier et al., 1999), macaques (Girard-Buttoz et al., 2009), sifakas (Fichtel et al., 2007), mouse lemurs (Perret, 1992), ring-tailed lemurs (Cavigelli and Pereira, 2000, Drea, 2007 , Starling et al., 2010), as well as some *Eulemur*, specifically the egalitarian *E. fulvus* or red-fronted lemur (Ostner et al., 2002, Ostner and Heistermann, 2003, Ostner et al., 2008). Additionally, like female *Lemur catta,* females of *Eulemur* species exhibit strictly seasonal estrous cycles (Evans and Goy 1968), and these cycles are shifted by six months in the Northern Hemisphere relative to those in Madagascar (Van Horn, 1975, Drea, 2007), suggesting some photoperiodic control of circulating hormone cycles. Moreover,

indicative of an activational role for steroid hormones on aggression in lemurs, in ring-tailed lemurs, androstenedione (A_4) and T in males, and A_4 and estradiol (E_2) in females, increase during the breeding season along with aggression (Drea, 2007). Likewise, in male *Eulemur fulvus,* androgen levels increase during seasonal periods defined by increased male competition, and those periods associated with increased male vigilance against infanticide (Ostner et al., 2008); a pattern consistent with both the challenge hypothesis, and an activational role of steroid hormones on behavior. The predictions that follow for female *Eulemur,* are: 1) seasonal changes in masculine behavior, particularly aggression and scent marking, should correlate with concurrent changes in androgen concentrations in female-dominant species; and 2) based on data from female rhesus monkeys that show no relationship between T and several reproductive behaviors, but do show a strong correlation between behavior and E_2 (Wallen et al., 1984), there should be little to no seasonal behavioral correlations with androgens in females from egalitarian species, and potentially greater correlations between behavior and E_2.

4.2 Materials and Methods

4.2.1 Subjects and housing

The subjects and housing were identical to those of the behavioral study outlined in Section 2.2.1, with the following alterations: The data presented in this section were collected during two 3-month periods, the first from June to September 2010 and the second from November 2010 to January 2011, allowing me to characterize hormone concentrations during the nonbreeding season (NBS) and breeding season (BS),

respectively. During the BS, 2 male-female pairs, one categorized as showing FSD and one as showing egalitarianism, were removed from the data set, due to contraception of the females under DLC breeding guidelines. Removing these two pairs resulted in a BS sample set of N = 9 FSD and N = 3 egalitarian, mixed-sex pairs.

4.2.2 Behavioral data collection

Behavioral data were collected following the methods outlined in Section 2.2.2.

4.2.3 Sampling procedures

Blood samples for hormonal analysis were collected following the methods outlined in Section 3.2.2.

4.2.4 Hormone assays

The blood samples were analyzed for 5-HT, A_4, T and E_2 following the methods outlined in Section 3.2.3.

4.2.5 Statistical analyses

The statistical analyses were conducted similarly to the analyses outlined in Sections 2.2.3 for behavior and Section 3.2.4 for hormones, with the following changes: To generate both a seasonal mean and a monthly mean of behavior and hormone concentration for each group, I averaged each individual's data points both across each season and across each month, within each season. Using the seasonal values, I tested for seasonal changes in behavior and hormones within females from each dominance group. Using the monthly values within each season, I calculated a Pearson's r, testing for statistically significant correlations, for each combination of variables within each

group. I used Graphpad Prizm v.6.0 (Graphpad Software, LaJolla, Ca) to calculate population means, standard deviations, standard errors, t-statistics, Pearson's r and p-values. I calculated the effect sizes by hand.

4.3 Results

4.3.1 Seasonal changes in behavior

Female-dominant and egalitarian *Eulemur* showed different patterns of behavioral change from the NBS to the BS (Figure 10; Table 9). As measured by behavioral effect sizes (d), female-dominant, female *Eulemur* (Figure 10; Table 9), showed an expectedly large (d > 0.8) increase in aggression (114.1% increase, d = 0.88, p = 0.09). These females also showed a relatively large (d > 0.65) decrease in rates of supplants (64.5% decrease, d = 0.7, p = 0.14) and scent marking (52.1% decrease, d = 0.68, p = 0.15) over this same period. Their small, BS increase (38% increase, d = 0.35, p = 0.46) in grooming rate was most likely due to chance (Welch's corrected Student's t = 0.75, d.f. = 14.49, p = 0.46). Despite the large effect sizes, due to high variance and the relatively small sample sizes, only the seasonal increase in aggression approached statistical significance (Welch's corrected Student's t = 1.85, d.f. = 11.07, p = 0.09).

By contrast, females from egalitarian species of *Eulemur* generally showed seasonal increases in all behavior (Figure 10; Table 9). Notably, they showed a small increase in rates of supplants, that is likely due to chance (66.7% increase, d = 0.22, p = 0.8), but relatively larger increases in aggression (280.1% increase, d = 0.72, p = 0.51) and scent marking (216.8%, d = 1.03, p = 0.36). Rates of grooming by egalitarian females, however, remained virtually unchanged between seasons (1.2% increase, d = 0.02, p =

0.98). As with the behavioral data from the female-dominant females, high variability and an even smaller sample size, reduced the power of my statistical tests, so that even the large mean change in scent marking rates (d = 1.03) failed to achieve statistical significance at the p = 0.05 level.

In the BS, the significant differences between female-dominant and egalitarian female behavior found during the NBS (detailed above in section 3.3) general disappear, however, the general pattern of increased supplants, aggression, and scent marking, and decreased grooming rates in female-dominant females relative to egalitarian females remains (Figure 10).

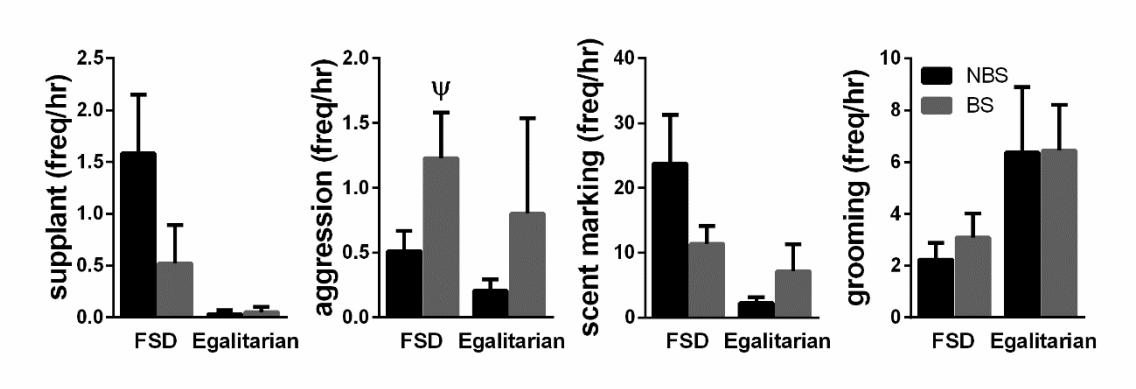

Figure 10. Seasonal changes in behavior by female *Eulemur* from species showing female-social dominance (FSD) versus egalitarianism. NBS, non-breeding season; BS, breeding season. $^{\Psi}P < 0.1$.

Table 9. Seasonal changes in behavior and hormones by female *Eulemur* from species showing female social dominance (FSD) versus egalitarianism (Egal).

Group		NBS mean (s.d.)	BS mean (s.d.)	p-value[a]	effect size (d)[b]	increase (I)/ decrease (D)[c]
FSD	**supplants**	1.58 (1.79)	0.52 (1.11)	0.14	**0.70**	**D**
	aggression	0.51 (1.11)	1.23 (1.06)	**0.09**	**0.88**	**I**
	scent marking	23.76 (23.77)	11.38 (8.32)	0.15	**0.68**	**D**
	grooming	2.23 (2.03)	3.09 (2.80)	0.46	0.35	**I**
	5-HT	1302 (446.9)	1319 (332.3)	0.76	0.04	**n/a**
	A_4	0.49 (0.28)	0.38 (0.30)	0.24	0.36	**D**
	T	0.28 (0.16)	0.92 (0.76)	**0.04**	**1.22**	**I**
	E_2	119 (58.22)	274.6 (231.6)	**0.07**	**0.94**	**I**
Egal	**supplants**	0.03 (0.07)	0.05 (0.09)	0.80	0.22	**I**
	aggression	0.21 (0.17)	0.80 (1.28)	0.51	**0.72**	**I**
	scent marking	2.26 (1.85)	7.16 (7.13)	0.36	**1.03**	**I**
	grooming	6.36 (5.06)	6.44 (3.08)	0.98	0.02	**n/a**
	5-HT	1755 (387.4)	1772 (504.0)	0.99	0.04	**n/a**
	A_4	0.18 (0.14)	0.09 (0.07)	0.39	**0.73**	**D**
	T	0.06 (0.07)	0.03 (0.02)	0.49	0.57	**D**
	E_2	73.1 (35.7)	53.3 (23.3)	0.46	0.63	**D**

a) p-value less than 0.1 in bold
b) effect size d greater than 0.6 in bold
c) change of less than 10% = n/a

4.3.2 Seasonal changes in reproductive endocrinology

Female-dominant and egalitarian *Eulemur* also showed different patterns of hormonal change from the NBS to the BS (Figure 11; Table 9). Female-dominant females showed almost no mean change in circulating concentrations of 5-HT (1.3% increase, d = 0.04, p = 0.76) and only a small decrease in circulating A_4 (22.4% decrease, d = 0.36, p = 0.24). Nevertheless, during the BS, they showed a statistically significant increase in T (228.5% increase, d = 1.22, p = 0.04) and also a strong, but statistically unreliable increase in E_2 (130.7% increase, d = 0.94, p = 0.07).

In egalitarian females, 5-HT concentrations remained relatively unchanged across season (< 1% increase, d = 0.04, p = 0.99); however, the concentrations of A_4 (50% decrease, d = 0.73, p = 0.39), T (50% decrease, d = 0.57, p = 0.49) and E_2 (27% decrease, d = 0.63, p = 0.46) all showed moderate (0.5 < d < 0.65) to moderately large (d > 0.7) decreases in the BS. Due to the small sample and high variability, Student's t tests of the transformed egalitarian female hormonal data also lacked the power to determine if these decreases were real or the products of chance and random sampling. Conservatively, these data suggest no major seasonal changes in the reproductive endocrine profiles of females belonging to egalitarian *Eulemur* species.

In the BS, the significant differences in circulating hormone concentrations between female-dominant and egalitarian females found during the NBS (detailed in Section 3.3) are maintained or increase (Figure 12).

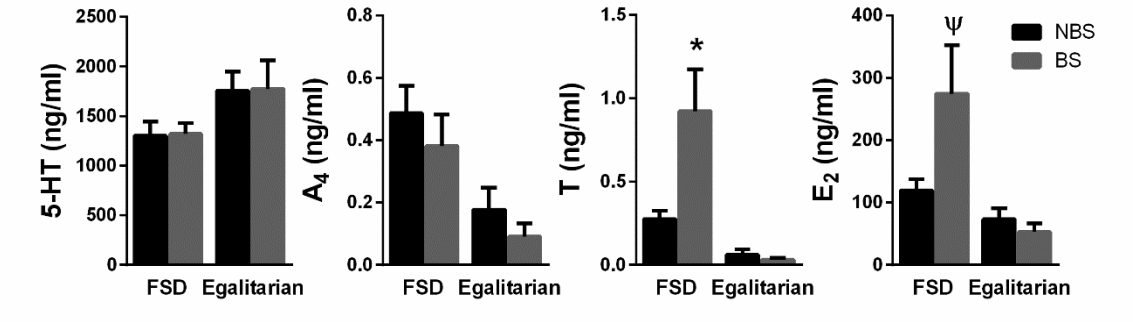

Figure 11. Seasonal changes in the hormones of female *Eulemur* from species showing female social dominance (FSD) versus egalitarianism. NBS, non-breeding season; BS, breeding season. $^{\Psi}P < 0.1$ and $^{*}P < 0.05$.

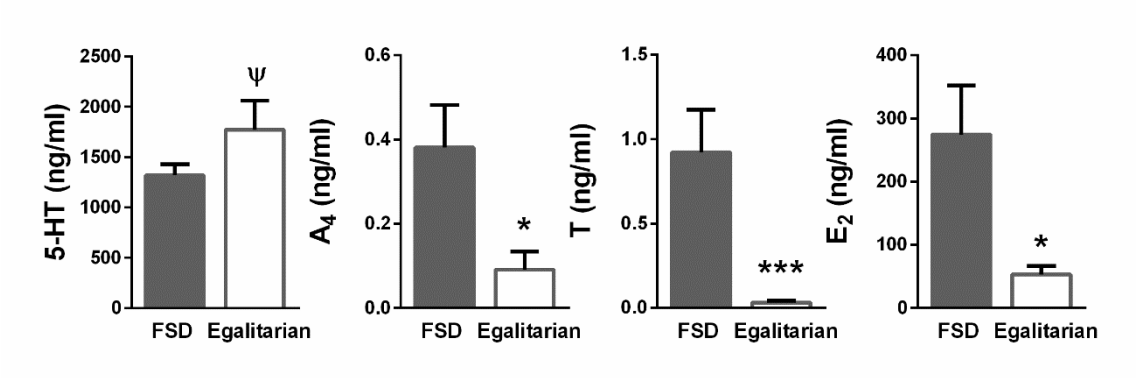

Figure 12. Breeding season differences in the hormones of female *Eulemur* from species showing female social dominance (FSD) versus egalitarianism (Non-breeding season differences shown in Section 3.3, Figure 8). $^{\Psi}P < 0.1$, $^{*}P < 0.05$ and $^{***}P < 0.005$.

4.3.3 Monthly correlations between hormones and behavior

The correlation matrices for the monthly behavioral and hormonal data are shown for female-dominant females in Tables 10 and for egalitarian females in Table 11. The monthly seasonal data are also shown in Figures 13 and 14 for female-dominant and egalitarian females, respectively.

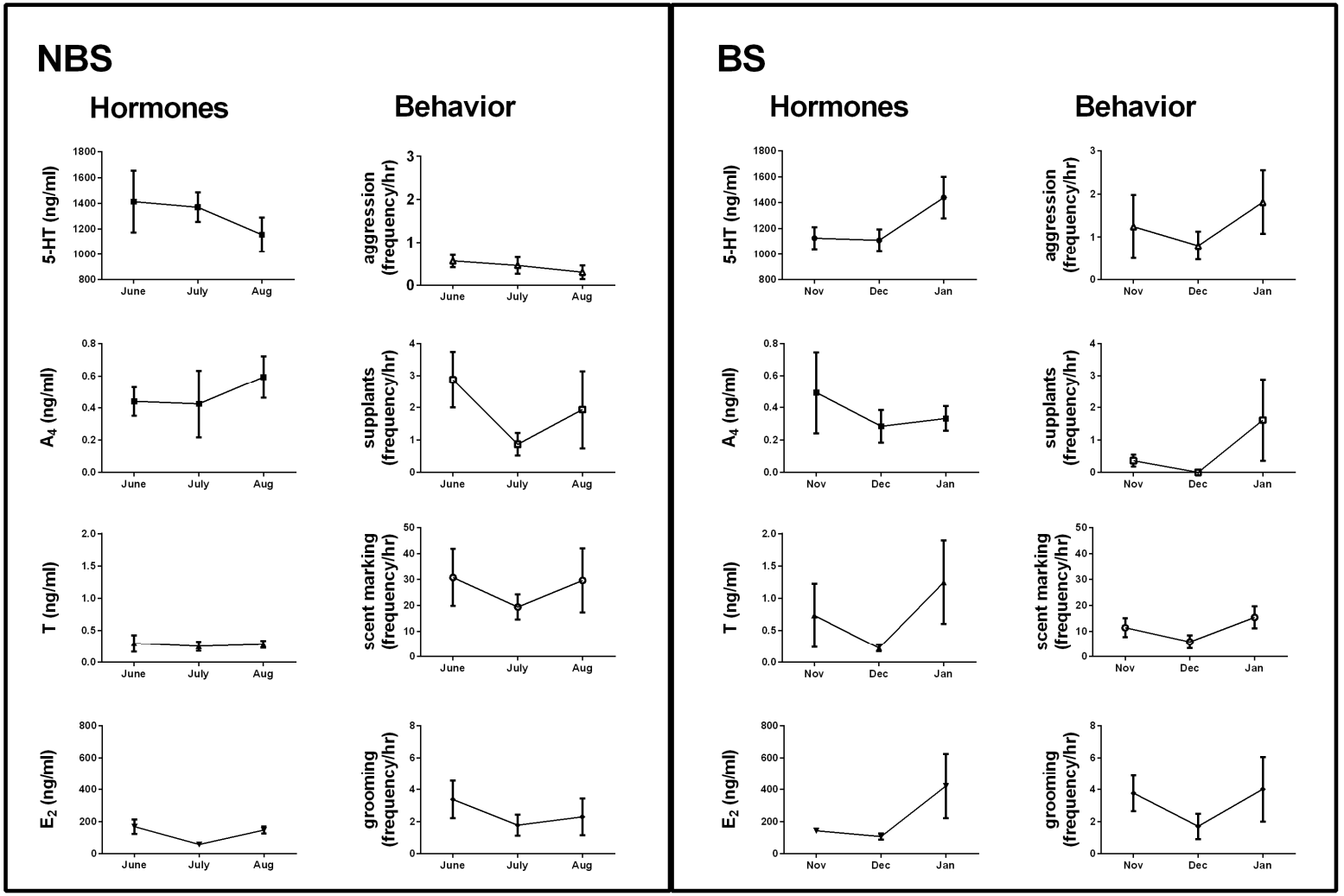

Figure 13. Female-dominant female *Eulemur* NBS and BS hormones and behavior by month.

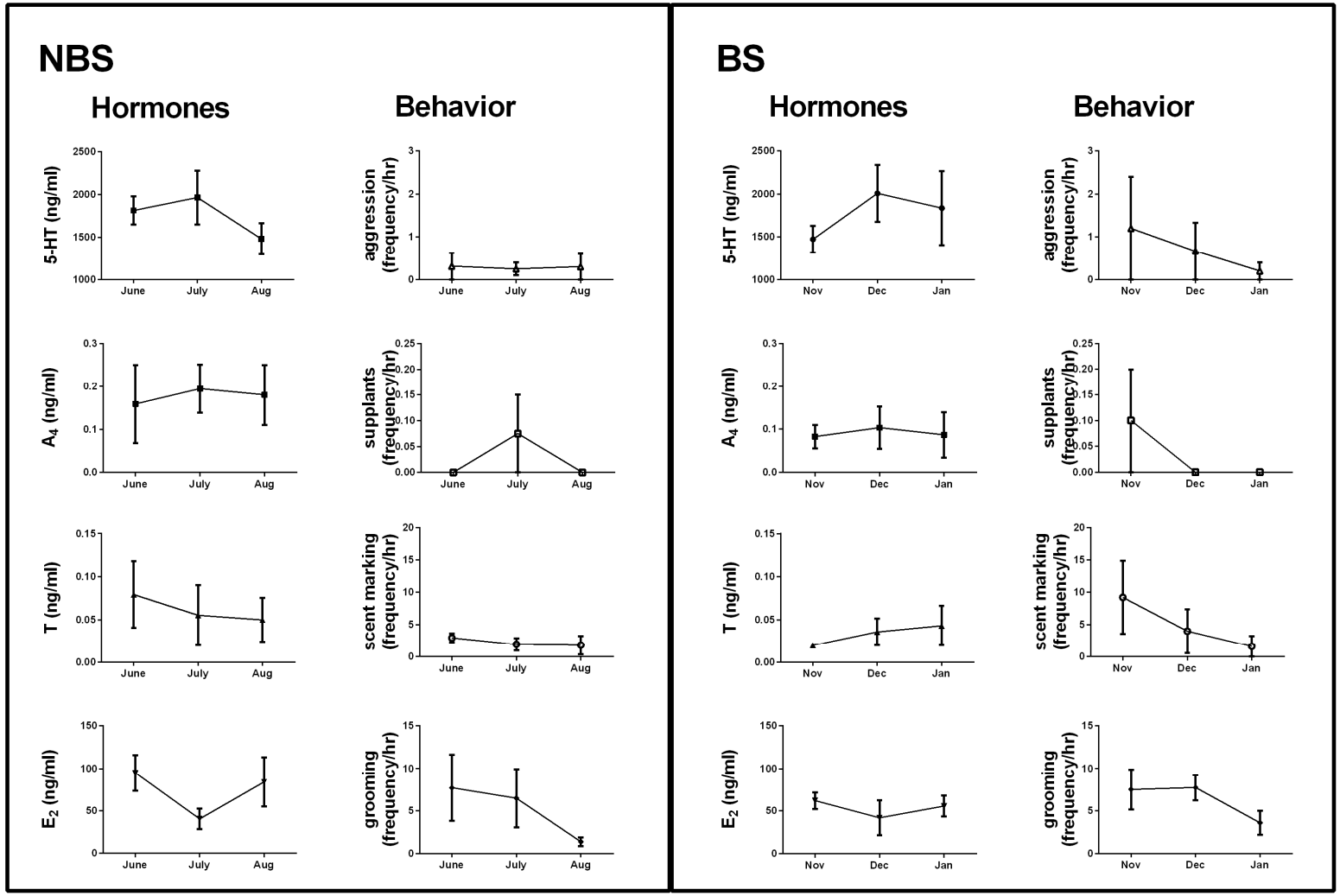

Figure 14. Egalitarian female *Eulemur* NBS and BS hormones and behavior by month.

4.3.3.1 Monthly patterns of behavioral and hormonal change for female-dominant females

During the NBS, in female-dominant female *Eulemur* there were several strong (Pearson's r > 0.8 or r < -0.8) relationships between variables (Table 10). T and E_2, which were highly correlated during the NBS (r = 0.99, p = 0.11), also were individually strongly correlated with grooming (T, r = 0.93, p = 0.24; E_2, r = 0.85, p = 0.35), scent marking (T, r = 0.97, p = 0.16; E_2, r = 1.0, p = 0.05), and supplants (T, r = 0.99, p = 0.08; E_2, r = 0.95, p = 0.19). Aggression showed little relationship to T or E_2 during the NBS, but correlated positively with 5-HT (r = 0.97, p = 0.16) and negatively with A_4 (r = - 0.88, p = 0.31). Most of these relationships failed to meet statistical significance; however, the relationships between E_2 and scent marking (p = 0.05) and between T and supplants (p = 0.08) were the least likely to be due to chance.

Table 10. Correlations between hormones and behavior during the non-breeding season (NBS) for female-dominant, female *Eulemur*. Pearson's r (above) and p-values (below, italics) Pearson's r with corresponding p-values < p = 0.12 highlighted in yellow.

NBS variables	5-HT	A_4	T	E_2	Groom Rate	Scent Mark	Supplants	Aggression
5-HT		-0.97	-0.01	-0.19	0.36	-0.27	0.1	0.97
A_4	*0.15*		0.25	0.41	-0.12	0.49	0.12	-0.88
T	*0.99*	*0.84*		**0.99**	0.93	0.97	**0.99**	0.24
E_2	*0.88*	*0.73*	***0.11***		0.85	**1.00**	0.95	0.07
Groom Rate	*0.77*	*0.92*	*0.24*	*0.35*		0.80	0.97	0.58
Scent Mark	*0.83*	*0.67*	*0.16*	***0.05***	*0.41*		0.93	-0.02
Supplants	*0.93*	*0.92*	***0.08***	*0.19*	*0.16*	*0.25*		0.36
Aggression	*0.16*	*0.31*	*0.85*	*0.96*	*0.61*	*0.99*	*0.76*	

During the BS, the relationships between variables changed and were less likely to be due to chance (Table 11). A very strong positive relationship appeared between T and scent marking (r = 1.0, p = 0.06) and between T and aggression (r = 1.0, p = 0.05). Likewise, supplants were strongly correlated with 5-HT (r = 0.99, p = 0.11) and E_2 (r = 0.99, p = 0.07). The strongest correlations between hormones and behavior occurred between T and scent marking, T and aggression, and E_2 and supplants, and were the least likely to be due to chance (all p < 0.1).

Table 11. Correlations between hormones and behavior during the breeding season (BS) in female-dominant female *Eulemur.* Pearson's r (above) and p-values (below, italics) Pearson's r with corresponding p-values < p = 0.12 highlighted in yellow.

BS variables	5-HT	A_4	T	E_2	Groom Rate	Scent Mark	Supplants	Aggression
5-HT		-0.26	0.89	**1.00**	0.62	0.84	**0.99**	0.92
A_4	*0.84*		0.22	-0.19	0.60	0.31	-0.09	0.15
T	*0.31*	*0.86*		0.91	0.91	**1.00**	0.95	**1.00**
E_2	***0.04***	*0.88*	*0.27*		0.66	0.87	**0.99**	0.94
Groom Rate	*0.58*	*0.59*	*0.27*	*0.54*		0.95	0.74	0.88
Scent Mark	*0.37*	*0.80*	***0.06***	*0.33*	*0.21*		0.92	**0.99**
Supplants	***0.11***	*0.94*	*0.20*	***0.07***	*0.47*	*0.26*		0.97
Aggression	*0.26*	*0.90*	***0.05***	*0.22*	*0.32*	***0.11***	*0.15*	

4.3.3.2 Monthly patterns of behavioral and hormonal change for egalitarian females

During the NBS several strong relationships between variables (Table 12) were also evident for egalitarian female *Eulemur*. In egalitarian females, 5-HT correlated the most strongly with grooming (r = 0.88, p = 0.32) but not significantly so. A_4 showed a strong

negative correlation with both scent marking (r = -0.87, p = 0.33) and aggression (r = - 0.9, p = 0.28) and a positive relationship to supplants (r = 0.81, p = 0.4). T showed a very strong positive relationship with scent marking (r = 1.0, p = 0.05) and E_2 showed a strong negative relationship with supplants (r = - 0.98, p = 0.12) and a very strong positive relationship with aggression (r = 1.0, p = 0.0003). Like the data for female-dominant females, many of these relationships failed to meet statistical significance at the $p < 0.05$ level. The positive correlations between T and scent marking and between E_2 and aggression however, were significant (p = 0.05 and 0.0003 respectively) with the relationship between E_2 and aggression during the NBS standing out as the strongest.

Table 12. NBS and BS correlations between egalitarian female *Eulemur*'s hormones and behavior visualized in Figure 13. Pearson's r (above) and p-values (below, italics). Pearson's r and corresponding p-values less than p = 0.1 highlighted in yellow.

NBS variables	5-HT	A_4	T	E_2	Groom Rate	Scent Mark	Supplants	Aggression
5-HT		0.20	0.38	-0.60	0.88	0.30	0.74	-0.60
A_4	*0.87*		-0.83	0.90	-0.29	-0.87	0.81	-0.90
T	*0.75*	*0.38*		0.51	0.77	**1.00**	-0.34	0.51
E_2	*0.59*	*0.28*	*0.66*		-0.15	0.58	-0.98	**1.00**
Groom Rate	*0.32*	*0.81*	*0.44*	*0.90*		0.72	0.33	-0.15
Scent Mark	*0.80*	*0.33*	***0.05***	*0.61*	*0.49*		-0.41	0.58
Supplants	*0.47*	*0.40*	*0.78*	*0.12*	*0.78*	*0.73*		-0.98
Aggression	*0.59*	*0.28*	*0.66*	***0.00***	*0.90*	*0.61*	*0.12*	

During the BS, very different correlational patterns emerge (Table 13). Many of the relationships found during the NBS disappeared or were completely reversed. T became strongly and negatively correlated with scent marking (r = -1.0, p = 0.003), supplants (r = -0.95, p = 0.2), and aggression (r = - 0.99, p = 0.11) and scent marking and aggression themselves showed a strongly positive relationship (r = 0.99, p = 0.11). E_2 lost its strong relationship with aggression (r = 0.34, p = 0.78) and the relatively strong relationships between A_4 and behavior also weakened. A_4 and E_2 became strongly and negatively correlated (r = - 0.99, p = 0.08). Of these relationships the strongest was found in the negative correlation between T and scent marking which was highly statistically significant (p = 0.003).

Table 13. Correlations between hormones and behavior during the breeding season (BS) in egalitarian female *Eulemur*. Pearson's r (above) and p-values (below, italics) Pearson's r with corresponding p-values < p = 0.12 highlighted in yellow.

BS variables	5-HT	A_4	T	E_2	Groom Rate	Scent Mark	Supplants	Aggression
5-HT		0.86	0.80	-0.91	-0.15	-0.81	-0.95	-0.69
A_4	*0.35*		0.38	-0.99	0.38	-0.38	-0.65	-0.22
T	*0.41*	*0.75*		-0.49	-0.71	-1.00	-0.95	-0.99
E_2	*0.26*	*0.08*	*0.67*		-0.26	0.50	0.74	0.34
Groom Rate	*0.90*	*0.75*	*0.50*	*0.83*		0.71	0.46	0.82
Scent Mark	*0.40*	*0.75*	*0.00*	*0.67*	*0.50*		0.95	0.99
Supplants	*0.20*	*0.25*	*0.20*	*0.47*	*0.70*	*0.20*		0.88
Aggression	*0.51*	*0.86*	*0.11*	*0.78*	*0.39*	*0.11*	*0.31*	

4.4 Discussion

4.4.1 Female-dominant and egalitarian female *Eulemur* show very different patterns of seasonal behavioral and hormonal change

The data presented here continues to support fundamental physiological and behavioral difference between female-dominant and egalitarian female *Eulemur*. Despite being closely related (Horvath et al., 2008) with similarly masculinized morphology (Hill, 1953; delBarco-Trillo et al., 2012), female-dominant and egalitarian females differed in patterns of both seasonal behavioral and hormonal change, as well as in seasonal correlations between the two. Moreover, these data continue to be consistent with the hypothesis of female masculinization in female-dominant species.

Both T and E_2 are correlated with some behaviors within both groups, supporting an activational role for these hormones. The specific activational roles of the steroid hormones, however, differ between groups and appear to change across seasons. Seasonal changes in the roles of steroid hormones in actively mediating behavior in female *Eulemur* are not unexpected. The HPG axis of seasonal breeders is often upregulated during the BS (Dawson et al., 2001) and chemical mechanisms for steroid action can vary seasonally (Wennstrom et al., 2001). Beyond simple increases in circulating concentrations of sex-steroid hormones, seasonal changes can occur in brain steroid receptors, in neuropeptides like GnRH, sensitivity to neuromodulators of reproductive behavior, activity of brain steroid synthesizing, and metabolizing enzymes (Callard et al., 1983, Pasmanik and Callard, 1988a, Pasmanik and Callard, 1988b, Wood

et al., 1995, Gahr and Metzdorf, 1997, Foidart et al., 1998, Soma et al., 2000a), each of which might affect the seasonal correlations between hormones and behavior.

There is also evidence from research on birds that the hormonal mechanisms underlying behavior, particularly aggression, often shift depending on the season (Soma et al., 2000a, Soma et al., 2000c, Soma and Wingfield, 2001, Soma et al., 2008). In male song sparrow for instance, although territorial aggression is exhibited during both the NBS and BS, the hormonal substrate regulating its expression changes from T during the BS to dehydroepiandrosterone (DHEA) during the NBS (Soma et al., 2000a, Soma et al., 2008). Interestingly, the aromatase inhibitor fadrozole greatly reduces NBS aggression in song sparrows. DHEA appears to serve only as a substrate for local conversion in the brain to E_2, which is then the likely proximate hormonal substrate regulating NBS aggression in these birds (Soma et al., 2000b, Soma and Wingfield, 2001). Although the mechanisms underlying the changes in seasonal patterns of hormonal and behavioral correlations in both groups of *Eulemur* are clearly not know at this time, what does appear to be substantiated by the current data is that these hypothetical mechanisms likely differ between female-dominant and egalitarian species.

Based on the reasoning that the function of a behavior often better predicts its hormonal basis than does its form (Adkins-Regan, 2005), the different behavioral and hormonal associations apparent between female-dominant and egalitarian females likely indicate that, in these two groups of species, scent marking and aggression, serve

different social functions. Scent marking (Ralls, 1971, Albers and Prishkolnik, 1992, Heymann, 2006a, Heymann, 2006b, Drea and Scordato, 2008) and aggression (Bouissou, 1983, Soma, 2006, Soma et al., 2008), can serve multiple social functions within mammals. Two of the most important functions of scent marking for instance, include intrasexual competition and the coordination and advertisement of reproductive state (Kappeler, 1993, Mertl-Millhollen, 2006, Drea and Scordato, 2008). In ring-tailed lemurs studied at the DLC, female scent marking was associated with resource ownership, asserting status, and maintaining intrasexual dominance hierarchies, as well as mediating reproductive behavior (Drea and Scordato, 2008). Scent marking in female ring-tailed lemurs peaks in early October coinciding with the beginning of the breeding season and increased female-female competition, and begins to decrease thereafter, reaching a nadir in late November (Drea and Scordato, 2008); the scent marking data from the female-dominant, female *Eulemur* are roughly consistent with this pattern, but the data from egalitarian, female *Eulemur* are not.

4.4.2 Support for increased competitive pressure on females from female-dominant species based on the challenge hypothesis

The challenge hypothesis (Wingfield et al., 1990) defines three theoretical levels of circulating T concentrations in male birds. Level 'A' is the NBS baseline concentration, level 'B' is the minimum concentration above 'A' needed to support gametogenesis and reproduction in males during the BS, and level 'C' is the physiological maximum. Correlations between aggression and T concentrations above level 'B', nearing the

maximum, should occur only during periods of increased male-male competition, such as during the establishment of breeding territories or mate guarding in the BS. Male birds in captivity often show lower concentrations of circulating T than their wild counterparts (Wingfield et al., 1990). This difference is due to the lack of the appropriate social cues, in the form of other competing males or the presence of receptive females that drive T concentrations above level 'B'. The exceptions to this pattern are polygamous, territorial bird species that lack male parental care. Changes in T concentrations in the males of these species are less dependent on social cues; the benefits of increased T appear to outweigh the costs in these species, and thus selection has acted to maintain elevated T at or near the theoretical maximum across the entire BS (Wingfield et al., 1990, Wingfield et al., 2001).

In female-dominant female *Eulemur*, aggression and circulating T increase and become highly correlated during the BS. No such pattern was evident in egalitarian species. As all of the females in this study were kept in captive, stable dyads, and experienced no additional competitive challenges during the BS, these seasonal patterns of behavioral and hormonal change suggest selection for elevated T and increased aggression (i.e. female masculinization) in female-dominant female lemurs, but not in egalitarian females. Based on the challenge hypothesis, this pattern in female-dominant females should only evolve if the competitive pressures are such that the increased benefit of elevated T outweighs its cost. In the wild, both breeding and birthing have

been characterized as periods of increased competition and heightened aggression in female lemurs (Evans and Goy, 1968, Pereira and Weiss, 1991, Sauther, 1991, Von Engelhardt et al., 2000, Drea 2007), consistent with these results.

In the context of the challenge hypothesis, the data presented here provide a link between the proximate hypothesis, that FSD is a consequence of female masculinization (Drea, 2007, 2009), and the ultimate hypothesis, that FSD evolved in response to increased female competition for limited resources in a highly seasonal environment (Hrdy, 1981, Jolly, 1998, Pereira et al., 1999). Female masculinization (i.e. increased adrogens and aggression) likely evolved in response to high levels of competitive pressure on females lemurs. That FSD appears to have been relaxed in egalitarian *Eulemur* suggests that the benefits of masculinization, or increased androgens, no longer outweigh the costs for females in these species. A decrease in competitive pressure experienced by females within egalitarian *Eulemur* species would explain these results. Egalitarian *Eulemur* have been reported to have a unique social system based upon 'special realtionships' between males and females (Pereira and McGlynn, 1997). These 'special' male-female realtionships have been suggested to decrease the need for dominance behavior and to decrease the opportunity for sexual promiscutity. Together these social traits may act to decrease female competition in these species. Agonism is reported to be rare and mild in egalitarian species (Kaufman, 1996), however, females in some egalitarian species do periodically aggressively evict other females from the group

(Kappeler and Fichtel 2012). These female evictions have been linked to reproductive competition. Unfortunately, changes in hormone concentrations during these eviction events have not yet been studied.

Since the females in the present study experienced no simulated or real intrasexual competition or challenge, it is unclear if T concentrations, in either group, are at, or near, their physiological or functional maximum (level 'C'). T concentrations in male lemurs shows some insensitivity to social cues (Ostner et al., 2002), in the much the same fashion as is seen in polygamous, territorial, non-parental male birds (Wingfield et al., 1990). Female *Eulemur* may have the ability to respond to seasonal competition and other social challenges with increased T, but I would predict, based on the data here, that this is more likely to be the case in egalitarian species, where T concentrations are low and competitive challenges (like female evictions) are not regular occurrences, than in female-dominant species, where T is already elevated and female intrasexual competition appears to be high.

5. Letrozole shows little effect on pair-wise behavior but does differently alter the response of females from female-dominant and egalitarian lemur species to conspecific odorants

5.1 Introduction

Both testosterone (T) and estradiol (E_2) are essential to many vertebrates, including mammals, for the expression of masculine behavior (Wu et al., 2009). This requirement also appears to be true for the expression of masculine behavior in the females of female-dominant, lemur species. As we saw in previous chapters, relative to egalitarian female *Eulemur*, female-dominant, female *Eulemur* express significantly greater circulating concentrations of T and E_2, as well as greater concentrations of the precursor, androstenedione (A_4). These patterns are evident year round, during both the non-breeding season (NBS) and the breeding season (BS), suggesting a function beyond maintaining reproductive physiology. There are also strong correlations both between T and E_2, and between either of these two steroids and certain sexually dimorphic behavior, including aggression and scent marking. These findings are consistent with high levels of female competition, and an activational role for these hormones in the expression and maintenance of female social dominance (FSD) among *Eulemur*.

Interestingly, in many species much of the organizational and activational role of androgens in the brain is mediated by local conversion, of either A_4 or T, to E_2 by the enzyme aromatase (Naftolin and Ryan, 1975, MacLusky and Naftolin, 1981, Soma et al.,

2000b, Trainor et al., 2006b, Wu et al., 2009) that catalyzes the final and rate-limiting step in the biosynthesis of E_2 (Ryan, 1959, Bhatnagar, 2007). Data from birds (Soma et al., 2000a, Soma et al., 2000b), rodents (Honda et al., 1998,Bakker et al., 2004a, Bakker et al., 2004b, Trainor et al., 2006a, Trainor et al., 2006b), and primates (Zumpe et al., 1993, Zumpe et al., 1996) all support an activational role for E_2 in mediating male behavior.

In mammals, evidence for the aromatization of androgens to estrogens in activating male behavior comes largely from transgenic mice (Bakker et al., 2004a, Bakker et al., 2004b) and the inhibition of aromatase activity in monkeys (Michael and Zumpe, 1970a, Michael and Zumpe, 1993, Zumpe et al., 1993). This research is largely focused on sexual behavior. Male aromatase knock-out mice show impaired mounting, intromission, and ejaculation, as well as a decrease in olfactory investigation of volatile conspecific body odor (Honda et al., 1998, Bakker et al., 2004a, Bakker et al., 2004b). Exogenous treatment of these mice with estradiol benzoate (EB) failed to reinstate or stimulate olfactory investigation of volatile body odor, supporting an organizational, rather than an activational, role of E_2 in the expression of this particular behavior (Bakker et al., 2004b). However, male sexual behavior was reinstated by EB treatment, indicating that these behaviors are actively regulated by estrogen (Bakker et al., 2004b). Likewise, compared to castrated males receiving T alone, castrated male cynomolgus monkeys treated with exogenous T and fadrozole, a potent aromatase inhibitor, showed significantly reduced ejaculatory behavior and reduced male sexual motivation in the presence of receptive

females (Zumpe et al 1993), evidence that the conversion from T to E_2 was necessary for the activation of these behaviors.

E_2 also actively mediates female behavior (Michael and Zumpe, 1970a, Zumpe and Michael, 1970b, Wallen, 1990, Michael and Zumpe, 1993, Woodley and Moore, 1999, Lonstein and Gammie, 2002, Lundstrom et al., 2006, Frynta et al., 2010, Renfro and Hoffmann, 2013, Martinec Nováková et al., 2014). Proceptive and sexual behavior in female primates, although strongly influenced by social and environmental conditions (Wallen, 1990, Michael and Zumpe, 1993), positively correlates with estrogen during the menstrual cycle (Wallen, 1990, Michael and Zumpe, 1970b), is reduced following ovariectomy, and can be reinstated by exogenous administration of EB (Zumpe and Michael, 1970b, Michael and Zumpe, 1993). E_2 also activates female olfactory behavior. Cyclic changes in estrogen during the menstrual cycle, treatment with exogenous E_2, as well as hormonal contraception, can all alter female preference of, response to, and sensitivity to male odors (Kelliher and Baum, 2002, Woodley and Baum, 2003, Lundstrom et al., 2006, Frynta et al., 2010, Renfro and Hoffmann, 2013, Martinec Nováková et al., 2014).

In both the males and females of many species, the regulation of aggression, in particular, seems to be influenced by estrogenic mechanisms (Michael and Zumpe, 1970a , Schlinger and Callard, 1989, Woodley and Moore, 1999, Soma et al., 2000b, Lonstein and Gammie, 2002, Trainor et al., 2006a, Trainor et al., 2006b). For instance,

territorial aggression in male birds is activated by E_2 (Schlinger and Callard, 1989, Soma et al., 2000b), particularly during the non-breeding season (NBS) (Soma et al., 1999, Soma et al., 2000b). Territorial aggression in female spiny mountain lizards is also likely to be mediated by E_2 (Woodley and Moore, 1999). Under a pregnancy-termination paradigm in which rats are hysterectomized and ovariectomized on gestational day 16, which is thought to mimic the hormonal changes that occur directly prior to parturition, females treated with E_2 become highly aggressive towards males (Lonstein and Gammie, 2002). Aggression in female macaques is also activated by estrogens (Michael and Zumpe, 1970a). Aggression increases across pregnancy in female macaques as E_2 increases, and ovariectomied female macaques administered E_2 express increased rates of aggression towards males. Because much of the masculinizing actions of T occur via its aromatization to E_2 (Naftolin and Ryan, 1975, MacLusky and Naftolin, 1981, Soma et al., 2000b, Trainor et al., 2006b, Wu et al., 2009), and E_2 is the major circulating hormone in females, in the present study I investigated the role of E_2 in activating behavior in female-dominant and egalitarian *Eulemur* species, by targeting the activity of the enzyme, aromatase.

I used the aromatase inhibitor, letrozole in this study, to inhibit the biosynthesis of E_2 in females from both female-dominant and egalitarian lemur species. Letrozole is a competitive aromatase inhibitor that effectively blocks production of E_2 from either A_4 or T (Bhatnagar, 2007, Forman et al., 2007). Letrozole is extremely selective and potent in

inhibiting aromatase. In female rats, for instance, letrozole inhibits aromatase with a 50% effective dose (ED_{50}) of 1 – 3 μg/kg (Bhatnagar, 2007). Likewise, in human females, 40 μg/kg/day reduces and maintains E_2 concentrations below the detection sensitivity of some assays (Geisler et al., 2002). In female baboons, 100 μg/kg/day of letrozole was found to decrease venous E_2 levels by 95% (Pepe et al., 2003).

Much of the research using letrozole has been medical in nature (Bhatnagar, 2007, Forman et al., 2007). For instance, letrozole is routinely used as an adjuvant therapy in the treatment of estrogen-dependent breast cancer (Bhatnagar, 2007), and as a treatment for infertility to induce follicle formation and ovulation (Forman et al., 2007). Researchers are, however, increasingly using letrozole in the same fashion as fadrozole, namely to probe the differential effects of T versus E_2 on such behavior as male parental care and aggression in hamsters (Hume and Wynne-Edwards, 2006, Wynne-Edwards and Timonin, 2007, Timonin and Wynne-Edwards, 2008), risk taking in men (Goudriaan et al., 2010), and mood disorders in women (Goodwin, 2006). Letrozole is typically more potent than fadrozole (Demers 1994), and has no effect on mineralocorticoid or glucocorticoid synthesis in the adrenal (Demers 1994, Deleo & Iamarca 1999), decreasing the adverse side effects of treatment. Letrozole's potency, selectivity, and lack of effect on the HPA axis make it ideal for investigating the activational role of E_2 and aromatization, on the expression of behavior in female lemur.

Treatment with letrozole may have a number of physiological and behavioral effects on female lemur. Physiologically, letrozole should significantly reduce circulating concentrations of E_2, as it does in other species (Geisler et al., 2002, Pepe et al., 2003, Bhatnagar, 2007). It is also likely that by blocking the conversion of androgens to E_2, letrozole treatment will generally increase circulating concentrations of androgens (Kumru et al., 2007, Gallicchio et al., 2011). If masculinized female behavior, like aggression and scent marking, in female-dominant lemur is mediated by estrogens, then reduced E_2, as a result of letrozole treatment, should reduce the expression of these behaviors. If on the other hand, aggression and scent marking are directly activated by androgens, then the increase in circulating concentrations of androgens, as a 'byproduct' of letrozole treatment, should lead to an increase in the expression of these behaviors. I generally expect to find a similar physiological response in egalitarian females to letrozole treatment as in female-dominant females, including decreased circulating concentrations of E_2, and potentially increased concentrations of circulating androgens; however, I expect more muted behavioral effects, potentially indicating less direct mediation of masculinized behavior by the endocrine system.

Unlike my previous studies, in the present study, in addition to *Eulemur*, I include subjects from the female-dominant lemur species *Lemur catta*. I do this for two reasons: 1) As the number of *Eulemur* females made available by the Duke Lemur Center (DLC) for hormonal manipulation was limited, the addition of several female *L. catta* provides

additional robustness to the subsequent analysis of my results; and 2) As much of the previous research on the mechanisms regulating female dominance in strepsirrhines has been conducted using *L. catta* as a model, particularly research related to olfactory communication and behavior (Scordato and Drea, 2005, Scordato et al., 2007, Scordato and Drea, 2007, Charpentier et al., 2008, Drea and Scordato, 2008, Boulet et al., 2009, Crawford et al., 2009, Boulet et al., 2010, Crawford et al., 2011), *L. catta* provides a vital framework and foundation for the behavioral and endocrine research proposed here. This is particularly that case for the hormone manipulations and behavioral bioassays (detailed below), which have not been performed in female *Eulemur* previously.

Because of the importance of olfactory communication in lemurs (Perret, 1992, Kappeler, 1998, Scordato and Drea, 2007, Drea and Scordato, 2008) and E_2's ability to mediate olfactory behavior (Kelliher and Baum, 2002, Woodley and Baum, 2003, Lundstrom et al., 2006, Frynta et al., 2010, Renfro and Hoffmann, 2013, Martinec Nováková et al., 2014), I also conducted a series of behavioral bioassays aimed at assessing the effect of letrozole treatment on the response of each individual to normal conspecific odors from the NBS. Previous researchers (Scordato and Drea, 2007, Drea and Scordato, 2008, delBarco-Trillo et al., 2012) have shown that the chemical complexity of scent secretions in female-dominant *Eulemur* and *L. catta* differ by sex, with females showing greater chemical complexity in their secretions than do males. In *Eulemur*,

however, males from egalitarian species produce more chemically complex odorants than do their female counterparts (delBarco-Trillo et al., 2012).

Although the response to a chemical signal can be influenced by a number of variables, including familiarity, detection threshold, and receiver motivation or preference (Dorries et al., 1997, Lundstrom et al., 2006, Renfro and Hoffmann, 2013, Martinec Nováková et al., 2014), in female-dominant lemurs, intrasexual competition appears to be a primary motivator of female interest in conspecific odorants (Drea and Scordato, 2008). In *L. catta,* when given a choice between odorants in a discrimination test, females showed a distinct preference for female odorants over male odorants (Scordato and Drea, 2007, Drea and Scordato, 2008). Male *L. catta*, on the other hand, show no preference for either male or female odors, attending to both equally (Drea and Scordato, 2008). Unlike male *L. catta,* in a similar test of egalitarian male *E. fulvus'* odor preference (Harrington, 1977), males preferred odorants from other males over female odorants. Unfortunately, Harrington (1977) did not test females in his study.

Based on this previous work and the dominance relationships within each group of species, in untreated female-dominant *Eulemur*, and *L. catta*, I expect to find increased interest in female odorants over male odorants. In untreated female egalitarian *Eulemur*, where intrasexual competition may be reduced and males and females form 'special relationships' within the larger social group (Pereira and McGlynn, 1997), I expect females to potentially show, on average, no preference for odors from either sex. In

female-dominant females, where odor preference should be more motivated by intrasexual competition, if estrogen is regulating this motivation, I expect female-dominant females to show a decrease in interest in female odorants under the influence of letrozole treatment. In egalitarian females, where odorant preference may be less motivated by competition, and potentially more motivated by the 'special relationships' that form between males and females (Pereira and McGlynn, 1997), I predict a potential decrease of interest in male odorants with letrozole treatment.

5.2 Material and Methods

5.2.1 Subjects and housing

My subjects were 12 reproductively intact, adult female animals representing six species of lemur. The animals in this study included 9 pairs from four species characterized by female social dominance or FSD (including *Eulemur rubriventer, N = 2; E. coronatus, N = 1; E. mongoz, N = 3;* and *Lemur catta, N = 3*) and 3 pairs from two species characterized as egalitarian (*E. fulvus collaris, N = 2;* and *E. f. rufus, N = 1*). Although only females were treated with letrozole, the animals were all similarly maintained with a partner male in 12 established, mixed-sex pairs at the DLC in Durham, NC, USA.

The housing was identical to that outlined in Section 2.2.1. To avoid interfering with either breeding or contraception, as part of the agreement with the DLC allowing the hormonal manipulation of endangered lemurs, the letrozole treatment was conducted during the NBS. The data presented in this section were collected over 3,

four-week periods, encompassing a pre-treatment, treatment, and post treatment period, during the NBS from June to September 2013.

5.2.2 Letrozole treatment

Using a within-subjects design, each female served as her own control during the pre-treatment, treatment, and post-treatment periods. During the first 4 weeks of data collection, females were untreated (pre-treatment period). Beginning on the fifth week of the study, all female subjects (N = 12) received letrozole at 40 μg/kg/day (treatment period). The letrozole was dissolved in dimethyl sulfoxide (DMSO) and administered orally via a food treat, consisting of either a grape or raisin. Letrozole has a terminal half-life of 42h and significant effects on E_2 concentrations are typically achieved within 2-3 days of treatment (Bhatnagar, 2007). Animals were dosed daily for 28 days. After letrozole treatment was terminated, data collection continued for a final 4 more weeks (post-treatment period).

5.2.3 Behavioral Data collection

Because only females were treated with letrozole, I focused on female behavior and female-initiated dyadic interactions, using continuous focal sampling. Observational data were otherwise collected in the manner outlined in Section 2.2.2.

5.2.4 Sampling procedures.

5.2.4.1 Blood sampling

Blood sampling was conducted once each during the pre-treatment, treatment, and post-treatment periods, following the sampling procedures outlined in Section 3.2.2.

5.2.4.2 Odor sampling

Odorant samples were collected from the combined genital/perianal (*Eulemur*) or genital (*Lemur catta*) glands of manually restrained, untreated donors during the pre-treatment period only. Thus, all odorants represented untreated conspecifics during the NBS. Odors were collected from all study subject females and their male dyadic partners. To provide enough presentation material for the bioassays, additional odorants were collected from one *E. rubriventer* pair and two *E. coronatus* pairs, which were not otherwise included in the study. A minimum of four odorant samples were collected from each female and their male dyadic partner using cotton swabs (precleaned with methanol and pentane). Samples were stored in similarly precleaned vials at −80 ◦C, until use.

5.2.5 Hormone assays

Concentrations of A_4, T, and E_2 were determined for each serum sample following the same procedures outlined in Section 3.2.3.

5.2.6 Odor presentation trials.

All three treatment periods were accompanied by behavioral bioassays to measure the response of females to untreated conspecific male and female odorants collected during the NBS. During each bioassay trail, subject females, temporarily isolated from their partner, were presented with three wooden dowels. For each trial, a 'control' dowel was the center dowel and was rubbed with plain cotton. The 'test' dowels, rubbed with conspecific odorant secretions, provided the choice of conspecific male odorant on one side, or conspecific female odorant on the other (presented at 'nose level,' as indicated by the arrows, Figure 15; following modified procedures, Scordato and Drea, 2007). Females were tested in this fashion four times, once during the pre-treatment period, twice during their treatment with letrozole, and once again during the post-treatment period, for a total of two control and two treatment trials. Placement of male and female odorants was alternated in consecutive trials, and subjects receive different sets of male/female odorants in consecutive trials. All trials were separated by at least two-weeks. Trails were observed and videotaped. Trails lasted 10 minutes and responses were scored for approaches, investigation (i.e., sniffing or licking the 'mark'), and scent marking (including competitive over-marking or adjacent marking).

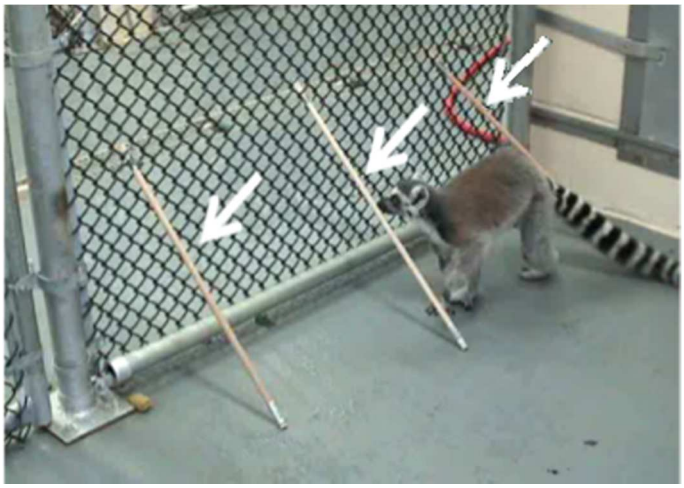

Figure 15. The behavioral bioassay setup. White arrows indicate the locations of control (center dowel) and odorant application (left and right dowels).

5.2.7 Statistical analysis

5.2.7.1 Behavior and hormones

To determine behavioral frequencies for each individual, I tallied the occurrences of each behavior for each subject during every observation period. Using these data I calculated for each behavior, in acts/hour, an average frequency for each individual for each treatment period. These values were then combined to create an average behavioral frequency for each group (female-dominant or egalitarian) for each treatment period. As each individual had only one hormone sample for each period these were averaged across each group to produce a group average for each treatment period that was used in subsequent analysis. In the event that an individual's hormone assay result was below the level of detectability, I used the minimum sensitivity value for that assay in our calculations.

As individual values could be matched across treatment periods I initially used the repeat measure one-way ANOVA to identify differences between treatment periods. Because of concerns about drug clearance affecting results during the post-treatment period, I subsequently re-analyzed just the pre-treatment and treatment periods using two-tailed paired t-tests. The results of the two test were comparable and here I present the statistical results of the pre-treatment and treatment paired t-tests but provide graphical representations of the data from all three treatment periods. To measure the correlation between hormones and behavior across the three treatment period I calculated a Pearson's r for each hormone or hormone ratio and each behavior. All statistics were calculated using Graphpad Prizm v.6.0 (Graphpad Software, LaJolla, Ca).

5.2.7.2 Odor bioassays

I averaged the results of the pre-treatment and post-treatment behavioral bioassay trials and, separately, the two letrozole treatment behavioral bioassay trials to generate a single control, or untreated, data set and a single treated condition data set, respectively. To assess the influence of letrozole treatment on subjects response to male and female conspecific odors, I used the repeat measure two-way ANOVA (letrozole treatment x odor source) to compare the two averaged data sets. I used Fischer's Least Significant Difference to conduct multiple tests for main effects of odor source, as well as to detect differences between responses to a specific odor (female, male, or control), during untreated or treatment periods. I use the un-paired Student's t-test to test for differences

between the responses of female-dominant and egalitarian females to specific odors in both the untreated and treated conditions. All statistics were calculated using Graphpad Prizm v.6.0 (Graphpad Software, LaJolla, Ca).

5.3 Results

5.3.1 Hormone patterns under letrozole treatment

In female-dominant female *Eulemur* and female *L. catta* (results combined), letrozole had no detectable effect on circulating concentrations of E_2 ($p = 0.68$, Figure 16, top row). Nevertheless, letrozole treatment did result in a significant increase in circulating concentrations of A_4 (paired t-test, $t = 3.4$ d.f. $=7$, $p = 0.011$), as well as a near significant increase in circulating T (paired t-test, $t = 2.255$ d.f. $=7$, $p = 0.059$) and a significant decrease in the circulating E_2/A_4 ratio (paired t-test, $t = 5.268$ d.f. $=7$, $p = 0.0012$; Figure 16, top row).

In egalitarian females, letrozole treatment resulted in a modest decrease in circulating E_2 (paired t-test $t = 3.47$, d.f. $= 2$, $p = 0.074$), but had no statistically measurable effect on any of the other hormone concentrations or on the E_2/A_4 ratio (Figure 16, bottom row).

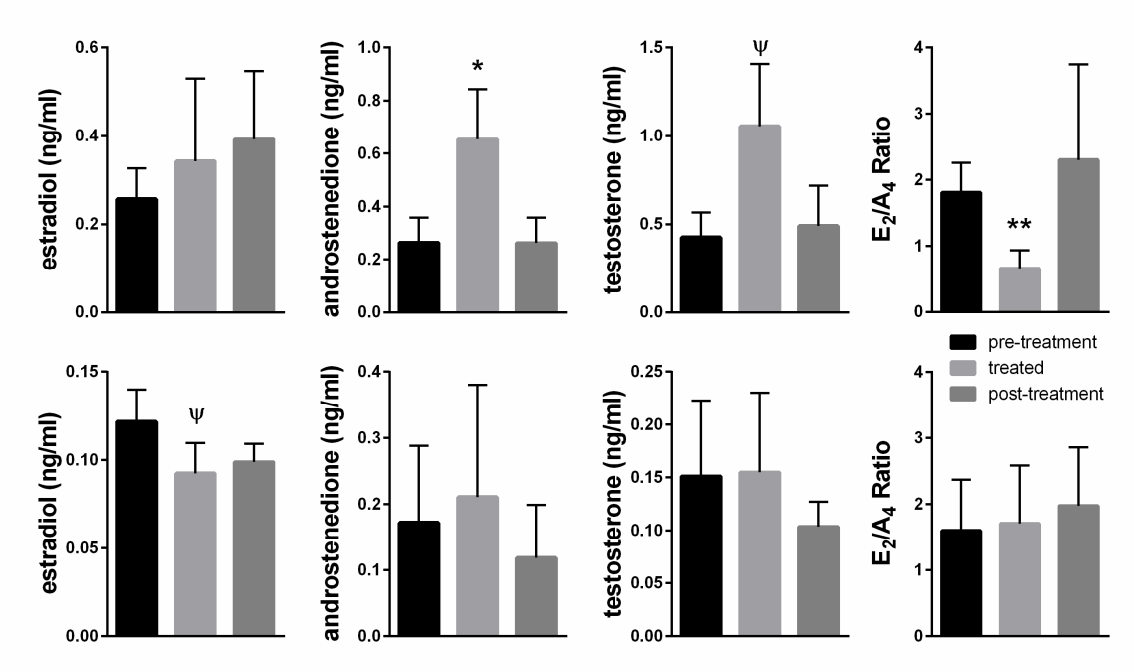

Figure 16. The effects of letrozole treatment on female endocrine parameters in female-dominant *Eulemur* and *L. catta* (top row) versus egalitarian *Eulemur* (bottom row). $^{\Psi}$ P < 0.1; *P < 0.05, **P <0.01.

5.3.2 Behavioral patterns under letrozole treatment

Despite its various effects on endocrine patterns, letrozole treatment had no statistically measurable effect on any of the behavior sampled in either group (Figures 17). Correlations between hormones and behavior were uniformly non-significant, with the strongest correlation being found between T and supplants (Pearson's r = 0.87, p = 0.35).

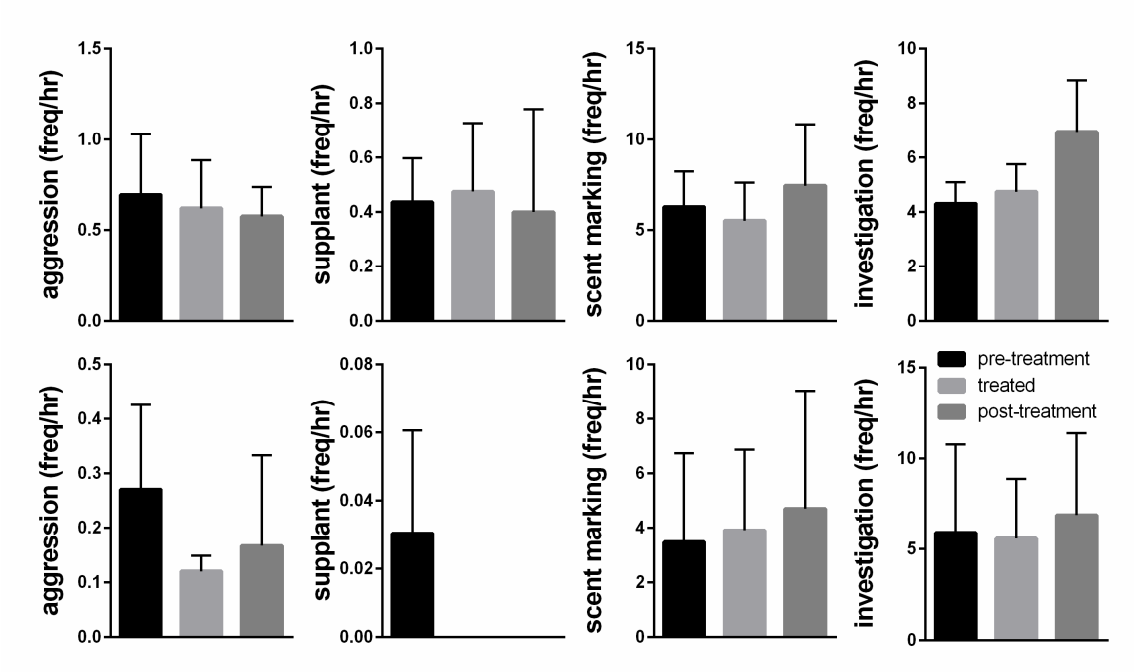

Figure 17. The effects of letrozole treatment on female behavioral parameters in female-dominant *Eulemur* and *L. catta* (top row) versus egalitarian *Eulemur* (bottom row).

5.3.3 Behavioral bioassays

5.3.3.1 Response to scented vs. unscented dowels

Under all conditions female-dominant females preferred poles scented with conspecific odorants, sniffing and licking them more often and for longer than they did blank, control poles rubbed with clean cotton swabs (female odorant vs. control: mean difference = 21.19 sec, $p = 0.0005$; male odorant vs. control: mean difference = 13.22 sec, $p = 0.015$; Figure 18A). Like female-dominant females, egalitarian females also preferred conspecific odorants over blank controls (female odorant vs. control: mean difference = 22.75 sec, $p = 0.007$; male odorant vs. control: mean difference = 20.08 sec, $p = 0.011$; Figure 18B).

5.3.3.2 Response to male vs. female conspecific scent under letrozole treatment

Letrozole treatment of female-dominant females resulted in a significant decrease in the sniffing response to female odorants, as predicted (untreated subject/female odorant vs. treated subject/female odorant: mean difference = 15.39 sec, $p = 0.011$; Figure 18A). Their average duration of investigation of conspecific female scent became equivalent to their average response to conspecific male scent (treated subject/female odorant vs. treated subject/male odorant: mean difference = 0.72 sec, $p = 0.89$; Figure 18A).

Letrozole treatment of egalitarian females resulted in a significant decrease in the average duration of the sniffing response to male odorants (untreated subject/male odorant vs. treated subject/male odorant: mean difference = 14.3, $p = 0.05$; Figure 18B), resulting in a modest, but nonsignificant, preference for female odorants during the treatment period (treated subject/female odorant vs. treated subject/male odorant: mean difference = 8.83, $p = 0.1$). The response to female and control odorants was unaffected by letrozole treatment (Figure 18B).

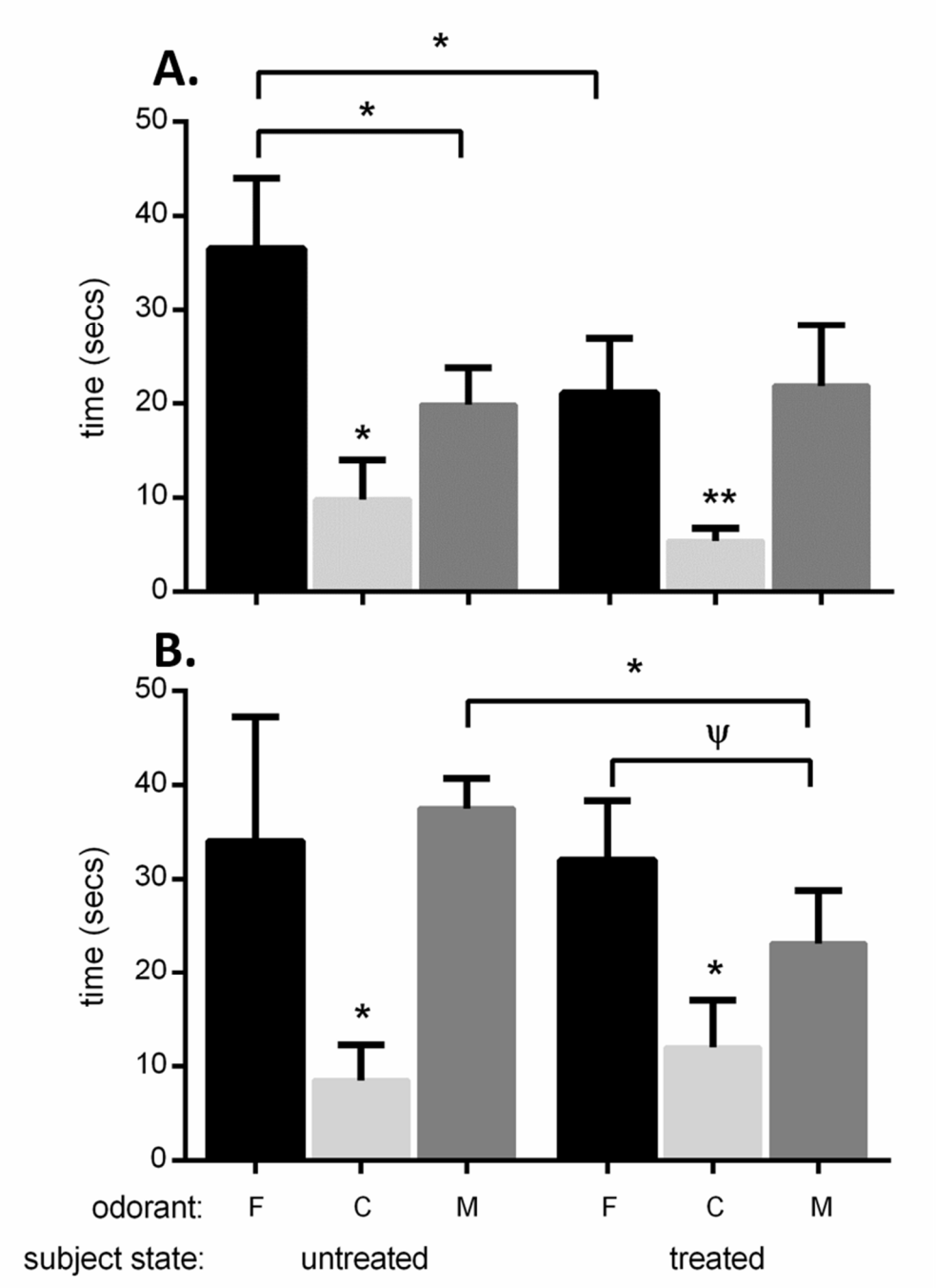

Figure 18. The behavioral bioassay results, presented as time (mean ± S.E.) spent sniffing each odorant (F = female, C = control/blank, M = male), for A.) female-dominant, and B.) egalitarian female lemur, while untreated and during letrozole treatment (treated). $^{\psi}$ P ≤ 0.1, *P ≤ 0.05, and **P < 0.01.

5.4 Discussion

5.4.1 Letrozole treatment altered hormone concentrations differently than expected

To discern the role of estrogens on the expression of behavior in female-dominant and egalitarian female lemur, I treated a cohort of female subjects (including female-dominant *Eulemur,* female-dominant *Lemur catta,* and egalitarian *Eulemur*), with the aromatase inhibitor letrozole. Surprisingly, letrozole treatment in this study appears to have had very little effect on measurable circulating concentrations of E_2, particularly in female-dominant female lemurs. These results are despite an administration of a dose similar to those given in studies of other species, including primates, which result in significant reductions of measurable E_2 within 2-3 days of treatment (Geisler et al., 2002, Pepe et al., 2003, Bhatnagar, 2007). Although I could not detect any change in overall E_2 concentrations in female-dominant lemurs with the current letrozole treatment, I did measure significant differences in circulating androgen concentrations. Letrozole treatment increased circulating A_4, in particular, by approximately 2.7x. Evidence of an effect of letrozole treatment was also found in the significant change in the E_2/A_4 ratio, which decreased by a factor of 2.6x. Despite a lack of change in measures of E_2, these other hormonal data are consistent with an inhibitory effect of letrozole on the conversion of A_4 to E_2. Thus, letrozole treatment seems to have triggered a physiological response in female-dominant female lemurs resulting in a dramatic increase in androgen, primarily in the form of A_4, which effectively overcame the competitive

inhibition of aromatase and maintained circulating E_2 concentrations at pre-treatment concentrations.

In egalitarian female lemurs, letrozole treatment appears to have had some efficacy against E_2, decreasing concentrations of circulating E_2 in egalitarian females by approximately 24%. These results, however, are not as dramatic as those reported in other studies in which letrozole treatment reduced E_2 to concentrations that were often below detection thresholds (Geisler et al., 2002, Pepe et al., 2003, Bhatnagar, 2007). Androgens also appear to have been less affected by letrozole treatment in egalitarian females compared to female-dominant females. As letrozole activity can differ between various species and cell types (Bhatnagar, 2007) the differences in response to letrozole could be indicative of differences in hormone physiology between female-dominant and egalitarian female lemurs.

A major shortcoming in the current endocrine data set, constraining the interpretation of these data, is the single measure of hormone concentrations taken during each treatment period. Circulating hormone concentrations are dynamic, reflecting both regular cyclic homeostatic fluctuations over time periods as short as hours, as well as acute changes in response to environmental, physiological, and social stimuli that may occur within a matter of minutes (Adkins-Regan, 2005). Thus, a single sample represents a 'snapshot' of an individual's hormonal state that may or may not reflect the endocrine state across a larger time period. The current study would have

been better served by a finer scale method of hormonal sampling. For instance, I have no way of determining if letrozole treatment resulted in a transient decrease in E_2 concentrations during the first week or two of treatment, before androgens concentrations rose to a level capable of counteracting its inhibitory effects. Fecal sampling for hormones or hormone metabolites would aid in addressing this issue in future studies.

The question remains as to why letrozole treatment failed to alter E_2 concentrations in female-dominant female lemurs. The answer may be as simple as insufficient dosing, however the dose selected and the oral route of administration have been shown to be effective in other species (Geisler et al., 2002, Bhatnagar, 2007). Furthermore, the observation of other significant hormonal changes in androgen concentrations suggests that letrozole had some effect on hormone biosynthesis. The possibility exists that female-dominant lemur species may have an altered biosynthetic pathway of estrogen synthesis. These alterations could theoretically be in the form of a mutation in the aromatase gene, or its promotor (e.g. Hemsell et al., 1974, Matsumine et al., 1991, Shozu et al., 2003). An analysis of the aromatase enzyme and its promotor genes in female-dominant lemurs would be a logical starting point for testing this hypothesis. Such a study would shed further light into the physiological mechanisms underlying female masculinization in lemurs.

Rates of dyadic behavior also remained relatively unchanged in both groups of female lemurs. Although this result is consistent with the hypothesis of estrogenic mediation of masculinized behavior (i.e. no change in estrogen and no change in behavioral rates), such a definitive interpretation of cause and effect between E_2 and behavior is likely premature. Social dynamics can have a powerful influence over the expression of behavior and the mediation of behavior by hormones (Wallen, 1990, Adkins-Regan, 2005). Experience too, can play an important role in the expression of behavior in the absence of hormonal stimuli. For instance, in many species, sexually experienced males that are then castrated frequently continue to express the full complement of sexual behavior and will mate when presented with a receptive female (Adkins-Regan, 2005). In the case of the current study, the male-female pairs used were all well established with a minimum tenure of at least 2 years together. Several pairs have been together for over 10 years. Thus, patterns of dyadic behavior within each pair are probably somewhat ingrained and therefore may be resistant to transient hormonal changes.

5.4.2 Letrozole treatment alters the response of females to conspecific odorants

Despite the inconclusive data concerning pair-wise behavior and hormone concentrations, letrozole treatment significantly affected the responses of both female-dominant and egalitarian female lemurs to extra-pair conspecific odorants, as seen in the behavioral bioassay trails. As has been previously reported for *L. catta* (Scordato and

Drea, 2007, Drea and Scordato, 2008), untreated females from female-dominant species preferentially investigate female odorants over male odorants. New data generated by this study, however, show that in a similarly untreated, control state, females from egalitarian species respond equally to both male and female odorants, preferring male odorants significantly more than did female-dominant females. These results are consistent with the patterns of chemical complexity of scent secretion reported for *Eulemur* (del Barco-Trillo et al., 2012) and *L. catta* (Scordato and Drea, 2007) males and females. These results are also entirely consistent with the hormonal and behavior data presented in Chapter 4. In Chapter 4, I suggest that the different seasonal changes in behavior and hormones found for female-dominant and egalitarian female *Elemur*, are the result of different levels of intrasexual competition experienced by females in these two groups of species. Intrasexual competition and the coordination and advertisement of reproductive state are two of the most important functions of scent marking (Kappeler, 1993, Mertl-Millhollen, 2006, Drea and Scordato, 2008). In female-dominant lemurs, female scent marking is associated with resource ownership, asserting status, and maintaining intrasexual dominance hierarchies (Drea and Scordato, 2008). Thus, the greater interest in female odorants by female-dominant females in these bioassay data, likely reflect a higher intrinsic level of female competition in species characterized by FSD than that found in egalitarian species. These results may also reflect differences in the importance of male-female relationships within the two social structures (e.g. the

'special relationships' hypothesized to characterize egalitarian *Eulemur,* Pereira and McGlynn, 1997).

Further differentiating female-dominant and egalitarian females, and again pointing to fundamental behavioral and physiological differences between the two groups, letrozole treatment resulted in measurable and opposite changes in female-dominant and egalitarian females as predicted. Both sex and hormonal state can have a measurable effect on the perception of and the response to olfactory signals (Dorries et al., 1997, Lundstrom et al., 2006, Renfro and Hoffmann, 2013, Martinec Nováková et al., 2014). Changes in hormonal state can also affect competitive and affiliative behavior. In the golden hamster (Payne and Swanson, 1971), E_2 ameliorates aggressive territorial behavior in females, and promotes female tolerance of conspecific males. Likewise, in female macaques, rates of proceptive behavior and affiliative interest in males correlates positively with E_2 (Michael and Zumpe, 1993). In the males of many species, however, E_2 seems to activate competitive behavior, particularly territorial aggression during the NBS (Schlinger and Callard, 1989, Soma et al., 2000b). In these males, the aromatase inhibitor fradrozole, decreases the rate and intensity of territorial aggression (Soma et al., 2000a).

The significant decrease in the attention paid by female-dominant females to female odorants during letrozole treatment suggest that, similar to the effects of

aromatase inhibition in male birds, competitive motivation may be under some hormonal control in these females. The lack of change in the response of egalitarian females to female odorants during letrozole treatment, indicates that hormones are likely playing a minimal role in the competitive dynamics between egalitarian females. The decrease in interest to male odorants seen for egalitarian females during letrozole treatment, on the other hand, suggests that hormones are regulating aspects of behavior associated with male-female social dynamics in this species. Additional work on the information content in *Eulemur* scent secretions as well as the transmission of this information, as has been done for *L. catta* (e.g. Scordato and Drea, 2007, Drea and Scordato, 2008), would provide further insight into the different function of scent marking and the social differences, particularly the extent and intensity of female intrasexual competition, between female-dominant and egalitarian species.

6. Conclusions

No behavior is completely understood without knowledge of its underlying physiological mechanisms (Tinbergen, 1963; Adkin-Regan, 2005). In species that express unique or rare behavior, knowledge of these underlying physiological mechanisms can test our understanding of normal processes, spanning the range from molecular biology to evolution, and provide novel insights into our understanding of organisms. In this dissertation I have addressed some of our lack of understanding about the proximate mechanisms underlying female dominance in lemurs. Specifically, I have tested the hypothesis that female masculinization is the primary explanation for the expression of female dominance in lemurs. Based on our current understanding of mammalian sexual differentiation (Goy and Young, 1957, Phoenix et al., 1959, Beach, 1975, Beach et al., 1982), this hypothesis presupposes the involvement of the endocrine system, specifically of the sex-steroid hormones that play a pivotal role in the development and expression of sexually differentiated traits. Thus, I conducted research on the behavior and hormones of several closely related lemur species within the genus *Eulemur*. The variation in the expression of female dominance by *Eulemur* species, including the presence of the only known lemur species reported not to show female dominance (Roeder and Fornasieri, 1995, Kaufman, 1996), allowed me to take a novel comparative approach that has been precluded in similar studies of female dominance in the spotted hyena and the ring-tailed lemur.

Based on previous investigations of species expressing female dominance (Glickman et al., 2006, Drea, 2009), I predicted that female-dominant female lemurs would be both behaviorally and hormonally masculinized relative to females from closely related egalitarian species. I observed and measured the rates of several behaviors that are known to show sex differences in many mammals; these behaviors include aggression (Darwin, 1871, Beach, 1975, French et al., 2013), scent marking (Rozenfeld et al., 1987, Hurst, 1990, Gosling et al., 1996, Allen, 1999, Gosling and Roberts, 2001), and grooming (Kaufman, 1967, Bernstein, 1970, Smuts, 1985, Gould, 1996). As predicted, when compared to egalitarian *Eulemur*, female-dominant *Eulemur* show greater reductions and even reversal of the sex differences that are typically found for these traits in other species. In female-female comparisons, female-dominant female *Eulemur* are more aggressive, scent mark more frequently, and groom their partners less than do egalitarian female *Eulemur*. Hormonally, female-dominant female *Eulemur* are masculinized relative to egalitarian females in that they show smaller sex differences in androgen concentrations between the sexes, as well as significantly greater circulating concentrations of androstenedione and testosterone compared to egalitarian females (Petty and Drea, 2015).

Despite differences in absolute concentrations of sex hormones, if the biosynthetic pathways are functioning similarly within the two groups, we would expect to find similar ratios of the precursor A_4 to its products T and E_2 in both groups. Thus, I

analyzed T/A_4 and E_2/A_4 ratios and compared them between the two groups. These data reveal that, while the ratios of T to A_4 are similar, suggesting similar T metabolism within each group, the E_2 to A_4 ratios differ dramatically between female-dominant and egalitarian females. These results lead me to suggest that there may be fundamental differences in the estrogenic pathway between female-dominant and egalitarian species. These differences could potentially include enzyme structure, function and/or number along the E_2 biosynthetic pathway. If true, such differences could potentially help explain the overall 'muting' of the reproductive endocrine system in egalitarian *E. fulvus* species, and provide a mechanism for explaining both female masculinization in female-dominant lemurs and the relaxation of female masculinization in the relatively young group of *E. fulvus* species. Although the existence of these physiologic differences in hormone biosynthesis remains to be validated, and their functional significance remain to be determined, the correlational data that I generated are consistent with such a hypothesis.

In my next analysis of female dominance in *Eulemur*, I sought to explore the connection between the targeted sex-steroid hormones and the expression of masculine behavior in these species. I did so first by correlating seasonal changes in behavior with concurrent seasonal changes in circulating hormones. Similar to previous results in ring-tailed lemurs (Drea, 2007), I found strong evidence for an activational effect of both T and E_2 on behavior in both female-dominant and egalitarian *Eulemur* females. There

were, however, differences between female-dominant and egalitarian females in the patterns of seasonal changes in behavior and hormones, and in the patterns of seasonal correlations between T and E_2, and their correlations to aggression and scent marking, in particular. During the BS, female-dominant females showed clear positive correlational relationships between T concentrations and both scent marking and aggression, consistent with female masculinization in FSD, a pattern that was absent in egalitarian females.

Because of the costs incurred with elevated T concentrations (Wingfield et al., 2001), the 'challenge hypothesis' (Wingfield et al., 1990) posits that the evolution of positive correlational patterns between T and competitive behavior, such as those found in female-dominant females, should only occur when the benefit outweighs the costs. The present study supports the hypothesis that female masculinization and FSD evolved due to the benefits derived from priority of access to resources in a harsh seasonal environment (Hrdy, 1981, Jolly, 1998, Pereira et al., 1999) and the ability to cope with increased intrasexual competition. This being the case, the benefits of msculinization must outweigh the costs. The relaxation of female masculinization and FSD in egalitarian *Eulemur,* indicate that the competitive pressure on females for resources must be diminished. This seems likely to be due to the 'special relationships' that are reported to exist between males and females in these species (Pereira and McGlynn, 1997).

Lastly, I manipulated the steroid biosynthetic pathway, and hence certain hormone concentrations, in female-dominant and egalitarian female *Eulemur* to provide further evidence of an activational role of hormones on behavior. Female-dominant females showed no change in circulating concentrations of E_2 in response to doses of letrozole that in other species can result in almost complete abolition of measurable E_2 (Geisler et al., 2002, Pepe et al., 2003, Bhatnagar, 2007). Female-dominant females instead seemed to respond to letrozole treatment with a significant increase in circulating A_4. Although an increase in androgens was not unexpected (Kumru et al., 2007, Gallicchio et al., 2011), in other studies this increase in androgens was accompanied by significantly lowered E_2 concentrations (Kumru et al., 2007, Gallicchio et al., 2011). In contrast to female-dominant females, egalitarian females showed a slight decrease in E_2 concentrations, but no apparent increase in androgens. These results are consistent with differences in E_2 metabolism in lemurs in general, and between female-dominant and egalitarian lemurs in specific. Differences in feedback mechanisms or the aromatase enzyme could explain these divergent results.

Evidence of fundamental behavioral and physiological differences between female-dominant and egalitarian lemurs was evident in the behavioral bioassays performed in conjunction with letrozole treatment. These assays tested the response of females, during both control and treatment periods, to conspecific male and female odorants. Consistent with earlier experiments in ring-tailed lemurs (Scordato and Drea,

2007, Drea and Scordato, 2008), female-dominant females attended more to female odors than to male odors, sniffing and licking them more frequently and for longer durations during the tests. In novel data generated by this study, egalitarian females, by contrast, show no preference for odors from either sex, sniffing and licking them at equal frequencies and for equal durations. These results are consistent with both the dominance structure of these species, and with what we know about the chemical complexity of *L. catta* and *Eulemur* scent secretions. In female-dominant species, consistent with their dominant social status, females produce more chemically complex odorants than do males (Scordato et al., 2007, delBarco-Trillo et al., 2012). In egalitarian species, males produce odorants that are more chemically complex than female odorants (delBarco-Trillo et al., 2012). Letrozole treatment resulted in different and opposite changes in the responses of female-dominant and egalitarian females to conspecific odorants. In female-dominant females, this meant a decreased interest in female odorants, and in egalitarian females, this meant a decreased interest in male odorants.

Hormones act to adjust behavior to physical and social circumstances and contexts (Adkins-Regan 2005), usually only actively regulating behavior associated with important social dynamics that may change and require a varied response. Based on the differential changes in behavior between female-dominant and egalitarian female lemurs in the bioassays during letrozole treatment, female intrasexual social dynamics appear to be more important in female-dominant species than in egalitarian species, and

thus appear to be under some hormonal regulation. In egalitarian species, it appears that the important social dynamic, under some hormonal regulation, is between males and females. These patterns are consistent with the expression of FSD, and the reported relationships between males and females (Pereira and McGlynn, 1997), in these species.

It has been suggested that monomorphism between the sexes, coupled with asymmetrical costs of reproduction, provides a simple parsimonious explanation for female dominance without relying on the masculinization of females or extremes in resource limitation or reproductive requirements (Dunham, 2008). This hypothesis is based on the game theory premise that, given equal ability, asymmetry in need determines which 'player' fights harder or gives up sooner in a contest of resource acquisition (Dunham, 2008). Although an interesting idea, I reject this hypothesis as a current proximate mechanism explaining female dominance in lemurs; it ignores a great deal of morphological and, as I have shown here, behavioral and physiological evidence that female-dominant lemurs are masculinized, differing both physiologically and behaviorally relative to egalitarian species. This game-theory hypothesis does, however, provide a starting point for conjecture about the evolutionary origins of female-dominance and female masculinization in lemurs, as well as the relaxation of these traits in egalitarian species. Given the evolutionary situation of asymmetry of need, it is possible that a female 'player', particularly one with an increased asymmetry in need, with a competitive edge, via slightly increased androgens, would have a distinct

advantage in any resource competition and be likely to realize increased fitness as a result. Via the dynamic and complex feedback and control mechanisms inherent to the endocrine system, a mutation of any one of a number of enzymes, including aromatase, or genetic regulatory regions, like transcriptional promotor regions, could theoretically result in increased circulating androgens. This androgenic increase could then have facilitated the evolution of a behaviorally and hormonally masculinized female.

The phylogenetic distribution of female masculinization and female dominance across Strepsirrhines suggests that female masculinization is the ancestral Strepsirrhine (and thus possibly the ancestral primate) condition (Petty and Drea 2015). The only Strepsirrhines known to lack female dominance and female behavioral masculinization are the most recently evolved group of species, the *E. fulvus* species (Petty and Drea 2015), whose behavioral and physiological differences from other lemurs are highlighted in the work presented within this dissertation. Potentially, as has been suggested (vanSchaik and Kappeler, 1996, Pereira and McGlynn, 1997, Pereira et al., 1999), the asymmetry in need for females in *E. fulvus* species has been reduced due to changes in social conditions or dietary behavior. The benefits of increased masculinization in these species would thus also be decreased, no longer outweighing the costs. Such socioecological change could then explain the relaxation of female dominance in egalitarian *Eulemur*, and explain the physiological and behavioral divergence from other lemur species in egalitarian *E. fulvus* species that I presented here.

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Biography

Joseph M.A. Petty was born on April 18, 1970 in San Salvador, El Salvador to Reverend Jess J. Petty and Gillian E. Petty. Joseph grew up in El Salvador, Ohio, Panama, New Jersey, and Ohio again, with his two sisters and his brother. He attended high school at the Mercersburg Academy, graduating in 1988 before spending a year studying at the Tonbridge School in Tonbridge, Kent UK. He received his B.A. in Biology from Oberlin College in 1993. After college Joseph spent a year in the Peace Corp teaching general science and chemistry at Avele College in Avele, Western Samoa. Upon returning to the States in 1995 he then worked as a laboratory technician at the University of Vermont (UVM) in the Department of Biochemistry until 1998. In 1998 he return to school and under the guidance of Dr. David Hirth and Dr. C. William Kilpatrick, studying the population genetics of Vermont's black bear population, he received his M.S. in Wildlife Biology in 2003.

Just prior to completing his Master's degree, he began working as the lab manager and senior laboratory technician for Dr. Benjamin Suratt at the Vermont Lung Center, in UVM's College of Medicine. There he conducted translation research on the biology of neutrophils and ran the day to day operation of the lab including mentoring and managing undergraduate and graduate research students. After 5 years in the Suratt lab, Joseph was accepted to Duke University as a Ph.D. student under the guidance of Dr. Christine Drea. As a Ph.D. student he has earned a number of rewards including a

National Science Foundation Doctoral Dissertation Improvement Grant ($23,553); Margot Marsh Biodiversity Foundation Grants ($10,000); Duke University Trinity Research Enhancement Mentorship Awards ($2,728); Duke Center for Science Education Student Science Education Outreach Grant ($980); and the James B. Duke Fellowship (additional stipend support of $20,000). For the past two years Joseph has been actively employed as a research scientist in the Drug Safety and Research and Development division of Pfizer, in the department of Investigative Toxicology.

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PETTY, J.M. & DREA, C.M. 2015. Female rule in lemurs is ancestral and hormonally mediated. *Scientific Reports*. Volume: 5: 9631. http://dx.doi.org/10.1038/srep09631.

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