

Integrating the Female Masculinization and Challenge Hypotheses: Female dominance, male deference, and seasonal hormone fluctuations in adult blue-eyed black lemurs (*Eulemur flavifrons*)

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Abstract

In the decades since female social dominance was first described in strepsirrhine primates, researchers have sought to uncover the proximate and ultimate explanations for its development. In the females of various female-dominant species, androgens have been implicated as regulators of behavior and/or predictors of seasonal fluctuations in aggression (the ‘Female Masculinization Hypothesis’). Males, more generally, respond to changing social demands via seasonal fluctuations in androgen-mediated behavior (the ‘Challenge Hypothesis’), that may also entail changes in activation of the hypothalamic-pituitary-adrenal axis. Here, we explore if androgens, glucocorticoids, and intersexual behavior fluctuate seasonally in the female-dominant, blue-eyed black lemur (*Eulemur flavifrons*), with potential consequences for understanding female aggression and male deference. Across two studies conducted during the breeding and nonbreeding seasons, we assessed rates of mixed-sex, dyadic social behavior (aggression and affiliation) and concentrations of fecal glucocorticoid metabolites (Study 1) and serum sex hormones (androstenedione, testosterone, and estradiol; Study 2). Our results align with several predictions inspired by the Female Masculinization and Challenge Hypotheses for intersexual relations: During the breeding season, specifically, both aggression and androstenedione peaked in females, while female-initiated affiliation decreased, potentially to facilitate female resource access and reproductive control. By comparison, all target hormones (androgens, estrogen, and glucocorticoids) peaked in males, with glucocorticoid concentrations potentially increasing in response to the surge in female aggression, and unusually high estrogen concentrations year-round potentially facilitating male deference via male-initiated affiliation. These results suggest complex, seasonally and hormonally mediated behavior in *Eulemur flavifrons*.

Keywords: female dominance ; Masculinization; Challenge Hypothesis; seasonal breeding; primates

Introduction

Female social dominance (the consistent ability of females to win intersexual disputes) is unusual in mammals (Ralls, 1976), yet it characterizes the Malagasy or lemuriform primates (Richard, 1987; Lewis, 2018). Historically, scholars have debated whether this social system is best attributed to male deference (e.g. Jolly, 1984) or female aggressiveness (e.g. Kappeler, 1990; Digby & Mclean Stevens, 2007); consilient perspectives (e.g. White et al., 2007) suggest that a combination of these complementary mechanisms operate across the sexes to generate lemur social structure. A comparison of sexually codominant versus female-dominant *Eulemur* species suggests a long history of androgen-mediated female aggression in lemurs, with androgenic ‘relaxation’ (i.e., reduced production in females) occurring only recently in codominant species (Petty & Drea, 2015). Here, we approach the topic of female social dominance and male deference from an integrative, mechanistic perspective that considers female masculinization (sensu Phoenix et al., 1959) in conjunction with changing seasonal demands in both sexes.

The Female Masculinization Hypothesis (Glickman et al., 1993; Drea, 2007; French et al., 2013; Drea et al., 2021), often applied to aseasonal species, posits that aggressively mediated social dominance in females is part of a suite of traits that are linked to androgen action and specifically associated with accruing benefits (including year-round resource access) via enhanced competition. Relatedly, the Challenge Hypothesis (Wingfield et al., 1990), originally developed for seasonally breeding species, posits that androgen concentrations fluctuate to meet social demands, augmenting to facilitate reproductive competition and becoming quiescent to facilitate parenting. A synthesis of these frameworks may underscore the value of examining integrated hormonal action. We thus investigate the possibility of multiple, seasonal relationships between intersexual social interactions and steroid hormones in a seasonally breeding, aggressively female-dominant strepsirrhine primate: the blue-eyed black lemur (*Eulemur flavifrons*).

Females of sex role-reversed and female-dominant species challenge typical assumptions regarding sexual selection, sexual dimorphism, and reproductive competition in vertebrates (Berglund & Rosenqvist, 2003; Stockley & Bro-Jørgenson, 2011; Clutton-Brock & Huchard, 2013). Female competition over access to mates or environmental resources implicate various endocrine mechanisms available to both sexes (Staub & DeBeer, 1997; Eens & Pinxten, 2000; Lipshutz & Rosvall, 2020; Drea & Grebe, in press). Framed within the Female Masculinization Hypothesis, enhanced female aggression (which facilitates achieving social dominance) is part of a suite of anatomical and behavioral traits typically observed or more strongly expressed in males, that become prominent in females via androgen exposure during critical periods of development. Androgens operate at both organizational and activational levels across generations (e.g., spotted hyenas (*Crocutta crocuta*): Glickman et al., 1987; Drea et al., 1998; Dloniak et al., 2006; Conley et al., 2020; meerkats (*Suricata suricatta*): Clutton-Brock et al., 2006; Davies et al., 2016; Drea et al., 2021; ring-tailed lemurs (*Lemur catta*): Drea, 2007; Grebe et al., 2019b; reviewed in French et al., 2013). Key reproductive hormones in the sexual differentiation of these species include androstenedione (A₄, the androgenic precursor to androgens and estrogens), testosterone (T), and estradiol (E₂), although patterns of adult sex differences in these steroids are highly variable across species (reviewed in Drea & Grebe, in press).

Support for the Female Masculinization Hypothesis in lemurids comes from a suite of atypical features, observed either within females or between the sexes. Behaviorally, these features include enhanced female aggression and intrasexual competition (e.g., Digby, 1999; Kappeler & Fichtel, 2012) that may require associated changes or accommodations in males (White et al., 2007), female rough-and-tumble play (Grebe et al., 2019b), female scent marking (Mertl-Millhollen, 2006; Drea, 2015), and male mate choice (Parga, 2006). Anatomically, they include clitoral elongation, with partial or complete traversal by the urethra (Drea & Weil, 2008), body size monomorphism or

reverse size dimorphism (Kappeler, 1990), and elaboration of female scent glands (delBarco-Trillo et al., 2012). Physiologically, they include the females' increased exposure to androgens *in utero* (Drea, 2011) and in adulthood (Petty & Drea, 2015), absence of bimaturation (Leigh & Terranova, 1998), and elaboration of female scent signals, with honest chemical ornamentation (Boulet et al., 2010).

Deemed the most endangered mammals (IUCN 2020), lemurs pose constraints on studies of organizational effects; nevertheless, there is much to learn from activational hormones and their mediation of adult behavior. For instance, the maintenance of female dominance over males in lemurs, expressed both via female physical aggression and male deference (e.g. Pereira & Kappeler, 1997; White et al., 2007), has been linked to seasonal activation of androgens (Drea, 2007) and glucocorticoids (Cavigelli et al., 2003; Starling et al., 2010). Potential seasonal differences in androgen action are also relevant to the Challenge Hypothesis, originally formulated for seasonally breeding male birds (Wingfield et al., 1990), but more recently expanded to other vertebrates, to aseasonal species, and to females (Hirschenhauser & Oliveira, 2006; Grebe et al., 2019c; Rosvall et al., 2020). This updated conceptualization of the Challenge Hypothesis posits that T secretion varies according to social environment or social stability more generally, such that increased T has activational effects associated both with increased aggression or mating competition (e.g., during the mating season) and with diminished parental investment (e.g., during the birthing season) (Gleason et al., 2009; Gerlach & Ketterson, 2013; Rosvall, 2013; Gettler, 2011). These effects may be moderated by glucocorticoid action, in either positive or negative directions: e.g., stress-induced activation of the hypothalamic-pituitary-adrenal (HPA) axis might interfere with the expression of mating behavior, but conversely, HPA activation can have metabolic effects that instead potentiate such behavior (Sapolsky et al., 2000; Goymann & Wingfield, 2004; Grebe et al., 2019a).

Blue-eyed black lemurs are a fitting species in which to synthesize these two hypotheses to examine intersexual relations. Rediscovered in northwestern Madagascar only in 1983 (Koenders et

al., 1985), this rare and understudied species occurs in male-skewed groups of 6-10 individuals, containing no more than three females per group (Randriatahina & Roeder, 2012). Females have a slight size advantage and are recognized for their unusual aggressiveness: Upwards of 81% of dominance interactions involve physical attacks (Digby & Kahlenberg, 2002; Randriatahina & Roeder, 2012), with females using targeted aggression to evict female competitors (Digby, 1999) and winning 99% of dominance interactions with males (Digby & Kahlenberg, 2002; Digby & Mclean Stevens, 2007). During the breeding season, females do not solicit mating; rather, males have been observed to aggressively solicit copulations, form coalitions to disrupt mating, and mate guard. Male-male agonism thus increases markedly during the mating period; nevertheless, females maintain reproductive control by aggressively rejecting potentially undesirable mates (Volampeno, 1999; Randriatahina & Roeder, 2012; Eschmann, 2019). Even among strepsirrhine primates, blue-eyed black lemurs exhibit an extreme form of female social dominance, but little is known about their seasonal patterns of female aggression and male accommodation, or potential mediating hormonal mechanisms in either sex.

Through two interrelated studies, conducted on different time scales, first we characterize sex and seasonal differences in behavior in relation to glucocorticoids and, second, we characterize sex and seasonal differences in reproductive hormones. In Study 1, we examine rates of aggression and affiliation (i.e., social modulatory behavior putatively mediated by androgens and/or estrogens) during the breeding season and a portion of the nonbreeding season for a given year. We also determine if entering the breeding season predicts activation of the HPA axis in males and females. Lastly, we test for covariation between fecal glucocorticoid metabolites and behavioral data. In Study 2, spanning over two decades, we provide a longitudinal (i.e., monthly) profile of circulating sex steroids and test for specific sex- or seasonally related patterns.

In accord with the Female Masculinization and/or Challenge Hypotheses, as applied to intersexual relations, we primarily expect dominant females to control resources and reproduction via androgen-mediated aggression, and subordinate males to be accommodating while subjected to the stress of social conflict. Accordingly, with respect to behavior, we expect that (1) females will initiate aggressive behavior more often than will males, particularly during the breeding season; (2) females will be less affiliative during the breeding season than the nonbreeding season; and (3) males will show increased affiliative behavior during the breeding season, in contrast to males in species expressing less extreme or no female dominance. With respect to hormones, the predictions for (4) glucocorticoid concentrations differ by hypotheses and/or sex. Following the Female Masculinization Hypothesis, glucocorticoids should increase less during the breeding season (if at all) in the sex controlling the interactions (here, the female), but should increase substantially in the submissive sex, (here, the male). On the other hand, the Challenge Hypothesis might instead predict seasonal increases in glucocorticoids for both sexes, in anticipation of increased aggression and/or mating competition. Given that social status and context mediate endocrine responses to conflict (e.g., Rose et al. 1975; Wingfield et al., 2001), in Study 1 we tested for, but did not necessarily expect, concurrent covariation between glucocorticoids and behavior, particularly not in the dominant initiator (i.e., female *E. flavifrons*). Lastly, (5) given known increases in female aggression and mating activity during the breeding season, and in line with an expanded Challenge Hypothesis, we expected that androgen concentrations will also increase in both sexes during this period. In Study 2, consistent with activational patterns in other lemurids (Drea, 2007; Petty & Drea, 2015), we did not expect a reversal of the traditional sex difference in adult androgen concentrations (i.e., male > female); nevertheless, consistent with proportional differences between male and female values in other lemurids, we might expect *E. flavifrons* to show reduced sex differences in androgen

concentrations relative to species expressing less extreme or no female dominance, particularly during the breeding season.

Methods

Subjects, Study Periods, and Housing

Our total study population consisted of 45 animals, socially housed in small cohorts at the Duke Lemur Center (DLC) in Durham, North Carolina. The DLC maintains the only breeding members of this species outside of Madagascar. Therefore, our sample size for such a rare and long-lived species only could be gained from additive, long-term study of consecutive cohorts, in this case, over a 22-year period (1998-2020).

Study 1 (on behavior and glucocorticoids) comprised 14 subjects (7F, 7M), including 12 adults (i.e., > 2.5 years old) and 2 juveniles, followed over a nearly four-month period (September 2 – December 20) in 2002 (see Figure S1 in SOM for sampling density by individual and month). In the northern hemisphere, this species' breeding season typically begins in October, with births occurring most frequently in March and weaning typically completed by May. Our study period thus encompasses the entirety of the six-week breeding season (October 1 – November 20), as well as ten weeks outside the breeding season (i.e., all other study dates immediately bordering the breeding season). These animals were housed, concurrently, as four social groups (consisting of two male-female pairs, a group of two females and one male, and a group of four animals, including a breeding pair and the two juveniles), two solitary males, and one solitary female (solitary animals were assessed for glucocorticoids only). Our one-male groups controlled for the potentially stressful environment of multimale social groups (as in *L. catta*: Starling et al., 2010). In addition, our study period included no changes in group composition, nor did we collect data from animals on the few days they were handled for routine veterinary care (for associations between these known stressors

and HPA activation in *L. catta*, see Seeley et al., 2021). The breeding pairs had been together for at least a year prior to this study, and most groups had been stable for at least 2 years. Four of the seven females in this study were contracepted via a melengestrol acetate implant (see our treatment of contraceptive status in *Data Analysis* section below).

Study 2 (on reproductive steroids) comprised 40 reproductively intact and non-contracepted adults (15F, 25M; 31 of which were unique relative to Study 1), ranging in age from 3-25 years. These animals contributed blood samples at any time of year during the full 22-year period.

All of the animals were individually known and recognizable (via microchip and unique collars or shave marks). Routine housing consisted of a temperature-controlled indoor area, connected to an outdoor pen (minimum enclosure area: 23.2 m²). Animals thus experienced the elements and local photoperiod, being confined indoors only when outside temperatures dropped below 4 °C (outside access was reinstated when temperatures exceeded 4°C for three consecutive days). Some of the subjects additionally gained summer access to one of three forested enclosures (1.5, 3.3, or 5.8 ha), where they could semi-free range, often in the presence of other species of lemurs. The subjects consumed a daily diet of fresh fruit, vegetables, and commercially available primate chow (Monkey Diet, LabDiet, St. Louis, MO, USA). Semi free-ranging animals could supplement their diet with plants and insects foraged from the forest.

Behavioral Observation and Fecal Sampling

For animals in Study 1, we conducted near-daily behavioral observations and/or fecal sampling. We observed the animals during 20-minute focal sessions that also included scan sampling at 5-minute intervals. Our schedule produced a total of 65 hours of observation (34 hr during the breeding season; 31 hr during the nonbreeding season). Two observers conducted behavioral observations on alternating days, using a comprehensive ethogram, from which we report counts of

affiliative behavior (allogrooming, receiving grooming, and huddling) and aggressive or dominance-related behavior (biting, grabbing, hair pulling; supplanting/withdrawal); for the full ethogram, see Supplementary Online Materials (SOM). We determined inter-observer reliability by having both trained observers record the same animals' behavioral events and then calculating the percentage of identical entries. Across two trials conducted at different times of the study, inter-observer reliability was 95%.

Opportunistic sampling of freshly voided feces, immediately placed on ice, produced a total of 372 samples; we collected 98% of these samples at 8:25-12:30 H and the remaining 2% at 12:30-16:30 H. We stored them at -80 °C within three hours of collection, and lyophilized, pulverized, and sifted them through a fine mesh within a year of sampling. The resulting powder then underwent an extraction method presented in Wasser et al. (2000). This assay is standardized using 0.2 g of dry feces; we recorded precise weights of fecal powder for each sample and later corrected the concentrations by this factor (i.e., sample weight / 0.2 g). After measurement, we extracted fecal powder in 2 mL of 90% methanol, after which we vortexed it for 30 minutes on a multi-pulse vortexer and twice centrifuged it. We extracted the supernatant after each centrifugation and discarded any sediment. We stored the resulting supernatant at -80 °C in 2 mL-polypropylene vials with O-ring caps to prevent evaporation.

Fecal Glucocorticoid Assays

We measured the concentrations of fecal glucocorticoid metabolites (FGCM) using Double Antibody Corticosterone I-125 Radioimmunoassay Kits specifically designed for rodents (MP Biomedicals, Cat. No. 07-120103, Costa Mesa, CA), validated by Wasser et al. (2000) for use in several mammalian and avian species, and validated by our group for use in ring-tailed lemurs (Starling et al., 2010). Corticosterone was the primary glucocorticoid metabolite recovered, with the

following cross-reactivities: < 1% cortisol, deoxycorticosterone, progesterone, testosterone, aldosterone, A₄, and 5 α -dihydrotestosterone; and < 0.01% 11-desoxycortisol, cholesterol, dehydroepiandrosterone (DHEA), DHEA-sulfate, 20 α -dihydroprogesterone, estrone, 17- α and - β estradiol, estriol, pregnenolone, 17 α -hydroxypregnenolone, 17 α -hydroxyprogesterone. The assay sensitivity was 0.2 ng/g of dry feces.

We validated all immunoassays according to the serial dilution procedure described by Khan et al. (2002) and assayed all fecal samples in duplicate. The maximum allowable limit for intra-assay variation was 6.0% between duplicates; if exceeding that count, we re-ran the sample. The mean inter-assay variations for high and low corticosterone controls were 10.1% and 13.9%, respectively. Twelve samples failed to yield a valid measurement after being assayed twice, and we discarded two FGCM measurements that were more than six standard deviations above the mean, likely resulting from assay error (final n = 358).

Blood Sample Collection

For Study 2, we obtained 87 banked serum samples (1 - 11 per individual) from the DLC (Figure 1). All of the subjects were habituated to the blood-draw procedure, which involves sequential, individual handling to minimize stress and the time interval between capture and blood draw. DLC veterinary staff drew blood from the femoral vein of awake, manually restrained animals, typically in the morning hours (mean time of collection \pm SE: 10:27 \pm 0:31 hr). These samples were immediately transferred to serum separator tubes (Vacutainer®, Becton Dickinson, Franklin Lakes, NJ, USA), allowed to clot at ambient temperatures, then placed into a cooler until centrifuged for 20 min at 1500 x g. Serum samples were decanted and stored at -80 °C until analysis.

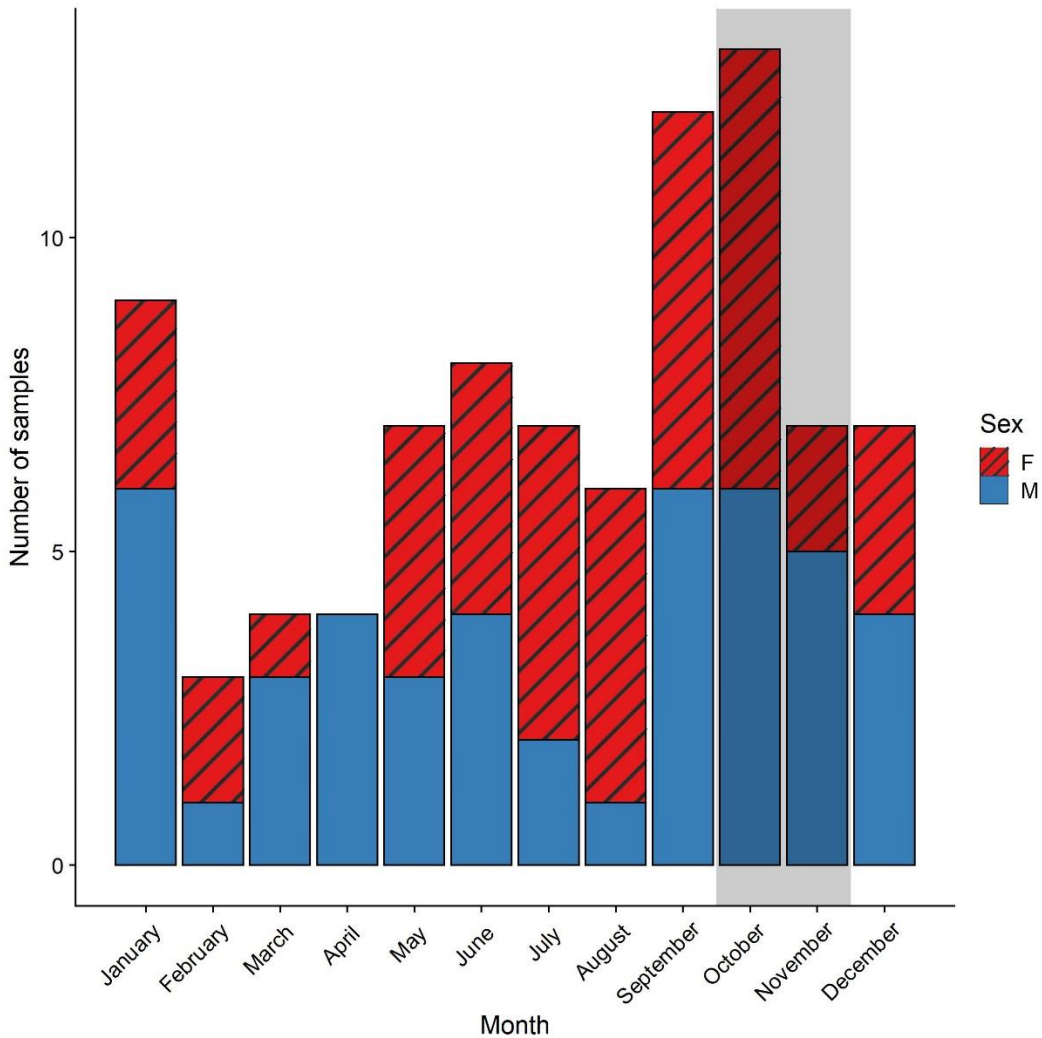


Figure 1: Blood sampling distributed by subject sex and by month across the 22 years of Study 2. Breeding season months in the Northern Hemisphere are shaded in gray.

Blood Hormone Measurements

In 2020 – 2021, we performed all of the enzyme immunoassays (ELISAs; ALPCO diagnostics, Salem, NH, USA) for A_4 , T, and E_2 , according to previously described methods (Petty & Drea, 2015; Grebe et al., 2019b). Sensitivities for the A_4 , T, and E_2 assays were 0.04 ng/ml, 0.02 ng/ml, and 10 pg/ml, respectively. The intra-assay coefficients of variation (CV) for these hormones

were 3.14%, 5.48%, and 7.34%, respectively; averages of the high and low inter-assay CVs were 15.3%, 3.7%, and 12.1%, respectively.

Data Analysis

Given differences in the density of data collection, our primary analyses (see below) modeled longitudinal data from Study 1 (behavior, FGCMs) on a day-by-day basis, and the data from Study 2 (A₄, T, E₂) on a month-by-month basis. Our social housing arrangements necessitated focusing on intersexual interactions and precluded examining intrasexual competition in our behavioral analyses. Moreover, the different study timelines precluded a test of any interaction between glucocorticoids and androgens.

In Study 1, there were thirteen instances in which we obtained two FGCM measurements from the same individual on the same day; we averaged these same-day measurements for analysis. In *E. macaco* (a close relative to *E. flavifrons*), researchers reported no difference in cortisol concentrations between control females and females treated with medroxyprogesterone acetate (Asa et al., 2007). By contrast, glucocorticoid concentrations in our contracepted females were lower than those in control females ($t(135) = -2.60, p = 0.011$; Table S1). Because these mean differences did not entail notably different seasonal patterns (see Table S1, Figure S2), we collapsed across contraceptive status in our primary analyses; results of models including this variable as a covariate were qualitatively similar and are reported in Table S1. We sampled FGCMs concurrently with behavioral observations on a subset of study days ($n = 134$) and used this subset to predict rates of aggressive and affiliative behavior from FGCM concentrations. We also considered possible associations between behavior and FGCMs 1-2 days following behavioral observation, to account for gut transmission time (Starling et al., 2010); these analyses yielded comparable results to those we present below (see Table S3 in SOM). We found no main effects or interactions linking hormone concentrations to either the date of sample collection or subject age, with one exception: for T,

concentrations increased with age in males only ($t(28) = 3.31, p = 0.003$). We thus include this interactive effect in our main analyses for T; see Table S2 in SOM for results of analyses including these covariates for all sex steroids.

In both Study 1 and Study 2, we followed up our longitudinal analyses with binary comparisons of behavior and hormone concentrations between the breeding and nonbreeding seasons. We delineated the breeding season with slightly different precision in our two studies. By observing reproductive behavior in only 1 year and subtracting the approximately 5-month gestation period from the subjects' parturition dates, we could narrow the breeding dates in Study 1 to October 1 – November 20. In Study 2, blood serum samples (but not detailed behavioral data) were collected across multiple years from animals that may have bred, but did not necessarily conceive, so we delineated the breeding season for this dataset as the entirety of October and November, following historical birth records at the DLC.

In light of ongoing debates regarding the appropriate treatment of hormone variables in statistical analyses (e.g., Roney, 2019; Gangestad et al., 2019), for all models concerning hormone concentrations, we report two separate models: one using raw, untransformed values, the other using log-transformed values. Log-transformed values may be useful to account for physiologically salient effects of hormones potentially resulting from proportional, rather than absolute, changes in hormone concentrations (Jones, 1996).

We analyzed longitudinal variation in aggressive and affiliative behavior, FGCM concentrations, and sex hormone concentrations using generalized additive models (GAMs) to regress these outcome variables on time of year, with separate trends estimated for males and females. We analyzed binary comparisons between the seasons using generalized linear mixed models (GLMMs). We included sex, age at the time of sampling, and group size as fixed covariates and animal ID as a random effect in all models. We conducted all GAMs using the R package *mgcv*

(Wood, 2017), and all GLMMs using the package *glmmTMB* (Brooks et al., 2017). GAMs fit smoothed splines to time-series data, and are thus well-suited to simultaneously identify non-linear associations among variables and account for repeated sampling from the same individual. For all of our GAMs, a “significant” spline can be interpreted as one that cannot have a horizontal line drawn through its 95% confidence interval (Ross, 2019). For the GAMs applied to sex hormones, we specified a cubic spline basis function to model seasonal patterns across multiple years without any discontinuity from December to January (Simpson, 2014).

For models predicting rates of behavior (i.e., counts of a relevant event per 20-minute focal observation), we specified a negative binomial family function to account for overdispersion of behavioral counts. For models predicting hormone concentrations, GAM and GLMM models were set to the Gaussian family default, except for analyses of A₄, in which the family was set to Tweedie to account for numerous values below the threshold of detection for females. See R code and data posted publicly at <https://osf.io/m2euy/> for the full results of all models.

Results

Study 1: Social behavior and fecal glucocorticoids

Social behavior. In models predicting intersexual behavior with sex and day/season as covariates, females initiated aggressive behavior significantly more often than did males ($p = 0.002$ and $p < 0.001$ in models examining day and season, respectively). There was no significant day-to-day variation in the frequency of aggressive behavior in females ($p = 0.174$), and only marginal variation in males ($p = 0.056$; Figure 2A). In binary seasonal comparisons, season interacted significantly with sex ($\chi = 2.80, p = 0.005$): whereas female intersexual aggression increased significantly during the breeding season ($p = 0.009$), male intersexual aggression was non-

significantly less frequent during the breeding season ($p = 0.133$). Prediction 1 on female aggression was thus met.

Unusually, rates of affiliative behavior did not significantly vary overall between the sexes ($p = 0.394$), although there were some notable temporal patterns. Day-to-day variation in intersexual affiliative behavior was significant for females ($p = 0.008$), but not for males ($p = 0.257$). Moreover, patterns of variation also differed by sex: females were least affiliative from October – November (i.e., during breeding-season months) and showed slightly higher rates in September and December (i.e., during nonbreeding season months); male rates of affiliation remained relatively flat across the 4-month study period (Figure 2B). In binary comparisons averaging across both sexes, rates of affiliative behavior were significantly lower during the breeding season ($\chi = 3.71, p < 0.001$); despite somewhat different trends for females and males, the binary interaction between season and sex was not significant ($\chi = -1.11, p = 0.266$). Considering the relation between both categories of behavior, intersexual aggression and affiliation were, unsurprisingly, inversely proportional within both sexes; however, these opposing fluctuations tracked the breeding vs. non-breeding season for females only. Prediction 2 on female affiliation was thus met, but prediction 3 on male affiliation was not met.

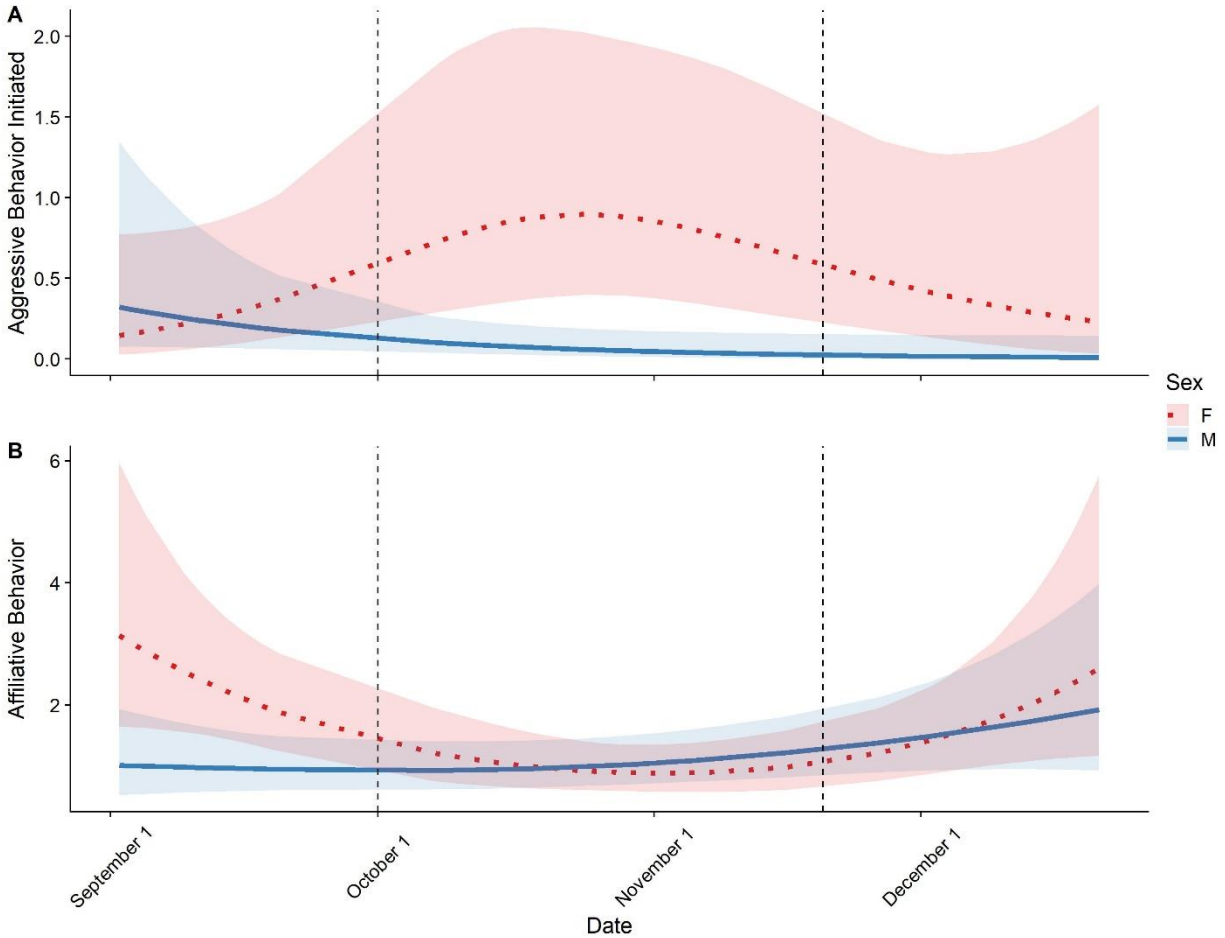


Figure 2: Patterns of (A) initiated aggressive behavior and (B) affiliative behavior in female and male blue-eyed black lemurs (*Eulemur flavifrons*) during the breeding season (delineated by vertical dashed lines) and adjacent periods of the nonbreeding season (September, December) in the Northern Hemisphere.

Fecal Glucocorticoids. We found that males had marginally greater average FGCM concentrations than did females across the 4-month study period ($p = 0.065$); this trend diminished when considering log-transformed values ($p = 0.229$). There was little evidence of day-to-day variation in FGCMs based on raw concentrations, both in females ($p = 0.094$) and in males ($p = 0.161$; Figure 3A), but stronger, significant evidence when considering log-transformed values both

in females ($p = 0.037$) and in males ($p = 0.033$; Figure 3B). Females showed their peak FGCM concentrations in September, followed by decreasing concentrations throughout the remainder of the study period, whereas males showed a slight peak around the beginning of November (Figure 3). In binary GLMM comparisons, sex did not significantly interact with season in predicting raw FGCM concentrations ($t(335) = -1.58, p = 0.116$), but this interaction was significant for log-transformed concentrations ($t(334) = -2.13, p = 0.034$). Decomposing this interaction into simple effects, in females, neither raw nor log-transformed FGCM concentrations ($p = 0.823$ and $p = 0.633$, respectively) supported a seasonal difference; in males, both raw ($p = 0.027$) and log-transformed ($p = 0.006$) FGCM concentrations supported a breeding season increase. These glucocorticoid findings are consistent with predictions generated by the Female Masculinization Hypothesis for both sexes, and with the prediction inspired by the Challenge Hypothesis for males only.

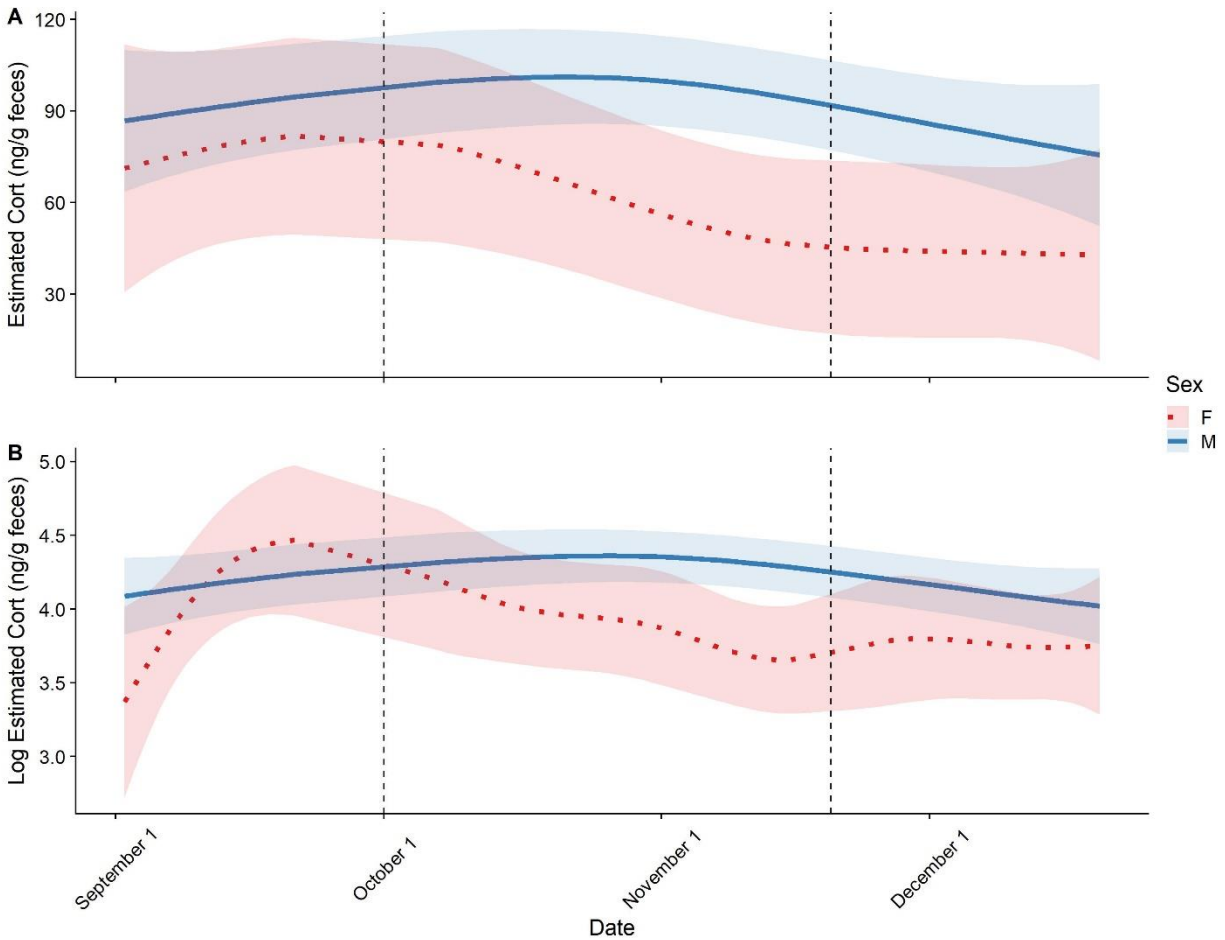


Figure 3: Trends for (A) raw and (B) log-transformed concentrations of fecal glucocorticoid metabolites in female and male blue-eyed black lemurs (*Eulemur flavifrons*), from September to December. Vertical dashed lines delineate the breeding season in the Northern Hemisphere.

Relationship between behavior and fecal glucocorticoids. Lastly, we examined if FGCMs predicted behavior on days when both behavioral data and fecal samples were collected for the same individuals. Whether raw or log-transformed, FGCMs failed to predict the frequency of aggressive behavior initiated by either sex (all p s > 0.13). By contrast, both raw and log-transformed FGCMs were significant predictors of affiliative behavior for females ($p = 0.031$ and 0.043 , respectively), although not for males ($p = 0.200$ and 0.273 , respectively). Affiliative behavior thus occurred most frequently in females expressing the highest FGCM concentrations (Figure 4).

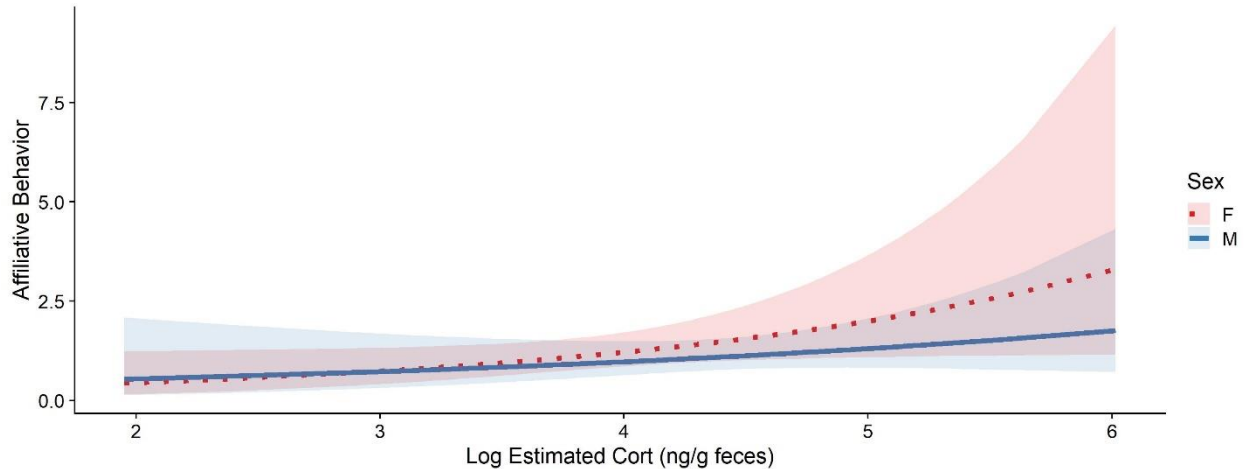


Figure 4: Frequencies of intersexual affiliative behavior between blue-eyed black lemurs (*Eulemur flavifrons*), as predicted by log-transformed fecal glucocorticoid metabolites.

Study 2: Reproductive Steroids

Androstenedione. Consistent with the typical mammalian sex difference, females had significantly lower raw A_4 concentrations than did males (male values were approximately 3.5x higher than those of females; $t(78) = 5.59, p < 0.001$). Females showed significant month-by-month variation for both raw and log-transformed values ($p = 0.001$ and 0.003 , respectively), with a trough around April - May and a peak in November and December (Figure 5); however, model uncertainty was pronounced around this peak owing to the small number of observations. Males also showed significant month-by-month variation ($p = 0.010$), with A_4 concentrations peaking from November to January (Figure 5A). For log-transformed values, this pattern was qualitatively similar, but not statistically significant ($p = 0.121$; Figure 5B). In binary seasonal comparisons for females, there was no significant difference observed in A_4 concentrations between the two-month breeding season compared to the remainder of the year, either for untransformed or log-transformed values ($t(81) = 0.19, p = 0.851$ and $t(81) = -0.62, p = 0.538$, respectively), perhaps because the breeding season slightly preceded, yet overlapped with peak values. Males, however, showed a significant breeding-

season increase in raw A_4 concentrations ($t(75) = 2.06, p = 0.043$), but not for log-transformed values ($p = 0.328$). Prediction 5 on androstenedione concentrations in females and males was thus partially met.

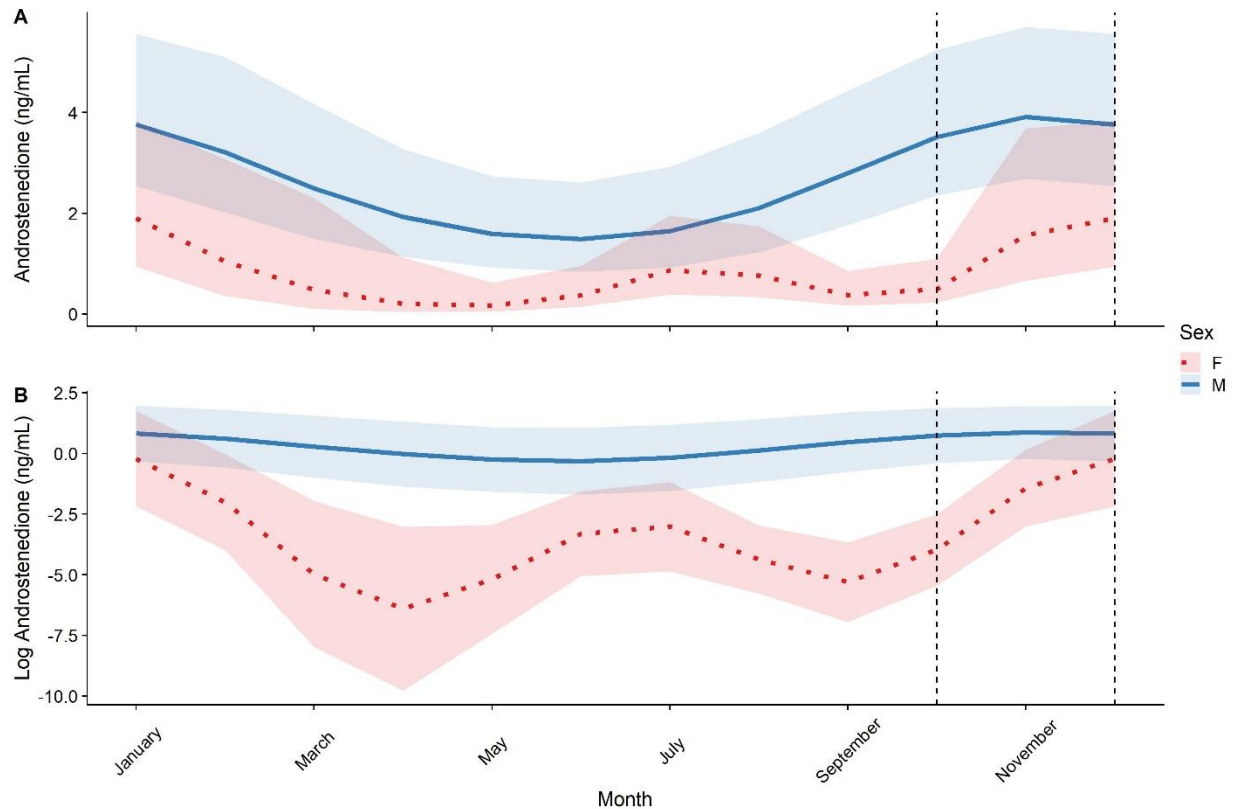


Figure 5: Annual trends in (A) untransformed and (B) log-transformed serum androstenedione concentrations in female and male *Enlemur flavifrons*. Shaded regions represent 95% confidence intervals. Vertical dashed lines delineate the breeding season.

Testosterone. Again, the typical sex difference in overall T concentrations was manifest in blue-eyed black lemurs, with females having significantly lower raw concentrations than males (male values were approximately 14x higher than those of females; $t(28) = 3.68, p = 0.001$). Indeed, T and A_4 concentrations were strongly correlated for both females and males ($r = 0.63$ and 0.59 , respectively; both $ps < 0.001$). Females showed no significant variation in T concentrations across

the year or by season, regardless of whether the concentrations were raw or log-transformed ($p > 0.50$). By contrast, males showed significant temporal variation in untransformed T concentrations across the year ($p = 0.002$), with a peak from August - October (Figure 6A), such that rising values preceded the onset of the breeding season. This variation, however, was not significant for log-transformed values ($p = 0.218$; Figure 6B), indicating that while males showed a substantial, absolute increase in T around the breeding season, the proportional increase was more modest. The peak in male T partially corresponded with our defined breeding season, such that in binary comparisons, males had significantly higher concentrations in the breeding season for raw, but not log-transformed, T concentrations ($t(56) = 3.02, p = 0.004$ and $t(54) = 1.52, p = 0.134$, respectively). Prediction 5 on testosterone concentrations was not met in females, although it was generally met in males.

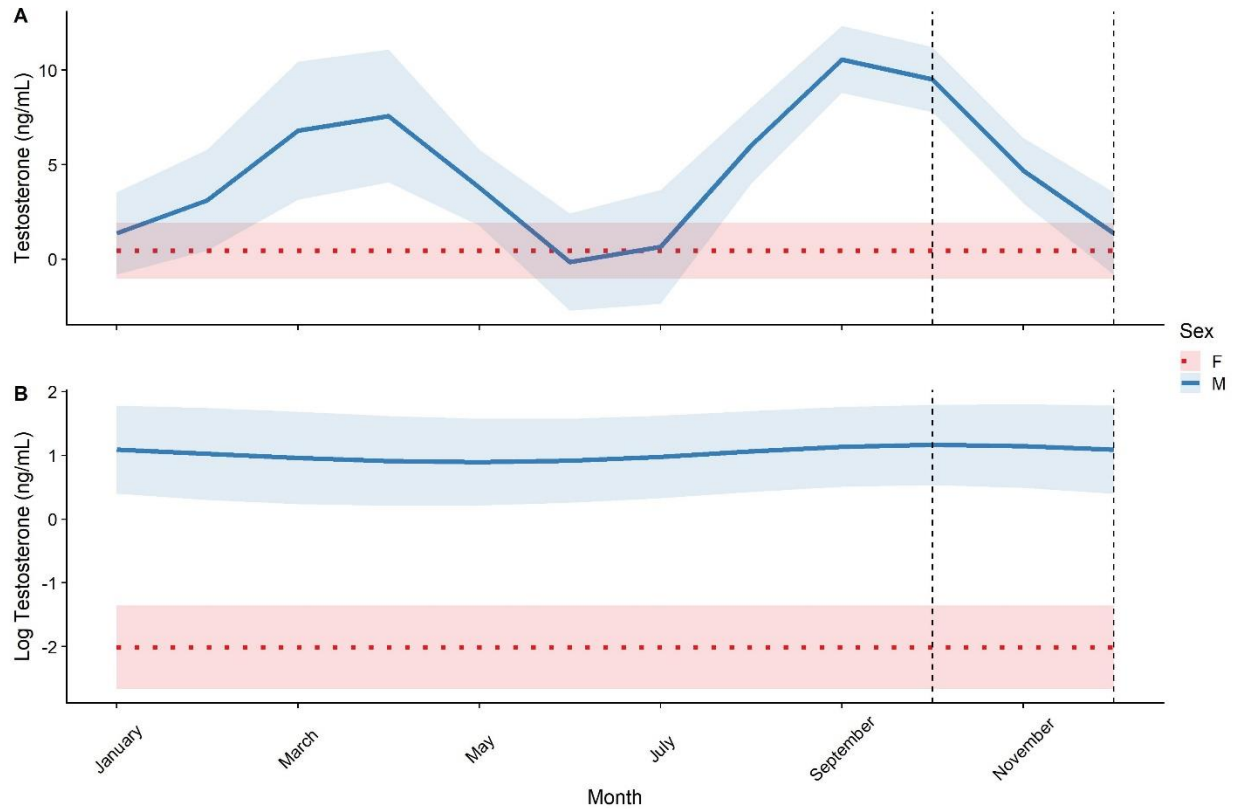


Figure 6: Annual trends in (A) untransformed and (B) log-transformed serum testosterone concentrations in female and male *Eulemur flavifrons*. Shaded regions represent 95% confidence intervals. Vertical dashed lines delineate the breeding season.

Estradiol. Unusually for mammals, E_2 concentrations in male lemurs, averaged across the entire year, were not significantly lower than those in females ($t(66) = -0.93, p = 0.354$). Female raw E_2 concentrations fluctuated significantly throughout the year ($p = 0.008$), with a peak in early summer or post weaning (Figure 7A), rather than during the breeding season. This pattern was qualitatively similar, but fell short of significance, for log-transformed values ($p = 0.097$; Figure 7B). Despite some fluctuation, males did not show significant month-by-month variation in either raw or log-transformed E_2 concentrations ($p = 0.227$ and $p = 0.192$, respectively; Figure 7). In binary seasonal comparisons, breeding season concentrations of E_2 in females were not significantly

different from nonbreeding season concentrations, for either raw or log-transformed values ($p = 0.118$ and $p = 0.617$, respectively). Males, however, showed significantly higher raw and log-transformed E_2 concentrations during the two-month breeding season compared to the remainder of the year ($t(61) = 2.07, p = 0.043$ and $t(61) = 2.18, p = 0.033$, respectively).

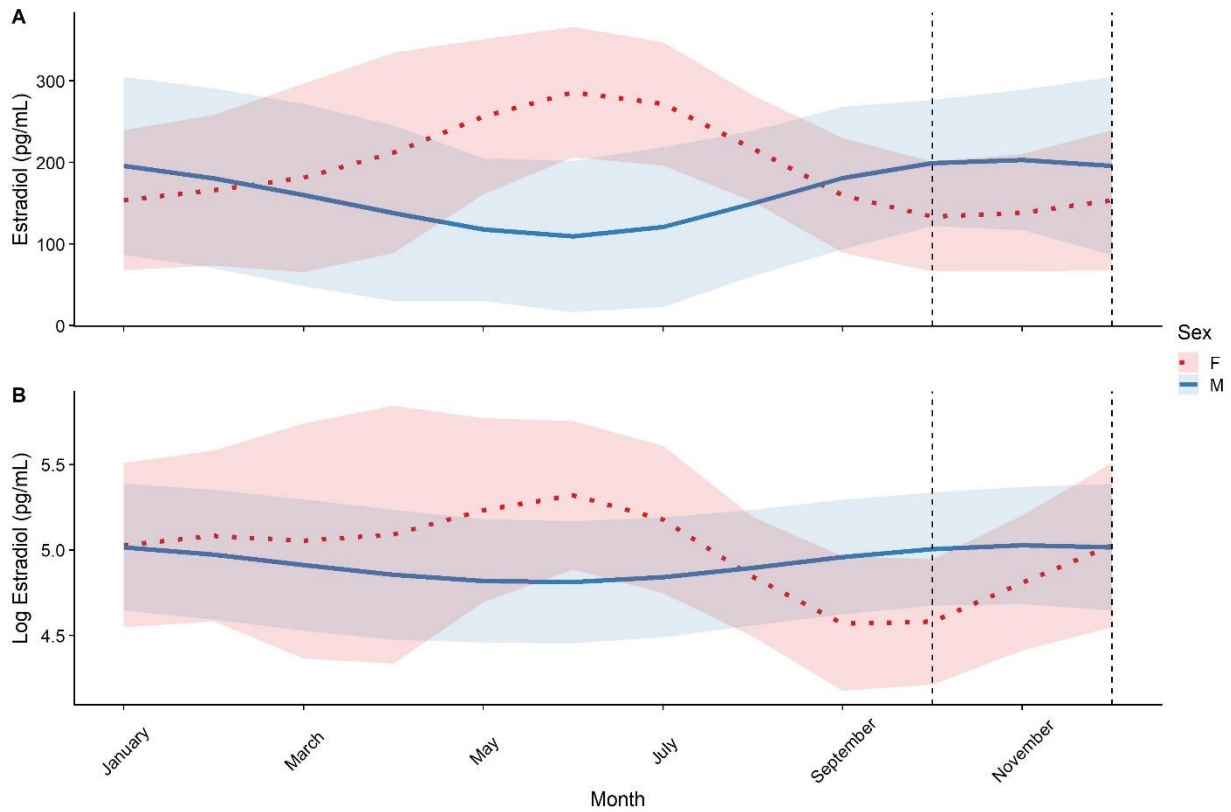


Figure 7: Female and male annual trends in (A) untransformed and (B) log-transformed serum estradiol concentrations. Shaded regions represent 95% confidence intervals. Vertical dashed lines delineate the breeding season.

Discussion

As part of a body of research aimed at understanding the evolutionary development of female dominance and male deference in lemurid primates, our findings on a rarely studied, female-aggressive species—the blue-eyed black lemur—speak to several predictions generated via the

Female Masculinization and/or Challenge Hypotheses. Notably, increased female aggression and decreased female affiliation in intersexual relations, particularly during the breeding season, coincided with increased concentrations of certain androgens, but not with glucocorticoids, consistent with the Female Masculinization Hypothesis of female dominance over males. Moreover, increased androgen and glucocorticoid concentrations in males during the breeding season met expectations of an adapted Challenge Hypothesis, in which interactions with aggressive females, whether in addition to or instead of male-male competition, creates social instability and stress for males. These findings reveal complex, seasonally mediated, intersexual relationships between hormones and dominance interactions in a female-dominant species.

Implications of results for females. The reverse sex difference in initiated aggression observed in female *E. flavifrons* was consistent with prior observations (Digby & Kahlenberg, 2002), and the documented breeding-season augmentation in female aggression was also comparable to observations in other female-dominant mammals (French et al., 2013). Both findings, together with the absence of a seasonal uptick in glucocorticoids, are consistent with female priority in access to resources, and with female reproductive control (e.g., rejecting potentially undesirable mates) in this species. We observed a traditional sex difference in androgens—with male values exceeding female values by a factor of 3.5 for A_4 and a factor of 14 for T—comparable to previous studies of these same hormones in *L. catta*, and intermediate within the range of differences reported across a variety of mammalian species (Drea, 2007). Despite fairly unremarkable activation patterns of female androgens, our observed peak in A_4 did coincide with a peak in female aggression (both occurring from October – December). The prolonged increase in A_4 concentrations, that extended beyond our two-month estimated breeding season, may reflect variation in the timing of breeding seasons across study years or some underestimation of season duration owing to a majority of first-cycle conceptions in polyestrous females (Asa et al., 2007), as also seen in *L. catta* (Drea, 2011). Although

we could not distinguish between potential functions of female increases in A_4 , the correspondence between female aggression and A_4 occurred here in intersexual contexts, potentially consistent with females competing with males over resources or aggressively rejecting undesired mates. Were this finding to extend to larger, mixed-sex groups, it might also suggest an androgen-mediated mechanism of female-female competition (Drea et al 2021). In contrast to A_4 , we found no evidence that T or E_2 were seasonally augmented in female *E. flavifrons*, despite significant covariation between A_4 and T, and the prominent role of E_2 in reproductive cycling. These patterns suggest a specific role of A_4 in this species' female dominance, consistent with findings in other female-dominant species (Yalcinkaya, 1993; Grebe et al., 2019b; Conley et al., 2020; Drea et al., 2021).

Researchers have treated transitional periods between seasons separately in previous longitudinal studies of other lemurids (e.g., Greene & Drea, 2014), specifically to maximize or better capture seasonal distinctions. In light of the 'nonbreeding season' in our Study 1 exclusively consisting of these transitional periods, we interpret high female FGCMs in September to potentially represent a peri-breeding season 'ramp-up' of energy mobilization among females, in anticipation of the breeding season (for a similar interpretation of cortisol patterns in male squirrel monkeys, see Schiml et al., 1996). Nevertheless, we could detect no systematic temporal relation between female aggression and glucocorticoids (cf. Cavigelli et al., 2003 and Starling et al., 2010 in *L. catta*, who report diverging results). More generally, our results highlight the benefits of appropriately modeling the transition between nonbreeding and breeding periods as a gradual, continuous process, in this case, revealing significant variation in FGCMs, and female A_4 , that was masked by more narrow and dichotomous analyses.

Implications of results for males. Based on a synthesis of results assessing non-linear trajectories across time, and binary comparisons of our defined nonbreeding vs. breeding season, we found fairly consistent support for breeding-season increases in male FGCM, A_4 , and T. An increase in

male FGCM concentrations during the breeding season is seemingly consistent with a male response to mobilize energy during times of anticipated increased competition (e.g. Muller & Wrangham, 2004). While some past work specifically implicates the presence of fellow male competitors in HPA responses (Sapolsky et al., 2000; Rogovin et al., 2003; Goymann & Wingfield, 2004; Starling et al., 2010), in our study, we observed a breeding-season increase in male FGCMs in the absence of adult male-male competition. While our results leave room for the role of endogenous rhythms in influencing HPA axis activation in males, we suggest that female aggression may play an important role as an elicitor of HPA responses.

Increased androgen concentrations in males during or slightly preceding the breeding season were consistent with patterns observed in other seasonally breeding primates (Rostal et al. 1986; Schiml et al., 1996; Cavigelli & Pereira, 2000) and align with expectations generated by the Challenge Hypothesis, in which increased concentrations of T should be associated with mating competition and social instability during the breeding season (Wingfield et al., 1990; Hirschenhauser & Oliveira, 2006). Positive associations between T concentrations and aggressive mating periods in *L. catta* corroborate our results (Cavigelli & Pereira, 2000; Drea, 2007; Gould & Ziegler, 2007). Nonetheless, because adult male *E. flavifrons* were not housed together, we could not directly address if increased androgen concentrations function to facilitate mating competition with other males (following Wingfield et al., 1990). The possibility remains that male fluctuations in androgen concentrations depend on photoperiod, endogenous rhythms, female semiochemicals or female presence. We suggest the latter, given evidence in anthropoid primates that the presence of cycling females can override photoperiod (Vandenbergh, 1969) and in lemurs that the male's reproductive condition primes his response to female scent (Scordato & Drea, 2007).

More consistent with male deference than with the Challenge Hypothesis, relatively elevated E_2 concentrations in male *E. flavifrons* is unusual and may explain the lack of a sex difference in the

initiation of affiliative behavior, though the nature of association between these features, including a potential functional mechanism, remains speculative. Previous evidence suggests that estradiol enables nurturing paternal behavior (e.g. Wynne-Edwards, 2001; Numan & Insel, 2003), providing one possible interpretation of our results—here, male estradiol might serve to promote affiliative behavior with mates. Alternatively, raised E₂ concentrations may owe to the aromatization of androgens (as a ‘byproduct’ of high androgen concentrations in males), although the absolute concentrations of androgens in male *E. flavifrons* are not particularly noteworthy. An additional increase in male E₂ during the breeding season is also concordant with the hormone’s importance in male reproductive function and mating effort (albeit often unrecognized). Indeed, E₂ is linked to breeding season increases in body size in squirrel monkeys and rhesus macaques (e.g. McCamant et al., 1987; Bercovitch, 1992) and is crucial to every stage of spermatogenesis, such that lowered E₂ has been linked to dilution of sperm, disruption of sperm morphology, and overall decreased fertility. Although E₂ production is localized to testicular cells and accounts for only 10-25% of circulating E₂ in human males (Hess, 2003; Akingbemi, 2005), if localized production of E₂ contributes to circulating concentrations in *E. flavifrons*, then the peak in circulating E₂ during the breeding season may be a result of increased spermatogenesis in advance of a breeding season that often involves multiple mating events. We suggest a fuller complement of sex hormones, including E₂ in males, should be considered in future research on seasonal fluctuations in endocrine function.

Limitations. Although we interpret our results as generally providing support for our predictions in both sexes, we also acknowledge some limitations to the strength of our conclusions. First, there were some discrepancies in the results yielded by the different modeling approaches. For instance, although there was no significant variation across the months for E₂, or for log-transformed A₄ and T in males, assessments of raw A₄ and T, and direct comparisons between the breeding and nonbreeding seasons, indicated that concentrations for all hormones peaked during the

breeding season. The discrepancy between results for raw versus log-transformed hormone concentrations suggests that absolute, rather than proportional, changes in sex hormones may more closely reflect seasonal organismal shifts. They also underscore the impact of subjective analytical choices in drawing conclusions from data (Gelman & Loken, 2014), and the need for sensitivity analyses to assess robustness of key results. Discrepancies between the GAM and GLMM results for sex hormones are likely due to multiple factors, including the monthly binning of sex steroid concentrations, the loss of information from dichotomizing the continuous variable of time of year, and the sample size limitations associated with studying a critically endangered species. Blood samples per month ranged from minimally one to maximally six per sex, such that variation between months and/or seasons would have to be particularly strong to reach statistical significance. Finally, as noted previously, captive housing arrangements of lemurs in both studies meant that certain functional interpretations of behavioral and hormonal shifts could not be tested. These features of our studies all act as important constraints to the generalization of our results, but they also represent trade-offs that entail benefits as well as drawbacks. For instance, our captive studies provide new information and insights on an exceptional, yet understudied species that could not be easily obtained in less controlled (i.e., wild) settings.

Conclusion

Based on the totality of our behavioral and endocrine findings, predominantly in intersexual dyads, we suggest that dominant females control resources and social interactions (including mating) via androgen-mediated aggression, and that subordinate males are behaviorally and physiologically deferent or accommodating, and unequally subjected to the stress of social uncertainty. Although fluctuations in male sex and stress hormone concentrations were, in most respects, consistent with predictions of the Challenge Hypothesis, considering results for both sexes together highlights the

value of integrating this hypothesis with a female masculinization framework. Our results concerning seasonal fluctuations in dominance interactions and their underlying hormone mediators, in aggregate, may provide a foundation for further exploring these links in other seasonally breeding and/or female-dominant species. In particular, our results suggest that a relationship between androgens and mating season, minimally, is not exclusive to males, and that females may alter their behavior and physiology in response to reproduction-related pressures to enforce intersexual dominance. We would expect a similar rationale to apply to other phases of the annual cycle, such as the birthing season. We have established that captive, intersexual social pairings are sufficient for the expression of certain seasonal shifts in behavior and physiology characteristic of female dominance; future researchers should consider how the nature of intersexual dominance may further respond to the broader socioecological variation available to wild animals, including in the face of intrasexual competition, same-sex coalitions, and resource limitations (e.g., Stockley & Bro-Jørgensen, 2011). In building towards this fuller understanding, however, a synthesis of multiple perspectives from behavioral endocrinology will continue to be invaluable.

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