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Female Social Rank and Steroid Production in Semi-Free Ranging Lemur Catta on St. Catherines Island, Georgia

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FEMALE SOCIAL RANK AND STEROID PRODUCTION IN SEMI-FREE RANGING LEMUR CATTA ON ST. CATHERINES ISLAND, GEORGIA

by

TIFFANY BURGESS

(Under the Direction of Michelle Cawthorn)

ABSTRACT

Many mammalian species live in complex social groups that profoundly affect the behavior and ecology of their members. In ring-tailed lemurs (*Lemur catta)* these groups are characterized by female dominance, where females have priority over males for food, grooming, and space. Among females there is also a linear dominance hierarchy. *L. catta* have a strong matrilineal social core due to females staying within their natal group and males dispersing at sexual maturity. Daughters “inherit” their mother’s social rank. However, it is not known if the matrilineal lines are maintained through learned dominant/aggressive behavior or inherited via genetically determined hormonal blueprints. I studied the intrasexual relationships among females, and correlated social status with the production of three steroid products: estradiol, progesterone, and testosterone. Biological samples (n=192) were taken in summer 2012 from a captive population (n=93) of semi free-ranging *L. catta* on St. Catherines Island, GA. Samples (n=15) were analyzed for steroids using enzyme-linked immunosorbent assays. Social status was determined via behavioral observations (50 hours). Due to the known negative relationship between cortisol and other steroids, its concentration was also analyzed to investigate a possible effect on agonistic behaviors. No significant differences in steroid concentrations or morphology were found between female ranks during the non-breeding season. Future studies should focus on determining the possible sex differences in androgen-receptor abundance and sensitivity in this and other female-dominant species.

INDEX WORDS: Ring-tailed lemur, *Lemur catta*, female dominance, steroids, androgens, morphology

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by

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B.S., Armstrong State University, 2010

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INTRODUCTION

Sociality has developed across the animal kingdom, ranging from eusocial invertebrate insects to high order primates, including multiple social hierarchies (i.e. home and work stratification) in humans. Animals form groups when there are more benefits, such as protection and generational learning, compared to a solitary life (Krause and Ruxton 2002). Group living can also increase the likelihood of winning territorial disputes with conspecifics or competing species. However, there are also consequences to social living in the form of competition for resources (i.e. food and mating opportunities) and increased exposure to parasites and disease (Kutsukake 2008). Social structure varies between species, especially among non-human primates that include: solitary orangutans, monogamous gibbons, polyandrous tamarins, polygynous gorillas, and multi-male/multi-female macaques (O'Neil 2012).

Social groups have evolved in many mammalian species for several independent reasons such as protection, limited resources, and reproductive availability. Individuals with differing preferences for resources and the use of aggression to attain these scarce reserves led to the formation of social rank, where dominant individuals fight for access to resources and subordinates settle for the leftovers at a lower energy cost (Weissing, 2011). In many cases, ranks form between sexes due to sexual selection of desired traits, as well as within sexes for access to mates. The dominant sex or individuals have primary access to food, mating, shelter, and grooming, as well as decision-making responsibilities for the group, such as dispersal initiation and direction (Young et al., 1990; Sauther et al., 1999). In some species, rank is inherited from one or both parents while in other species rank is earned through age, experience, and aggression (Cant et al., 2006). In mammals, the most common transmission of rank is through vertical inheritance from the previous generation, but inherited rank must be maintained.

Female Dominance

In mammals, males are usually the dominant sex and are larger and showier than females in sexually dimorphic species. Large, showy males then compete for access to or to be chosen by females (Andersson, 1994; Short and Balaban, 1994; Darwin, 1871). A role reversal between sexes is less expected due to high reproductive costs for the female. Therefore, female dominance in mammalian societies is much less common and is only consistently found and well documented in three social groups: bonobos, *Pan paniscus* (Parish, A.R., 1994); Malagasy lemurs, including *Lemur catta* (Sussman, 1991; Jolly, 1966); and spotted hyenas, *Crocuta crocuta* (Drea et al., 1998; Glickman et al., 1992, 1987; Frank et al., 1990). Bonobos are extremely non-aggressive, solving disputes through physical contact and sex between group members. Maternal social rank is important for priority of access to food and bonding within the female natal group, while males disperse after sexual maturity. Due to the lack of female enforcement, bonobos have been considered "female-centered" but sexually co-dominant, where all females are dominant to and rank above all middle and low-ranked males (de Waal, 1995; Smuts and Smuts 1993). Spotted hyenas lie at the other end of the female dominance spectrum. Sex roles in this species are completely reversed. Females dominate both males and other females with elevated female acts of aggression, reproductive dominance between females, and masculinized anatomical features (Goymann et al., 2001; Drea et al., 1998; Licht et al., 1998, 1992). The "hyperaggressiveness" and masculinization of females is suggested to have evolved in species like the spotted hyena, because of competition with males for access to dangerous feeding opportunities (Goymann et al., 2001; Glickman et al., 1987; Frank, 1986).

There are three main hypotheses to explain the evolution of female dominance: 1. *the Energy Conservation Hypothesis*, 2. a *male behavioral strategy*, and 3. a *female behavioral* *strategy* (Young et al., 1990). The Energy Conservation Hypothesis (Hrdy, 1981) argues that males in female-dominated societies “give up” their dominance and aggression to the females in order the save energy for male-to-male competition during the breeding season. On the other hand, Jolly (1984) argues that males might have more to gain year-round by being dominant. The male behavioral strategy hypothesis (Pollock, 1979) suggests that male subordination is a form of parental investment, where the male accepts a lower feeding priority in order for the females and offspring to have access to more resources. This hypothesis predicts a high level of paternal investment and depends on the certainty of paternity in semi-promiscuous species such as *L. catta*. This theory is best supported in monogamous species, such as the lemur *Indri indri* (Young et al., 1990). The female behavioral strategy hypothesis (Jolly, 1984; Richard and Nicoll, 1987) implies that females fight and win against the males for resources because they have more at risk reproductively because resources are limited. This hypothesis is supported by the fact that in female-dominated societies, such as *L. catta*, the females do receive primary access to food in low-resource territories (Jolly, 1984).

In addition to differences in dominance between males and females, many species have dominance hierarchies within either one or both genders (e.g., chickens; Croney et al, 2007). Social structure and dominance hierarchies in wolves (*Canis lupus)* have been extensively studied (Creel, 2005; Sands and Creel, 2004; Frank 1987). Within a wolf pack, there is a single dominant male (alpha). Other males are subordinate to the alpha, but also maintain a social stratification. Additionally, social stratification occurs among the females. This status stratification is important for resource allocation and mating priority among pack members. Alpha male and females are responsible for territory protection and hunting decisions, as well as the majority of the pack’s reproductive success. Beta individuals, usually a male position, are considered the alpha male's 'enforcer' and are responsible for intra- and inter-pack conflict resolution, maintaining order and cohesion in the pack. Omegas, usually the most submissive individuals within a wolf pack, relieve tension in the pack by taking the bulk of the intra-pack aggression from both alpha and beta males (Frank 1987). Ring-tailed lemurs have a similar social structure, except that females are dominant to males and all females within the troop are able reproduce. Lemur female betas, mid-rankers in this study, play a similar role and act as 'enforcers' for their troop's dominant female. Because of this, mid-rank and low-rank females experience the majority of the troop's intra-conflict, some-what relieving the dominant female from the stress of altercation.

At this point, there have been no single discoveries of a proximate mechanism to explain female dominance. Ultimate explanations (as described above) for this role reversal are attributed to agonistic behaviors in the form of aggression, but what is the physical cause of heightened frequencies and intensities of aggressive behaviors? A more proximate view of female dominance may look into the role of aggression-associated hormones for clarification.

Androgens and Cortisol

Masculinized traits and genitalia in female spotted hyenas led researchers (e.g., Von Engelhardt, 2000 and Drea et al., 1998) to look into the possibility that androgens- masculinizing sex hormones (testosterone, androstenedione, etc.) usually more abundant in males but still present in females- might be elevated in this species and responsible for this role reversal. In both sexes of mammals, androgens are largely produced in the gonads, although the adrenal glands account for minor androgen production. In fact, androgen exposure has been found to be developmentally important in pregnant female adults as well as at the fetal stage in spotted hyenas (Drea, 2011; Drea et al., 1998; Licht et al., 1998, 1992). Specifically, elevated androstenedione (A_4), a precursor to testosterone in the Δ^4-biosynthetic pathway in the ovaries, has been directly associated with female dominance in the spotted hyena (Drea, 2007). A_4, being molecularly similar to testosterone, has the same physical and behavioral effects as testosterone in a mammalian body. Although the other members of the Δ^4-biosynthetic pathway have known roles in sexual development and behaviors, it is unclear how they might additively affect mammalian behavior. In the Δ^4-biosynthetic pathway (Figure 1), steroids are usually converted unidirectionally starting with progesterone, which in the presence of specific enzymes is converted into A_4 that can then be quickly transformed into testosterone and then dihydrotestosterone or estrogens. All of these chemicals are associated with aggressive behaviors. In female mammals, A_4 is converted into testosterone and then estradiol (E_2) (Norris, 2007). With elevated levels of testosterone precursors and enzymes, a female mammal would be hormonally equipped for agonistic encounters. The purpose of my study was to investigate the relationship between steroid hormones and female dominance in the ring-tailed lemur, *Lemur catta.*

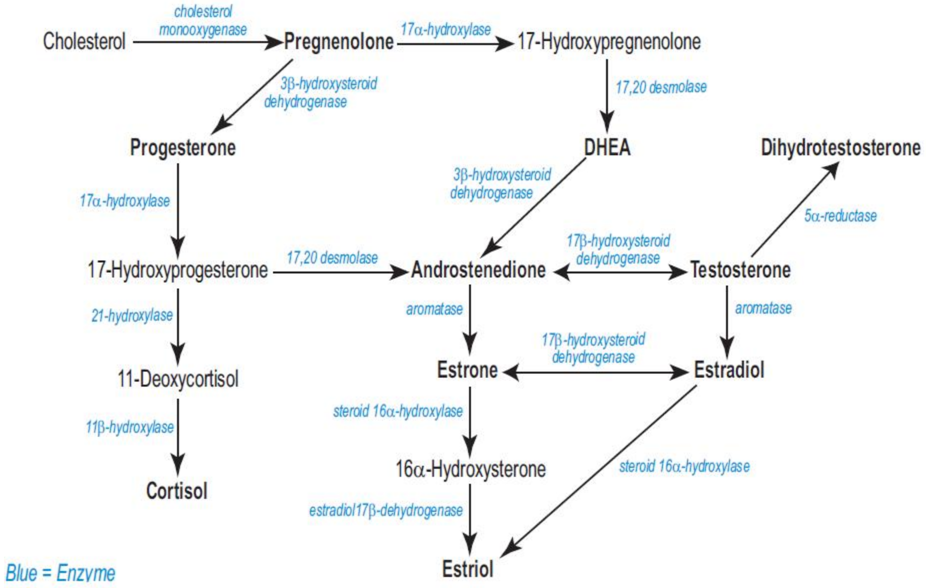


Figure 1 Delta-4-biosynthetic pathway of steroids (Bernstein, 2015)

In male-dominated societies, subordinate animals often experience higher levels of stress than dominant animals, due to constant attacks by more dominant individuals and lower fitness associated with low resource priority. Measuring levels of cortisol, the “stress hormone” is a common technique used to assess stress in individuals (Gesquire et al., 2011; Mehta et al., 2008; Pride 2005; Cavigelli, 1999). In these societies, lower rank correlates with higher levels of cortisol due to encounters of aggression by more dominant individuals and decreased health with limited access to food, while higher ranked individuals experience lower levels of the stress hormone (Gesquiere, 2011). The opposite trend is said to be true for female-dominated societies. High-ranked females hold the burden of high levels of stress due to increased intra- and intersexual agonistic encounters and elevated cortisol concentration year-round (Cavigelli, 1999). Cortisol is known to have negative effects on the concentration of circulating androgens in a mammalian body (Mehta et al., 2008). For this reason, the relationship between female dominance and androgens is complicated by cortisol, and needs to be accounted for in hormonal studies of dominance.

Prenatal exposure to elevated levels of androgens has been documented in hyenas and has proven to be a crucial step in the masculinizing development of females (Drea, 2009; Drea et al., 1998). There are many ways to test for prenatal androgen exposure, most of which involve invasively taking biological samples of both mother and fetus during gestation. An alternative less invasive technique is the use of a non-invasive digit measurement. This technique has been successfully tested in humans and is currently being tested in many other primate species to confirm the exposure of an individual to elevated concentrations of androgens while in the womb (Roney et al., 2004; McFadden and Bracht, 2003). By measuring the second and forth digits of the right forelimb, researchers can develop a feasible androgen estimate for analysis. The presence of a low second to forth (2:4) digit ratio indicates high fetal androgen levels during gestation and early development (Lutchmaya et al., 2004; Roney et al., 2004).

Study Species

Ring-tailed lemurs (*L. catta*) are a species of Malagasy lemur native to the island of Madagascar off the southeast coast of Africa. Due to habitat destruction and degradation all 70+ taxa of lemur on the island are at risk. Because *L. catta* is adaptable and reproduces successfully in captivity, it is ranked as “nearly threatened” on the IUCN red list, even though the numbers of wild lemurs are small. Although lemurs have several attributes such as scent marking and seasonal breeding that have been well-studied, female dominance has not been extensively investigated (Sauther et al., 1999; Young et al., 1990; Jolly, 1966).

Like spotted hyenas, female *L. catta* are moderately masculinized (Drea and Weil, 2008), exhibiting “masculinized” genitalia (Drea and Weil, 2008) and more aggression than males (Kappeler, P.M., 1990a). However, there is no sexual size dimorphism (Kappeler, P.M., 1990b). Only a handful of researchers have attempted to study proximate causes of female dominance in *L. catta* (Drea, 2007; Von Engelhardt et al., 2000), and they have reached different conclusions. Von Engelhardt et al. (2000) specifically focused on testosterone levels and concluded that there was no correlation with female dominance. Drea (2007), however, included A_4 in her study and found elevated concentrations in females compared to male lemurs. Furthermore, levels were higher than many other female mammals, and were comparable to the low range of A_4 found in female spotted hyenas. Drea concluded that A_4 could be responsible for female dominance in *L. catta* but suggested further future study.

In a more recent study, Petty and Drea (2015) compared lemurs from two clades: female-dominant species (dom-E) and egalitarian or co-dominant species (co-E). They found that dom-E females exhibit more dominant and aggressive behaviors towards males than did co-E females. This study also explored steroid concentrations between the two groups, concluding that dom-E females had significantly higher concentrations of A_4 and testosterone, along with a trend of elevated E_2, compared to the females of co-E species. The authors hypothesize that this behavioral difference between dom-E and co-E *Eulemurs* with relaxed androgen-mediated behavior is relatively recent in origin and suggests that female social dominance and masculinization is an ancestral lemur condition. Prenatal androgens may play a role on the masculinization of female *L. catta*, another similarity to spotted hyenas (Drea 2011). Petty and Drea (2015) hypothesized that female-dominant species of lemurs, such as *L. catta*, may exhibit sex differences in the sensitivity or abundance of androgen-receptors located in the brain, accounting for the lower concentrations of circulating androgens found in females. Drea's (2007) findings provide evidence for the importance of androgens in female dominance, but as these are the only studies examining this relationship, the role of androgens in female dominance remains unclear in *L. catta*.

A population of *L. catta* is maintained on St. Catherine's Island, Georgia (SCI). Lemurs are allowed to move and forage on the island without the restriction of fences, although their diet is supplemented and they are confined during extremely cold weather (Keith-Lucas, et al., 1999; Savage 2005). On SCI, formation and maintenance of troop structure closely resembles that of *L. catta* in the wild, and behaviors are species typical (Keith-Lucas, et al., 1999). Previous captive *L. catta* studies in behavioral endocrinology have been limited to smaller populations that were restricted in large netted enclosures. Thus, the animals on SCI provide an opportunity to further explore the relationship between female dominance and androgens with a large sample size of animals that live in a state similar to their wild environment.

In the northern hemisphere lemurs breeds during the fall months, give birth beginning in March, and are reproductively inactive during the summer months. Female aggression peaks during the breeding season but occurs year-round. Increasing and decreasing levels of steroid concentrations correlate with the breeding season, but are present during all life stages. The relationship between steroids and dominance during lactation remains unstudied, but Drea (2007) suggested that this period might be important in the maintenance of female dominance in the non-breeding season.

Previous studies have focused on the comparison of androgen concentrations between *L. catta* sexes to provide support for the role of androgens in female dominance, with less than conclusive results. This could be for several reasons, including the method of analysis, the androgens tested, or unknown gaps in the information. To limit previous confounding factors and simplify this study, I focused my study on the adult females of the SCI population. Further, this study focused on hormone concentrations during the non-breeding season to eliminate the confounding effect of breeding hormones.

The primary objective of this study was to quantify the relationship between female social status and three major steroid products of the Δ^4-biosynthetic pathway: estradiol, progesterone, and testosterone, in *L. catta* during the non-breeding season. My prediction was that higher-ranked females would possess higher concentrations of these steroids compared to other females. A second objective was to analyze the cortisol concentrations of the samples to determine the relative stress levels of each individual and relate that to rank, along with taking into account cortisol’s negative effects on androgen concentrations (Mehta et al., 2008;

Cavigelli, 1999). I predict that low-ranking, subordinate females would experience more stress and have higher cortisol concentrations. A third objective was to compare fecal and blood sample steroid concentrations to further understand the relationship between circulating and excreted steroid levels. I expected that excreted hormone concentrations would be substantially higher than circulating values and that cortisol would increase in blood plasma samples, due to the stress of capture. A fourth objective was to test the relationship between morphological differences in female scent gland and external clitoral measurements with social rank and with androgen levels (Drea and Weil, 2008; Scordato, 2007). I predicted that more dominant females would have more masculinized glands and genitalia. The final objective was to test for the evidence of possible prenatal exposure to androgens using a non-invasive manual 2:4 digit ratio measurement of the right forelimb to distinguish any possible variation within social hierarchy (Lutchmaya et al., 2004). This is the first study to examine the 2:4 digit ratio in *L. catta*, I predicted that higher-ranked females would possess a lower, more masculine digit ratio compared to other females.

METHODS

Study Animals

The subjects of this study were 93 captive, semi-free ranging *L. catta* found on St. Catherines Island (SCI), Georgia (31° 42” 38’, -81° 8” 2’). The original population of lemurs was donated from Duke University in 1985, as part of a reintroduction program for Madagascar. The population was later expanded with lemur donations from the Bronx Zoo and maintained through natural breeding. At present, SCI is a research facility that provides an environment where the lemurs can behave and reproduce in a natural setting, similar to their native habitat. At the time of this study, the population included 28 adult females, 23 adult males, and 42 juveniles and infants. These lemurs were split up into six troops with between 5-23 individuals per troop. All adults wore color-coded radio collars for individual identification and radio-tracking, and all lemurs were reproductively intact and exhibited species-typical behaviors (Keith-Lucas et al., 1999). Although my study focused on the adult females, all individuals were included during behavior observations and morphological measurements taken during annual exams, with males and juveniles serving as comparison groups.

Behavioral Assessment

SCI staff provided troop lists with updated social rank for both females and males. These were used to identify individuals and their ranks (SCI primatologist, pers. comm.). In the summer of 2012, I confirmed and recorded the social structure of each troop using traditional instantaneous and focal methods, including ‘all-occurrences’ sampling (Altmann, 1974). Females were observed for specific behaviors such as movement initiation, scent marking, grooming, feeding priority, and dominance-associated aggression. Frequencies of these dominant behaviors were used to confirm female status for each troop. In addition to observation, family structure records were used to place dominant females into a social hierarchy. Due to the varying sizes of troops, each female received a social rank between one and three: (1) low rank or most subordinate, (2) medium rank, and (3) high rank or the dominant female.

Biological Sample Collection and Storage

Fecal samples were collected from all free-ranging individuals of five troops in summer 2012. Each troop was visited for behavioral observation and fecal collection prior to its scheduled physical examination, along with additional random visits for additional fecal sample collection throughout the non-breeding season. Each adult female (identified by the colored radio collar) provided at least four, and up to nine, non-stressed fecal samples that were labeled and held on ice during transportation to Georgia Southern. Samples (n=330) were stored at Georgia Southern at -20 C until assayed and quantified in the lab.

SCI veterinarian, Dr. Terry Norton, collected blood samples during SCI summer annual exams. These samples were provided as a circulating steroid comparison against the excreted volumes found in fecal matter. Each adult provided one 5 mL heparinized vial of whole blood that was centrifuged down for plasma (1 mL) separation, before being stored on ice for transportation. Samples were stored in a Georgia Southern laboratory at -20 C until assayed and quantified in the lab. Fecal concentration means were exclusively used for the steroid-rank analysis due to the possible cortisol interference in plasma samples, caused by the stress of capture for the examination process, along with only having one plasma sample collected from each adult animal.

Fecal Steroid Extraction & SPE Cleanup

Multiple fecal samples, ranging from four to nine, were taken from each female during the non-breeding sampling season. From those samples, two were selected for each ranked adult female: one from early and the other from late in the collection period, at least a month apart, to test for seasonal change between late-lactation and pre-breeding periods. Fecal samples were dried for twenty-four hours and sifted through 1 mm mesh, resulting in homogenized fecal powder. Liquid-liquid extraction was performed on 0.10g of the dried feces, using 5 mL of 50/50 ethanol and water solution. Each mixture was vortexed, centrifuged, the supernatant decanted, and dried with nitrogen in a warm water bath at 34°C. Solid phase extraction (SPE) was then performed by first reconstituting samples in 1 mL of 70/30 water and methanol solution. Samples were vortexed and then sonicated for 30 minutes in a warm water bath. SPE columns (Phenomenex Strata-X 33u Ploymeric Reversed Phase, 200 mg/6 mL) were primed with methanol and ultra-pure water prior to running samples. Samples were loaded into SPE columns and allowed to briefly air-dry, before flushing the column with 2 mL of methanol and centrifuging at 1500 rpm for one minute. The collected elute was dried again using nitrogen gas. Dry samples were sealed with Parafilm and stored at 4° C until reconstitution for the steroid assay (Zeigler and Purett, pers. comm.).

Plasma Steroid Extraction

Plasma samples were allowed to thaw before performing liquid-liquid extraction on 10-20 µL using 1 mL of diethyl ether. Samples were vortexed for 30 seconds and plasma was isolated using dry ice. Ether supernatant was decanted into new tubes and allowed to air-dry over night in fume hood at room temperature before reconstitution for the steroid assay.

Enzyme-linked Immunosorbent Assays

Four Δ^4-biosynthetic pathway analytes (estradiol, progesterone, testosterone, and cortisol) were tested using two fecal samples, early and late non-breeding season, from each ranked female (n=15). Each steroid, except estradiol, was also measured from blood plasma samples.

The EIA kits were done based on a modified version of the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI) and all samples were run in duplicate. Fecal estradiol was measured using enzyme immunoassay (Cat. No. 582251). The antibody used in the estradiol EIA shows a cross reactivity to estradiol-3-sulfate of 14.5%, estradiol-3-glucuronide of 14%, and estrone of 12%, (estradiol 100%). The intra-assay coefficient of variation (%CV) was 9.19 and inter-assay %CV was 18.10. The antibody used in the progesterone EIA (Cat. No. 582601) shows a cross reactivity to 17β-estradiol of 7.2%, pregnenolone of 2.5%, and testosterone of <0.05% (progesterone 100%). Intra-assay %CV was 25.40 and inter-assay %CV was 16.67. The antibody used in the testosterone EIA (Cat. No. 582701) shows a cross reactivity to 19-nortestosterone of 140%, 5α-dihydrotestosterone of 27.4%, and 5β-dihydrotestosterone of 18.9% (testosterone100%). Intra-assay %CV was 6.92 and inter-assay %CV was 8.15. The antibody used in the cortisol EIA (Cat. No. 500360) shows a cross reactivity to prednisolone of 4% and cortexolone of 1.6% (cortisol 100%). Intra-assay %CV was 7.88 and inter-assay %CV was 10.82. Varying dilution ratios (volume of plasma: volume of EIA buffer) were used for different samples, depending on sample type (either fecal or blood plasma) and volume of available samples, so that the percentage of bound analyte fell within the most sensitive portion of the standard curves (20-80% B/B_0). Female plasma was pooled using 200 μL from several random adult females and used to confirm parallelism of the species' against the standard curve for each steroid kit, ensuring accurate quantification (Figure 2).

Androgen-estrogen (A/E) ratios were also calculated using each female's fecal testosterone and estradiol concentrations to test for relative, rather than absolute steroid values that may be moderately responsible for female masculinization in this species of lemurs (French et al, 2013).

Morphological Measurements

Standard morphological measurements of mass and body length, along with scent gland and female genitalia dimensions were taken at the time of routine annual examinations coupled with digital photographs of some individuals. Manual straight-line caliper measurements of height and width were taken of each animal's antebrachial glands, and each male's spur and brachial glands (Scordato, 2007). External clitoral measures were taken of the organ length and width of the ventral side using straight-line caliper measurements (Drea and Weil, 2008). Each animal was assigned a body condition score (BCS) between one and five (one being severely underweight and five extremely obese) by the SCI veterinarian based on the presence or absence of body fat, muscle mass, and/or bone exposure. A body-mass index (BMI) was calculated for each lemur using its' total body length (cm) and mass measurements (kg). All length and width measurements were converted into total area dimensions for statistical analysis.

Digit Ratio Measurement

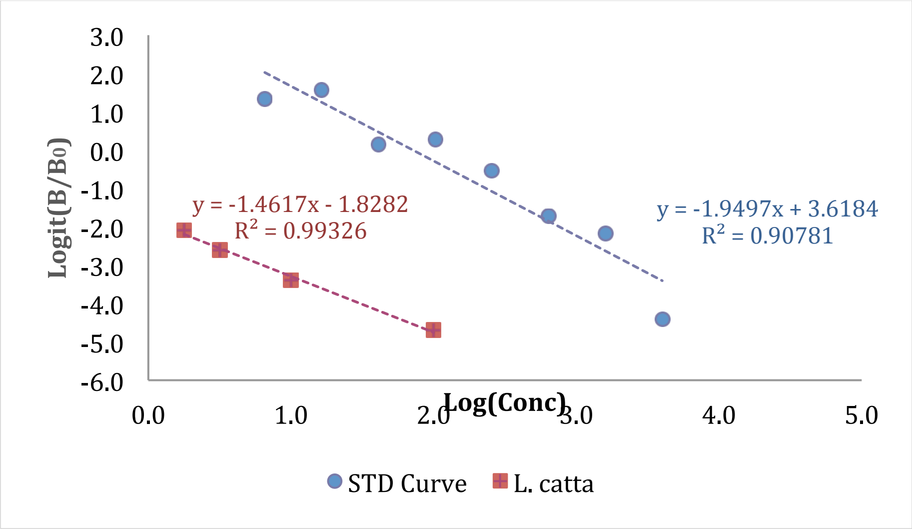
Digit ratios were determined by manual straight-line caliper measurements of the second and fourth digits of the right forelimb of each animal during SCI routine lemur annual examinations conducted under general anesthesia by the St. Catherines veterinary staff. This provided a basic estimate of the individual's prenatal exposure and possible sensitivity to androgens. This ratio was considered along with the individual's collected steroid levels during the analysis of all data.

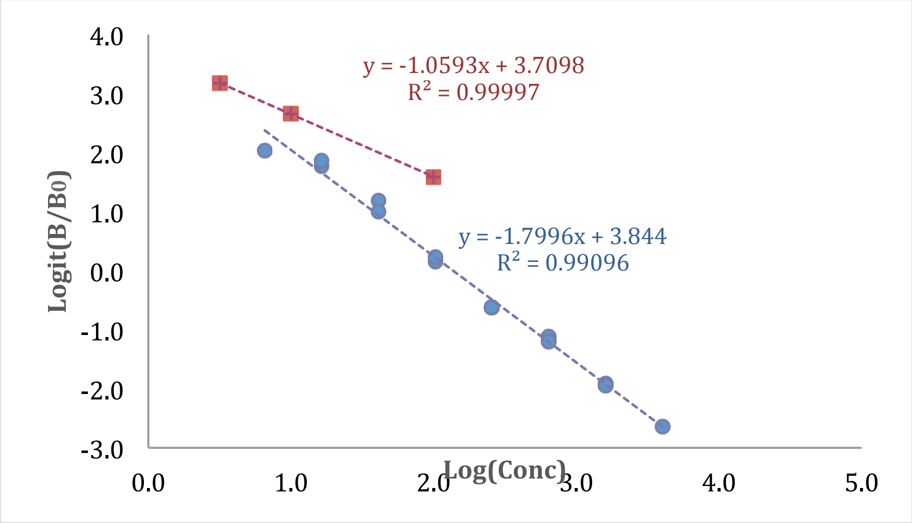
Statistical Analyses

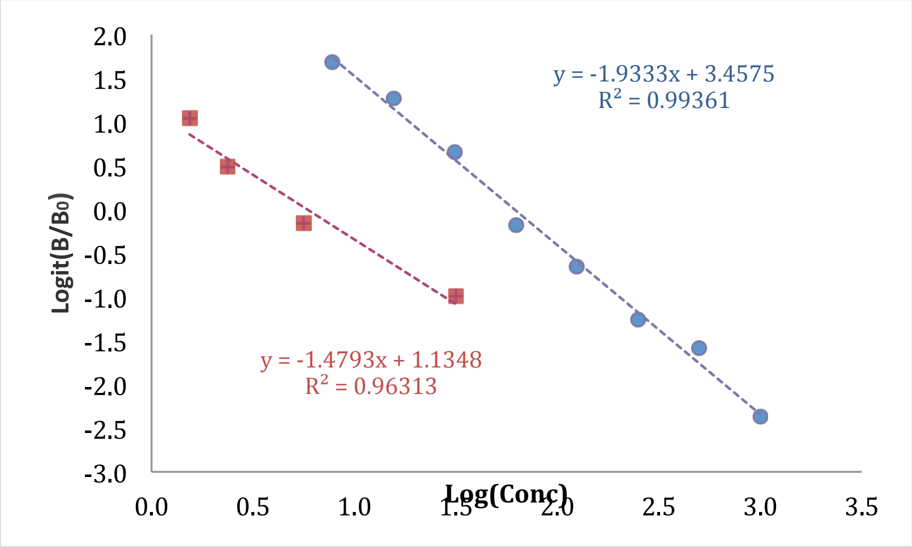
Statistical analyses were performed using JMP Pro 10 with statistical significance set as $\alpha= 0.05$. Mean steroid concentrations were averaged for each ranked female and morphological measurements were compared back to each female's social status. Statistical analysis for fecal steroid concentrations, morphological measurements, and female social rank were performed on all data using one-way ANOVAs.

Table 1 Behaviors observed to determine female *Lemur catta* social rank.

Behavior	**Description**
Grooming	Removing parasites from fur (allo-, mutual, or self-groom)
Feeding/Foraging	Eating at food station or searching in tree canopy
Scent Marking	Using wrist or genital scent glands to mark trees and other objects
Stink Fighting	Using wrist glands to scent tail and waves towards competitor (passive form of male-male competition)
Approach	One animal moves towards another
Displace/retreat	When approached by another, moves to another location
Chase	When approached by another, moves away quickly
Fight (play)	Interacting without aggression
Fight (aggression)	Interacting with aggression
Vigilance	Looking around and alert
Vocalize	Communication through calling to others (warning, locating, moving)


3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
-4.0
-5.0
-6.0
Logit(B/B0)
y = -1.4617x - 1.8282
R² = 0.99326
y = -1.9497x + 3.6184
R² = 0.90781
0.0
1.0
2.0
3.0
4.0
5.0
Log(Conc)
● STD Curve
■ L. catta


4.0
3.0
2.0
1.0
0.0
-1.0
-2.0
-3.0
Logit(B/B0)
y = -1.0593x + 3.7098
R² = 0.99997
y = -1.7996x + 3.844
R² = 0.99096
0.0
1.0
2.0
3.0
4.0
5.0
Log(Conc)


2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
-3.0
Logit(B/Bo)
y = -1.9333x + 3.4575
R² = 0.99361
y = -1.4793x + 1.1348
R² = 0.96313
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
Log(Conc)

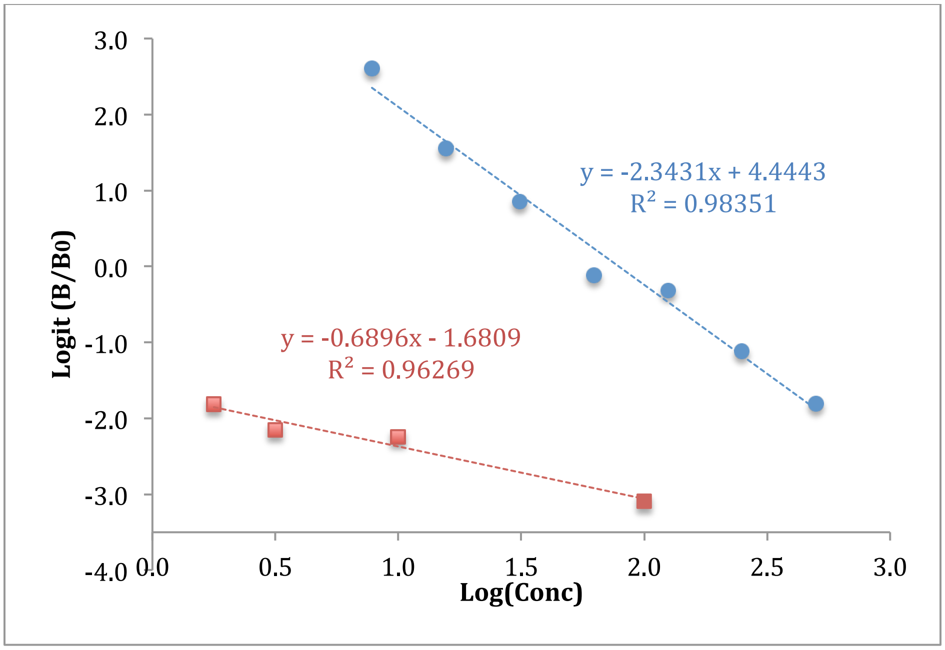


Figure 2 *Lemur catta* plotted for parallelism by EIA standard curve for each steroid (A-D). A: cortisol, B: estradiol, C: progesterone, D: testosterone

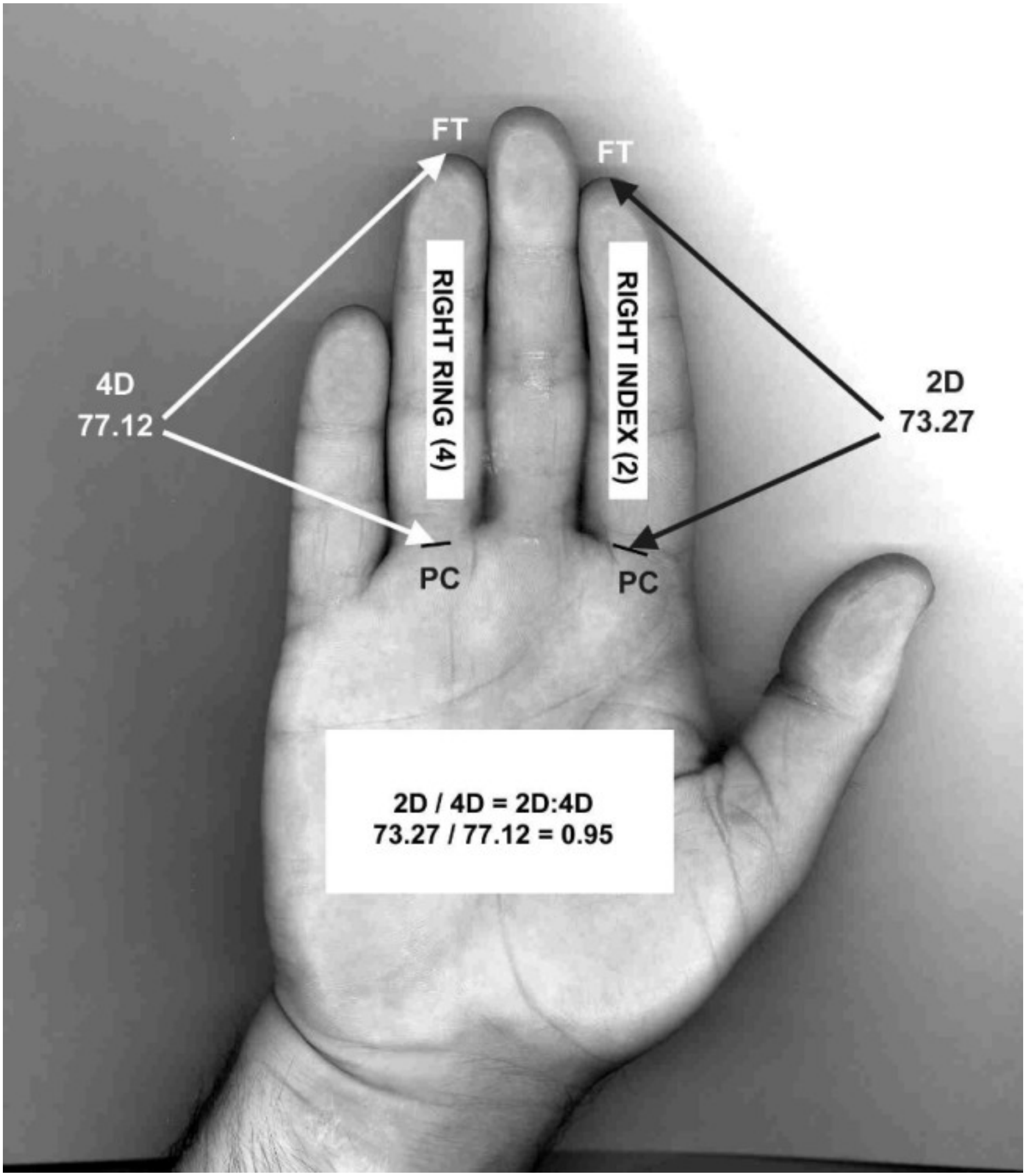


Figure 3 Digit ratio measurement. 2015. New York City. *Fingering cheaters science ties finger length to promiscuity*. Massapeal.com

RESULTS

Steroids

Multiple fecal samples, ranging from four to nine, were taken from each female during the non-breeding sampling season. From those samples, two were selected for each ranked adult female: one from early and the other from late in the collection period, at least a month apart, to test for seasonal change between late-lactation and pre-breeding periods. No significant differences were discovered between early and late season, therefore the data was averaged for each female before further analysis.

Low ranking females showed trends in having higher levels of estradiol ($F_{1,14} = 1.97$, $p = 0.18$) and testosterone compared to high and mid-rank females. Low and mid-rank females both had slightly elevated, but not significant, progesterone concentrations compared to high-ranking females (Figure 4).

No significant differences were found between female ranks for fecal cortisol concentrations.

Table 2 shows the relationship between circulating blood plasma and excreted fecal sample concentrations of each tested steroid, excluding plasma estradiol due to extremely low concentrations that were under detectable limits. Excreted fecal values are far more concentrated than the circulating equivalent; for example plasma progesterone is 20x lower than fecal progesterone (Table 2). Plasma testosterone is 7x lower than excreted testosterone. However, due to the stress of capture, plasma cortisol is considerably elevated compared to 'natural' or 'non-stressed' fecal values and varied between high and low ranks ($F_{1,9} = 3.46$, $p = 0.099$). This concentration reversal for cortisol may also relate back to an individual's 'stress frequency'. Plasma estradiol concentrations were extremely low and under the EIA kit's sensitivity.

Androgen-estrogen (A/E) ratios were also calculated (testosterone/estradiol) but were not found to be significant between ranked females, although high-ranked females possessed higher, more masculinized ratios.

Morphological Measurements

Statistical analysis for morphological measurements was performed on all data using one-way ANOVAs. Significant relationships within all ranked females (n=15) included only age and rank (Table 3: $F_{1,14} = 8.9136$, p= 0.0042). No significant differences were found between ranks in female scent gland or external clitoral measurements.

Although 2:4 digit ratios showed a slight negative trend for high-ranking females (Tables 3 and 5), as a species *L. catta* possess a low, more masculinized, 2:4 ratio compared to most documented primate digit ratios.

During the SCI 2012 annual physical examinations, it was discovered that five of the 28 adult females in the population possessed at least one wrist spur, which is considered to be only a male trait in this species. These females were not immediately related and varied in age, with the youngest being a two-year old subadult.

Table 2 *Lemur catta* mean (+/- SE) fecal and blood plasma steroid concentrations by female social rank.

Rank	Biological Sample	Progesterone	Testosterone	Cortisol	Estradiol
High (n=5)	Fecal (ng/g)	132.9 (+/- 48.7)	21.1 (+/- 2.77)	7.31 (+/- 1.19)	33.44 (+/- 5.41)
	Plasma (ng/mL)	5.43 (+/- 1.07)	2.6 (+/- 0.46)	33.56 (+/- 7.08)	Under detectable limits
Mid (n=5)	Fecal (ng/g)	184.3 (+/- 18.7)	21.31 (+/- 1.71)	8.62 (+/- 2.19)	35.93 (+/- 2.46)
	Plasma (ng/mL)	7.68 (+/- 1.25)	2.85 (+/- 0.29)	36.08 (+/- 7.27)	Under detectable limits
Low (n=5)	Fecal (ng/g)	183.2 (+/- 25.5)	26.17 (+/- 4.42)	7.49 (+/- 1.68)	65.53 (+/- 21.2)
	Plasma (ng/mL)	10.48 (+/- 2.15)	3.87 (+/- 0.50)	50.5 (+/- 5.01)	Under detectable limits

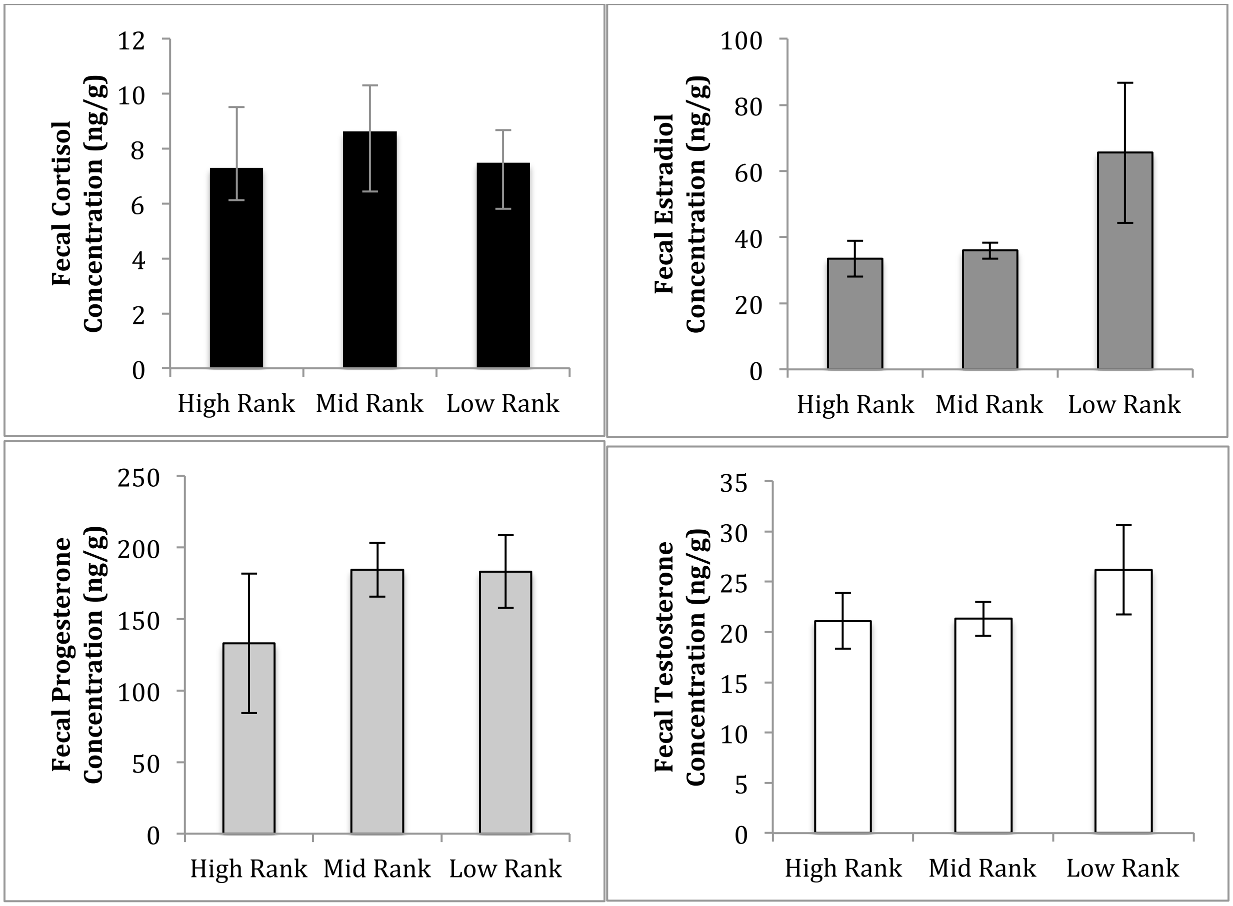


Figure 4 *Lemur catta* mean (+/- SE) fecal steroid concentrations (A-D) by female social rank. A: fecal cortisol, B: fecal estradiol, C: fecal progesterone, D: fecal testosterone (n=15).

Table 3 *Lemur catta* mean (+/- SE) morphological values by female social rank.

	Rank			
Variable	**Low (n=5)**	**Medium (n=5)**	**High (n=5)**	**p-value**
Age	5.4 (+/- 1.47)	5.4 (+/- 0.93)	13.4 (+/- 2.04)	**0.0042****
Mass (kg)	2.49 (+/- 0.15)	2.68 (+/- 0.11)	2.76 (+/- 0.18)	0.4488
Body length (cm)	61.8 (+/- 0.9)	61.1 (+/- 0.64)	59.8 (+/- 1.2)	0.3464
BMI (kg/cm^3)	0.00065 (+/- 3.6×10^{-5})	0.00072 (+/- 3.1×10^{-5})	0.00077 (+/- 5.1×10^{-5})	0.1472
BCS	2.24 (+/- 0.12)	2.48 (+/- 0.09)	2.62 (+/- 0.25)	0.2933
Digit ratio	0.755 (+/- 0.02)	0.800 (+/- 0.02)	0.802 (+/- 0.02)	0.1992
Gland area	45.9 (+/- 7.83)	46.7 (+/- 6.10)	44.8 (+/- 8.92)	0.9846
Clitoral area (cm^2)	329.36 (+/- 59.8)	361.34 (+/- 72.1)	395.84 (+/- 49.3)	0.7492

Table 4 *Lemur catta* clitoral values and social rank position for each adult female (n=15).

Individual	Age	Rank	Clitoral Length (mm)	Clitoral Width (mm)	Clitoral Area (mm^2)
Cassidy	2	2	34.1	11.2	381.92
Frida	3	1	37.5	5.9	221.25
Polli	3	1	21	10	210
Delilah	5	1	28	15	420
Magdalene	5	2	35.2	9	316.8
Ezra	5	1	34.5	8.1	279.45
Jayde	6	2	41.3	6.1	251.93
Melia	7	2	37	17	629
Autumn	7	2	31.1	7.3	227.03
Holly	9	3	21	13	273
Sue Rae	10	3	43	7.2	309.6
Tyann	11	1	50.6	10.2	516.12
Nikki	12	3	41	10	410
Jen	16	3	46	12	552
Newport	20	3	53	8.2	434.6
Mean	8.07		36.95	10.01	362.18
STD Error	1.304		2.428	0.827	33.47

Table 5 *Lemur catta* morphological values for each adult female grouped by social rank (n=15).

Rank	Individual	Age	Mass (kg)	Body length (cm)	BMI (kg/cm^3)	BCS	Digit ratio	Gland area	Clitoral area (cm^2)
High	Holly	9	3.06	58	0.00091	3	0.87	42.8	273
	Sue Rae	10	2.4	56	0.00077	1.8	0.75	52.8	309.6
	Newport	20	2.5	62	0.00065	2.3	0.8	29.4	434.6
	Jen	16	3.3	62	0.00086	3	0.79	74.4	552
	Nikki	12	2.5	61	0.00068	3	0.81	24.5	410
Mean		13.4	2.76	59.8	0.000772	2.62	0.802	44.78	395.84
STD Error		2.04	0.183	1.20	5.12×10^{-5}	0.246	0.019	8.92	49.30
Mid	Melia	7	2.9	60	0.00081	2.5	0.84	39.2	629
	Jayde	6	2.6	62	0.00067	2.3	0.78	60	251.9
	Cassidy	2	2.4	59.5	0.00067	2.3	0.79	35.3	381.9
	Autumn	7	2.9	61	0.00078	2.8	0.76	63	227
	Magdalene	5	2.7	63	0.00068	2.5	0.83	36	316.8
Mean		5.4	2.7	61.1	0.000722	2.48	0.799	46.7	361.32
STD Error		0.927	0.106	0.640	3.10×10^{-5}	0.092	0.016	6.10	72.13
Low	Delilah	5	3	61.5	0.00079	2.5	0.71	72	420
	Frida	3	2.1	59	0.0006	1.8	0.77	34.9	221.3
	Ezra	5	2.4	63.5	0.0006	2.3	0.8	40.2	279.5
	Polli	3	2.3	61	0.00063	2.3	0.8	28	210
	Tyann	11	2.6	64	0.00063	2.3	0.7	54.3	516.1
Mean		5.4	2.49	61.8	0.00065	2.24	0.756	45.87	329.37
STD Error		1.47	0.151	0.903	3.63×10^{-5}	0.117	0.022	7.83	59.80

Figure 5 Female *L. catta* with wrist spur. Photo taken by T. Burgess.

DISCUSSION

Steroids

This project adds to the current understanding of social hierarchy within a female dominated society by determining the relationship between androgenic hormones and female rank in *L. catta* during the non-breeding season. This non-reproductive period is less studied due to lower levels of aggression, but can provide a reproductively unbiased view into female dominance. This study includes a larger sample size of individuals in a more natural environment than has been previously studied. This study will hopefully further the understanding of a chemical proximate mechanism of dominance in the species, as well as determine the possible effects of androgens on a primate's social behaviors.

The primary objective of this study was to test for the relationship between female social status and three major steroid products of the Δ^4-biosynthetic pathway: estradiol, progesterone, and testosterone, in *L. catta* during the non-breeding season. My prediction that higher-ranked females would possess higher concentrations of these steroids compared to other females was not completely supported. These data support a negative trend between fecal testosterone concentrations and female status, confirming Engelhardt's (2000) and Drea's (2007) findings that female dominance is not the direct result of elevated female testosterone. However, female plasma testosterone concentrations for this study appear elevated (mean: 3.11 ng/mL, range: 1.24-5.08 ng/mL) in comparison to Drea's findings (2007) where female *L. catta* averaged 0.2 ng/mL with a range of 0.1-0.7 ng/mL. This difference may be due to the difference in the captive environment between the two studies. In Drea's (2007) study, captive lemurs are housed in large netted enclosures, whereas in my study captive lemurs are largely free ranging, possibly allowing for more 'wild' behaviors. A recently published article (Petty and Drea, 2015) comparing a female social dominant (FSD or dom-E) species of lemur and a more egalitarian species (co-E) concluded that dom-E females have higher levels of androgens, including testosterone and A_4, than the female egalitarian species, supporting the hypothesis that some lemur species are more masculinized than others and that male-female androgen concentration ratios vary between species and are closer between sexes in dom-E species, such as *L. catta*. The authors also suggested that females may have a higher sensitivity to circulating androgens resulting in highly aggressive behaviors (Petty and Drea, 2015).

My results support the hypothesis that lower ranked females are subjected to aggressive attacks by higher ranked individuals, usually the medium-ranked or "enforcer" females, and show a trend of elevated testosterone and estradiol as a result of these aggressive encounters, although the data was not found to be significant. Similar to low-ranking lemurs, subordinate female African wild dogs (*Lycaon pictus*) have higher estrogen levels, along with higher estrogen/progestin ratios, in the non-mating period resulting in the prevention of ovulation. During the mating season, however, subordinate females exhibit lower concentrations of estradiol and mate less often than dominant females (Creel et al., 1997). Mid-ranked female *L. catta*, along with low-rankers, also showed a trend in elevated fecal progesterone further reinforcing the low-medium rank steroid-inducing aggressive cycle (Figure 4). Each group uses their steroid reservoir, either progesterone or testosterone, to quickly convert metabolites into circulating androgens and estrogens, resulting in more aggressive behaviors for both medium and low ranking females during the non-breeding season and possibly year-round. These results are similar to hyenas, where high-ranking females possess higher levels of circulating androstenedione than lower-ranking females and even males (Drea 1998; Glickman et al. 1987). It is also possible that as in hyenas, there is a facilitative effect or positive feedback loop, where by engaging in more agonistic behaviors, individuals can indirectly elevate future androgen concentrations (French, 2013).

A recent study on male chacma baboons (*Papio hamadryas ursinus*) found positive correlations between testosterone concentrations and changes in rank, where males rising in rank had higher levels of testosterone than males falling in rank, but results varied depending on the group's hierarchy stability (Beehner et al., 2006). The authors also concluded that testosterone could be used as a predictor for a male's future rank. Another study on savannah baboons (*Papio cynocephalus*) concluded that high-ranking males had elevated testosterone levels compared to other males regardless of the group's stability (Gesquiere et al., 2011). However, a study that manipulated androgen (testosterone, dihydrotestosterone, and estradiol) concentrations in male white-throated sparrows concluded that the conversion of both androgenic and estrogenic metabolites, and not testosterone alone, are required for the hormonal control and maintenance of aggression and dominance in that avian species (Archawaranon and Wiley, 1988). This suggests a larger interconnected relationship between Δ^4-biosynthetic pathway products, their conversion, and their eventual impact on aggressive behavior.

My second objective was to analyze the cortisol concentrations of the samples to determine the stress of each individual and its rank. It was predicted that low-ranking, subordinate females would experience more stress and have higher cortisol concentrations. No significant differences were found between ranks for fecal cortisol measures, although trends can be seen in plasma samples indicating that all animals become stressed (at least a four-fold increase) during the examination process. However, dominant females have lower levels of cortisol and thus appear to be less stressed than males and lower ranked females (Table 2). This trend could suggest a stress threshold difference between ranks and sexes that might trace back to the frequency of that individual's agonistic encounters.

Previous studies focusing on cortisol levels and stress in wild *L. catta* show that high-ranking females have higher glucocorticoid (cortisol and its metabolites) levels than subordinate females, due to frequency of agonistic encounters and aggressive attacks experiences by individuals (Cavigelli et al., 2003; Cavigelli 1999). However, the present study does not support this hypothesis and instead suggests that populations with few hierarchical turnovers, possibly due to access to plentiful resources, have little variation in fecal cortisol concentrations across ranks during the non-reproductive season. One study in wild lemurs found that high fecal glucocorticoid concentrations could serve as a mortality predictor, although the exact causes of death for individuals in this study are unknown (Pride 2005). Fecal cortisol levels in this study did not exceed these predicted parameters (> 41 ng/g), although plasma cortisol concentrations were within this 'stress landscape' range but are not considered a long-term threat due to long durations between stressful examination captures.

Although reproductive suppression is considered to be caused by high levels of stress in subordinate individuals, a study on African wild dogs concluded that both dominant females and males held higher basal corticosterone levels than lower-ranking individuals, suggesting that other steroids, possibly progestin, could be responsible (Creel et al., 1997). On SCI, dominant female lemurs go into estrus first at the beginning of the breeding season and have priority in mate choice, usually mating with the dominant resident male. After the dominant female mates, lower-ranked females ease into estrus and breed until successful fertilization or up to three cycles. This suppression could account for the slightly elevated levels of progesterone found in both mid and low-ranked females during the period prior to the breeding season and the lack of variation found in fecal cortisol between ranks.

A study in wild savannah baboons found that high-ranking males had lower glucocorticoid levels compared to low-ranking males, with the exception of the alpha (highest-ranking) male having significantly higher stress hormone than the beta male, possibly relating back to aggressive activity differences (Gesquiere et al., 2011). These studies as a whole suggest that stress and cortisol levels are relative to agonistic encounter frequency, rather than the direct result of an animal's social position, accounting for the variation across mammalian species.

A study observing wild graylag geese (*Anser anser*) determined that stress and social dominance differs between species, rather it be high- or low-ranking individuals that exhibit more stress, and that social stress is more associated with male-male competition frequency, access to females, and parental investment, depending on the season (Kotrschal et al., 1998). A study conducted on data gathered on free-ranging animals (species ranging from birds to primates) concluded that the relative allostatic load, or physiological consequences of chronic stress to the body, predicts a dominants or subordinants expression of glucocorticoid levels, either high or low, in both sexes independent of species (Goymann and Wingfield, 2004). Although stress is widely documented across several species with varying social systems, the data still seems inconsistent and exact causes and relationships remain unclear.

My third objective was to compare fecal and blood sample steroid concentrations to further understand the relationship between circulating and excreted steroid levels. I expected that excreted hormone concentrations would be substantially higher than circulating values and that cortisol would increase in blood plasma samples, due to the stress of capture. Fecal concentration means were exclusively used for the steroid-rank analysis, therefore, blood plasma data was isolated and used for comparison only. Fecal samples were collected to provide a large sample size from free-ranging individuals that were also non-invasive and resulted in minimal stress to the animals. Excreted fecal values are far more concentrated than the circulating equivalent, with the exception of a distinct increase in plasma cortisol, indicating a large spike of stress hormone during the examination process, which is an expected and temporary response (Balcombe et al, 2004). Future studies should focus on liquid-chromatography mass spectrometry techniques that have greater detectability of low concentrations, along with examining other estrogens, androstenedione, and DHT to get a better understanding of the full impact of the Δ^4-biosynthetic pathway. Wild lemur fecal samples should also be collected to further compare steroid profiles against captive populations to determine the predictive accuracy that captive model animals provide.

Morphological Measurements

A forth objective was to test the relationship between morphological differences in female scent gland and external clitoral measurements with social rank and androgen levels (Drea and Weil, 2008; Scordato, 2007). I predicted that more dominant females would have more masculinized glands and genitalia. Most morphological relationships found in this study can be logically explained and accounted for, especially age and clitoral area to rank. In this population of *L. catta*, matrilineal relationships are fairly stable and troops are each composed of one primary family tree, with the few unrelated females given lower ranks. For this reason, eldest females hold top social positions with little status turnover and few fission events, probably accounting for the significant association between age and rank. Due to plentiful resources and a relative lack of predators, breeding success in this population is high; each female typically produces offspring each year, and multiple births (twins and triplets) are common. Infant mortality is also relatively low, usually less than 10 percent annually, depending on the year. Measurements of clitoral size in a previous study of *L. catta* were the same between younger and older females; in fact, there appeared to be a trend that clitoral size slightly decreased with age (Drea and Weil, 2008). The clitoral measurements for this study are larger in both length and width, than previously published values but were not found to be different between ranks (Table 4). This difference in recorded measurements could relate back to the population's increased fertility success and multiple births, or possibly the season in which the data was collected, in the non-reproductive season roughly three months after the last birthing season.

The unexpected morphological discovery that five out of the 28 adult females measured during the 2012 annual examinations possessed at least one wrist spur is only the second documented occurrence of females possessing wrist spurs (Figure 5). Sauther & Cuozzo (2006) documented one wild female with the right spur present and a captive mother/daughter pair with both spurs present that were observed engaging in stink fighting, usually a male-only behavior observed during the breeding season as a passive form of male-male competition. The authors used this as an example of virilization, the development of a male secondary characteristic exhibited by a female. It is also important to note that these three females were not, in a physical way, negatively impacted by this masculinized trait and observed to be reproductively successful. One of the SCI females has also been observed attempting to stink fight, suggesting that these females have functional use of their spurs and are using them similarly as males. Further observations of these females should be done to determine if all spur-possessing females use their spurs for scent marking and stink fighting, along with the frequency of these behaviors to compare to their male counterparts.

The final objective was to test for the evidence of possible prenatal exposure to androgens using a non-invasive manual 2:4 digit ratio measurement of the right forelimb to distinguish any possible variation within social hierarchy (Lutchmaya et al., 2004). This is the first study to examine the 2:4 digit ratio in *L. catta*, I predicted that higher-ranked females would possess a lower, more masculine digit ratio compared to other females. Digit ratios, although not found to be significant in this study, did provide valid information not previously discussed in this context. As a species, *L. catta* possess a low 2:4 digit ratio that does not significantly vary within females or even between sexes, although males seem to have slightly lower ratios. Digit ratios have been found to co-vary with social systems across nonhuman primate species. Nelson and Shultz (2010) propose that both male and female 2:4 digit ratios decrease with increasing levels of intrasexual competition. High-ranking female rhesus macaques possess low 2:4 digit ratios, similar to *L. catta* as a species, suggesting that prenatal androgen effects may assist in the maintenance of social rank in several species of nonhuman primates, but vary between species (Nelson et al., 2010). This could support the hypothesis of prenatal exposure to androgens in both sexes, or even the feminization of male offspring during late gestation as proposed in previous studies (Ostner et al, 2003). It is also possible that relative rather than proximate steroid concentrations may play a role in early female masculinization and that the androgen-estrogen ratio should also be considered (Ostner et al, 2003; French, 2013). Ostner et al. (2003) found that female-carrying mother *L. catta* possess high, more masculinized, A/E ratios compared to male-carrying mothers, suggesting that absolute values of circulating steroids are not the complete picture and possible physiological differences, such as androgen-receptor abundance or distribution could account for observed differences among females or between sexes.

Although many of the morphological traits measured did not prove to be significant for this study, factors such as age and body condition still seem to be important in establishing and maintaining social rank in female *L. catta*. The steroid data supports trends that females with higher frequency of agonistic behaviors, both mid and low rank positions, could possibly have a steroid reservoir (either progesterone or testosterone) that can be quickly converted into circulating androgens and estrogens that then result in more aggressive behaviors. High rank females might not have the highest steroid concentrations in the troop to account for their status, but they also do not have the need to participate in the agonistic congregations, because the 'beta' (in this case mid-rank) female enforces the alpha's position. This may also account for the high-rank females' lower stress and plasma cortisol levels. Future studies should focus on determining the possible sex differences in androgen-receptor abundance and sensitivity in female dominant species such as the spotted hyena and *L. catta* to further understand the role of androgens and other Δ^4-biosynthetic pathway products in female dominance. The hypothesized prenatal feminization of male offspring during late gestation should also be considered and further examined.

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