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Progesterone levels in seasonally breeding, free-ranging male *Macaca thibetana*

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Abstract Progesterone, a “female” hormone, modulates sexual behaviors in male mammals. This modulation is well documented in laboratory animals, but it is as yet unknown whether progesterone is associated with testosterone and/or sexual behaviors in primates living in their natural environments. In this study, we collected a total of 426 fecal samples and approximately 453 h of behavioral data from five male Tibetan macaques (*Macaca thibetana*) to study relationships between males’ progesterone and testosterone levels and sexual behaviors. Our results showed a negative correlation between fecal testosterone and progesterone levels. Both hormones seasonally varied. For three of the five subjects, we also found that copulatory and sexually motivated behaviors were negatively correlated with fecal progesterone levels. While not significant for the other two males, this study provides insight into the relationship between progesterone and the sexual behaviors of male primates living in a natural environment.

Keywords *Macaca thibetana* · Male · Progesterone · Copulatory behavior · Sexually motivated behavior

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Introduction

The importance of androgens in the regulation of male sexual behavior is well documented in birds (Ball and Balthazart 2004), mice (Schneider et al. 2005), non-primate mammals (Arteaga-Silva et al. 2008; Bakker et al. 2004; Gastal et al. 2007; Li et al. 2004; Wallen 2001), non-human primates (Barrett et al. 2006; Wallen 2005), and humans (Isidori et al. 2005; Mong and Pfaff 2003).

Progesterone, named for its pro-gestational role in maintaining pregnancy (Wagner 2006), is traditionally regarded as a “female” hormone, and studies have mainly focused on its influence on female-typical behaviors (Bercovitch 2001; Hanke et al. 1996; Hoffman et al. 2008). Previous studies have documented that progesterone plays a critical role in the maintenance and development of female reproductive functions (Adkins-Regan 2005). Progesterone also has an impact on male sexual behavior, and data on this is becoming more available. These studies, though currently few in number, have provided insights into the role of progesterone in modulating male mammals’ sexual function (Andersen et al. 2007; Ball and Balthazart 2004; Barrett et al. 2006; Crews et al. 1996; Friess et al. 1997; Schneider et al. 2003).

Progesterone is essential in the synthesis of other hormones, especially testosterone (Andersen and Tufik 2006). In males, progesterone influences spermatogenesis, sperm capacity, and testosterone biosynthesis (Andersen and Tufik 2006). For example, treatment with progesterone reduces males’ testosterone levels (Gordon et al. 1970). Additionally, progesterone has a role in the regulation of male sexual behavior (Phelps et al. 1998; Scott et al. 2002; Zumpe et al. 2001; Zumpe et al. 1997). It decreases sexual behavior in many male mammals (Bercovitch 1999), including non-human (Barrett et al. 2006; Zumpe et al. 2001) and human (Bradford 1983) primates. For example, in a study of rats, Witt

et al. (1995) showed that progesterone-dependent mechanisms influence neurochemical pathways involved in copulation. Progesterone may also be fundamental for the regulation of male mating behavior in non-human primates (Barrett et al. 2006). For social primate species, it is noteworthy that the population's local ecology and social structure could be important factors influencing hormone production and sexual behaviors (Wallen 2005). Testosterone regulates male sexual motivation in free-living, non-human primates (Wallen 2001). Progesterone appears to exert its behavioral effects by first reducing the male's sexual urges, thereby decreasing his sexual activity (Wagner 2006).

Studies of captive and semi-free ranging rodents (Andersen et al. 2007; Hanke et al. 1996; Roof et al. 1996; Schneider et al. 2005; Scott et al. 2002; Witt et al. 1995) and non-human primates (Mishra et al. 2005; Zumppe et al. 2001; Zumppe et al. 1997) provide a basic understanding of progesterone's role in regulating male sexual behavior. Most of these studies have focused on laboratory animals, such as rodents, while fewer have been conducted on non-human primates. In these captive settings, progesterone treatment could be easily manipulated by the experimenters (Barrett et al. 2006; Scott et al. 2002; Zumppe et al. 1997), but the animals' housing was unlike the socioecology more typical for these species (Wallen 2005). Therefore, although the physiological function of progesterone in males has been documented, it is as yet unknown whether progesterone modulates testosterone and/or male sexual behaviors in primates living in a natural environment.

We focused our study on a primate population living in its natural setting to explore the relationships between and among progesterone, testosterone, and male sexual behavior. This is the first such study to investigate these relationships in a group of Tibetan macaques (*Macaca thibetana*). Tibetan macaques live in multi-male, multi-female groups with linear dominance hierarchies (Li 1999). *M. thibetana* has seasonal mating and birth, with the mating season lasting from July to January. During the mating season, higher frequencies of copulatory and sexually motivated behaviors (i.e., sexual chase, grimace, and sexual inspection) have been observed (Li et al. 2005; Xia et al. 2010). Seasonal variations in testosterone have been well documented in this population: testosterone was higher in the mating season and lower in the non-mating season (Xia et al. 2008), and copulatory behaviors were positively correlated with fecal testosterone levels (Xia et al. 2015). Based on these past findings, we hypothesized in this study that in free-living male Tibetan macaques, progesterone would be associated with testosterone and/or the occurrence of sexual behaviors. We predicted that the adult males' fecal progesterone levels would negatively correlate with testosterone levels (P1), and that males' fecal progesterone levels would be significantly different in the non-mating (higher level) and mating (lower level) seasons

(P2). Finally, we predicted that adult males' copulatory (P3) and sexually motivated (P4) behaviors would both negatively correlate with progesterone levels.

Methods

Study site and subjects

We conducted this study at Mt. Huangshan, which is located in Anhui Province, China. This area is a World Cultural and Natural Heritage site and is well known as a tourist destination and research site for the study of Tibetan macaques (*Macaca thibetana*). For more details of the study site, see Xia et al. (2010). The focus group of Tibetan macaques in the present study was the Yulinkeng 2 (YA2) group, which fissioned from the Yulinkeng 1 (YA1) group in 1996 (Li et al. 1996).

We collected behavioral data from five YA2 adult males. During the study period, the group increased from 38 to 43 animals and was composed of seven adult males, seven adult females, 22 subadults/juveniles, and two to seven infants (including five infants born during the study period). The five males (GBZ, CT, YX, JT, and BL) selected for study were thoroughly habituated to humans and readily distinguishable by facial/body characteristics. Two other adult males were not included as subjects because one was not well-habituated to humans and was difficult to collect fecal samples from, and the other had lost one testicle during a fight several years earlier.

Behavioral data collection

One of us (DPX) collected all of the behavioral data during an intensive study period of 303 days from October 2005 to September 2006 (mean = 25 days/month, range = 15–31). DPX began behavioral observations at approximately 08:00 and continued them until 17:30 each day. DPX maintained a distance of 5–10 m from monkeys. He used focal animal sampling and continuous recording (using a digital voice recorder) to score copulatory and sexually motivated behaviors (Altmann 1974). The order of daily focal animal observation was done via random selection and the duration of the focal animal sample was set at 20 min (Li et al. 2005; Li et al. 2007). If a given focal monkey could not be found at its sampling time or was lost during sampling, DPX chose the next monkey in the order and returned to the previous monkey when he reappeared (Li et al. 2005; Li et al. 2007).

Focal sampling yielded a total of 452 h of data. The focal samples (20 mins) were evenly distributed between the mating [226 h (monthly $X \pm SD = 45.2 \pm 2.43$ h, $n = 5$)] and non-mating seasons [226 h (monthly $X \pm SD = 45.2 \pm 3.73$ h, $n = 5$)] and were approximately evenly distributed among the five adult males (range: 80–94.3 total hours per male).

During focal samples, DPX recoded copulatory and sexually motivated behaviors, the latter of which included sexual chase, grimace, and sexual inspection (Li 1999; Xia et al. 2010). As described by Li (1999) and Li et al. (2007), we defined copulatory behavior as an instance in which a male mounted a female with intromission and thrusting, with or without ejaculation. We could determine whether the male ejaculated based on the post-copulation behavior—sperm-eating. Sperm-eating occurred after copulations with ejaculation, but not after copulations without ejaculation (DPX and LKS, personal observations). We defined sexual chase as occurring when a male chased a female and the female ran away, sometimes stopping but without screaming (Li 1999; Xia et al. 2010). Grimace was scored when a male bared his teeth and raised his eyebrows while oriented toward a female. Sexual inspection occurred when a male touched, licked, or sniffed a female's genital area (Li 1999; Xia et al. 2010).

Fecal sample collection and hormone analysis

We non-invasively collected a total of 426 fecal samples (of known identity) from the five adult males (mean = 86/individual, range = 74–111/individual). We collected one to two samples per week from each male using a predetermined collection schedule. For most weeks, we obtained a fecal sample from each male before any male was sampled twice. The total number of samples from each male varied (GBZ = 81; CT = 74; YX = 74; JT = 86; BL = 111), but fecal samples were evenly distributed across the five males for any given weekly period.

We extracted testosterone from feces following the methods described by Barrett et al. (2002) and Beehner and Whitten (2004). In cases where we obtained multiple samples from the same individual on the same day, we analyzed only the first sample. We placed fecal samples in a methanol solution, and each sample was homogenized, filtered, and loaded onto a reverse-phase C18 cartridge. Following solid-phase extraction, we immediately froze (−20 °C) all samples until they were transported to Anhui University for analysis. There, we eluted steroids from cartridges with methanol and subsequently froze (−70 °C) the samples until the time of radioimmunoassay (RIA). Immediately prior to RIA, we evaporated sample aliquots under nitrogen and reconstituted them at a fivefold dilution in working buffer.

Radioimmunoassay

We assayed all samples for testosterone and progesterone using the testosterone and progesterone RIA kit (Equate RIA ¹²⁵I Testosterone and progesterone Kit, Beijing North Institute of Biological Technology, Beijing, China), which Xia and colleagues previously validated for use in Tibetan macaques (Xia et al. 2008; Xia et al. 2015). We conducted the RIA at the

Nuclear Medical Laboratory of Anhui Medical University. We used the GC-911 radioimmunoassay gamma counter (Zhongjia Company of University of Science and Technology of China) and recorded radioactivity counts for 2 minutes per sample. For testosterone analysis, inter-assay coefficients of variation were $9.13 \pm 0.89\%$ (high control, $N = 30$) and $13.95 \pm 0.09\%$ (low control, $N = 30$). The intra-assay coefficient of variation was $2.79 \pm 0.66\%$ (fecal extract pool, $N = 6$). For progesterone analysis, inter-assay coefficients of variation were $1.73 \pm 0.17\%$ (high control, $N = 30$) and $0.85 \pm 0.09\%$ (low control, $N = 30$). The intra-assay coefficient of variation was $0.68 \pm 0.14\%$ (fecal extract pool, $N = 6$). We ran all samples in duplicate, and in this article we express values as ng/g dry feces.

Data analysis

We report data as mean (\pm SE) rates of copulatory behavior (events/h) and progesterone/testosterone levels (ng/g). We used a one-sample Kolmogorov-Smirnov test to examine whether the data conformed to a normal distribution ($P > 0.05$).

Modified from Barrett et al. (2002), we separated the study period into 1-month blocks, which yielded 12 distinct “sampling periods.” We calculated monthly (12 months) average values for both behavioral and progesterone data for each of the five males. We used Pearson correlation tests to analyze the relationship between fecal testosterone and progesterone levels. We used paired-samples *t* tests to compare males' progesterone levels during the mating and non-mating seasons (each male in both seasons, $n = 5$ males). We analyzed the relationships between sexual behavior (i.e., copulatory behavior, sexually motivated behaviors) and fecal progesterone at both the group and individual levels. We used Spearman's correlation tests to test the relationship between each male's monthly (12 months) sexual behavior (i.e., copulatory behavior, sexually motivated behaviors) and his progesterone levels (each male per month, $n = 5$ males).

We used the software SPSS 13.0 (SPSS Inc., Chicago, IL, USA) (Norusis 2005) to analyze our data, with the levels of significance set at 0.05 for all statistical tests. Statistical tests were all two-tailed.

Results

P1: progesterone and testosterone

The fecal progesterone levels of the five adult male Tibetan macaques' aggregated data negatively correlated with their testosterone levels during the study period (Pearson $r = 0.623$, $N = 12$, $P < 0.05$; see Fig. 1). When we analyzed the data for each monkey, however, only three monkeys

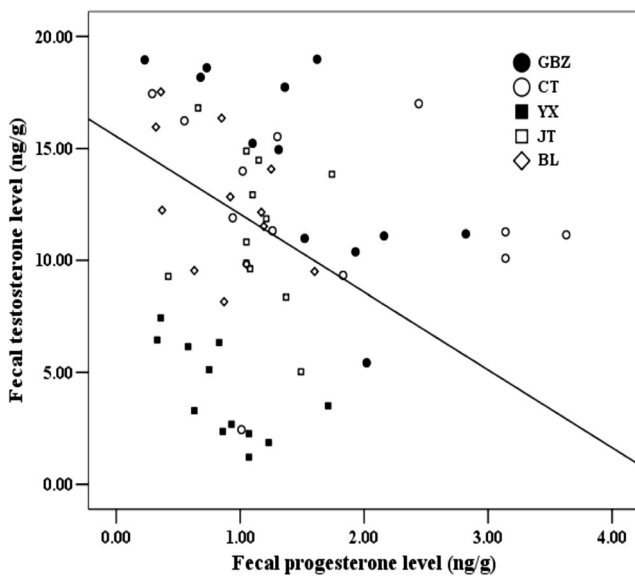


Fig. 1 Scatterplot of fecal progesterone and testosterone levels in male Tibetan macaques. The dotted line indicates the estimated slope of the regression among five adult males. The dotted line is a linear trend line based on ordinary least-squares (OLS) regression in Microsoft Excel

(GBZ, CT, and YX) had negative correlations for their progesterone and testosterone levels (GBZ Pearson $r = -0.726$, $N = 12$, $P < 0.01$, CT $r = -0.619$, $N = 12$, $P < 0.01$, and YX $r = -0.657$, $N = 12$, $P < 0.05$). The results were not significant for two monkeys (JT and BL) (JT Pearson $r = -0.201$, $N = 12$, ns , BL $r = -0.464$, $N = 12$, ns).

P2: seasonal variation of progesterone

The monkeys' fecal progesterone levels were significantly higher in the non-mating season (1.594 ± 0.081 ng/ml) than in the mating season (0.998 ± 0.489 ng/ml) ($t = 6.632$, $df = 4$, $P < 0.01$) (see Fig. 2).

P3: progesterone and copulatory behavior

The five males' aggregated copulatory behaviors were negatively correlated with fecal progesterone levels (Spearman's $r = -0.832$, $N = 12$, $P < 0.01$; see Fig. 3). However, this result was driven by three adult males: CT, YX, and BL (CT Spearman's $r = -0.621$, $N = 12$, $P < 0.05$, YX $r = -0.836$, $N = 12$, $P < 0.01$, and BL $r = -0.619$, $N = 12$, $P < 0.05$). We found no correlation for two of the males (GBZ Spearman's $r = -0.518$, $N = 12$, ns and JT $r = -0.303$, $N = 12$, ns).

P4: progesterone and sexually motivated behaviors

Each of the three sexually motivated behaviors was negatively correlated with fecal progesterone levels when the data for all of the monkeys were combined (sexual chases Spearman's $r = -0.769$, $N = 12$, $P < 0.01$, grimace $r = -0.671$, $N = 12$,

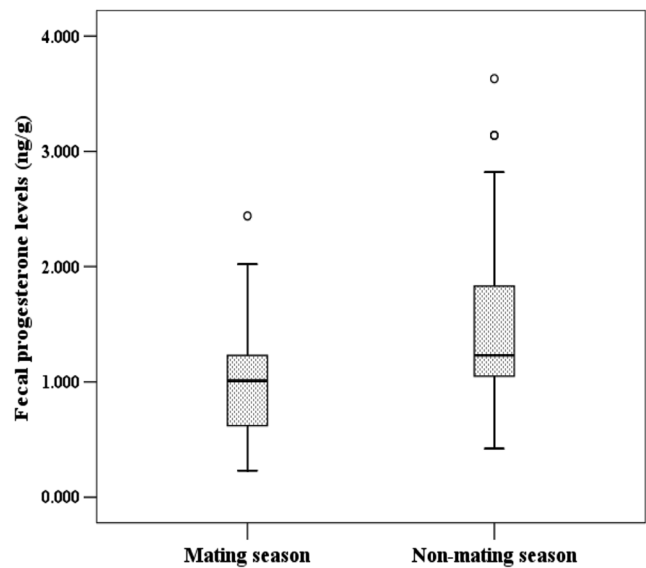


Fig. 2 Seasonal variation in fecal progesterone levels in adult male Tibetan macaques. The variance is shown here as standard deviation. Outliers are given as dots, which were identified based on the standard deviation

$P < 0.05$, and sexual inspection $r = -0.676$, $N = 12$, $P < 0.05$) (see Fig. 4). However, when we analyzed the data for each male individually, the results were more variable. We found significant negative correlations for the three highest-ranking adult males for sexual chase (GBZ $r = -0.721$, $N = 12$, $P < 0.01$, CT $r = -0.636$, $N = 12$, $P < 0.05$; YX $r = -0.797$, $N = 12$, $P < 0.01$), grimace (GBZ $r = -0.654$, $N = 12$, $P < 0.05$, CT $r = -0.760$, $N = 12$, $P < 0.01$, YX $r = -0.694$, $N = 12$, $P < 0.05$), and sexual inspection (GBZ $r = -0.666$, $N = 12$, $P < 0.05$, CT $r = -0.645$, $N = 12$, $P < 0.05$, YX: $r = -0.750$,

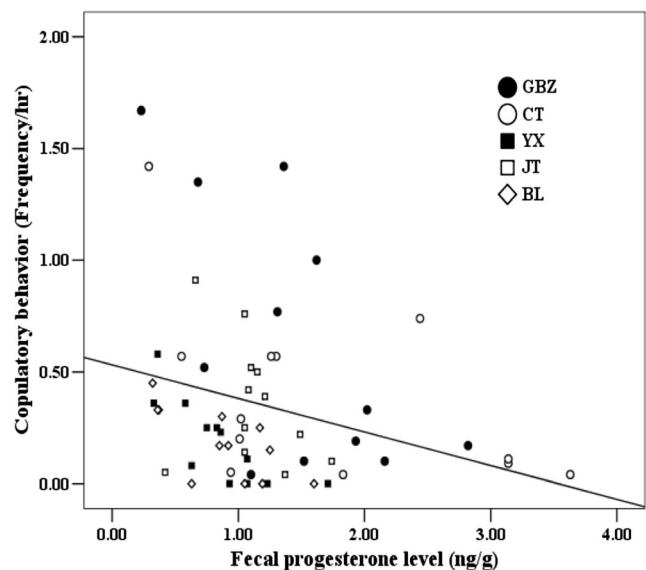


Fig. 3 Scatterplot of fecal progesterone and copulatory behaviors in male Tibetan macaques. The dotted line indicates the estimated slope of the regression among the five adult males. The dotted line is a linear trend line based on ordinary least-squares (OLS) regression in Microsoft Excel

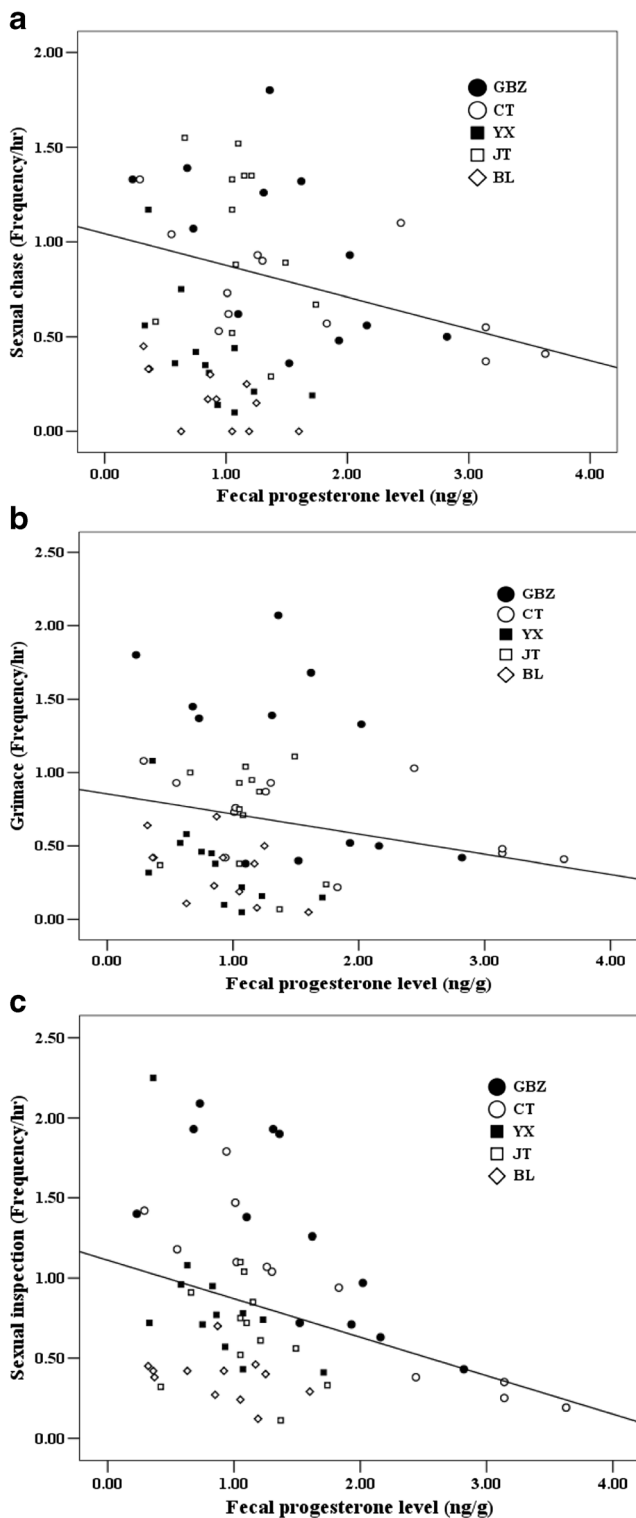


Fig. 4 Scatterplot of fecal progesterone and sexually motivated behaviors in male Tibetan macaques (A: sexual chase; B: grimace; C: sexual inspection). The dotted line indicates the estimated slope of the regression among the five adult males. The dotted line is a linear trend line based on ordinary least-squares (OLS) regression in Microsoft Excel

$N = 12$, $P < 0.01$). For the two lowest-ranking males (JT and BL), sexual chase (JT $r = -0.138$, $N = 12$, *ns*, BL $r = -0.537$,

$N = 12$, *ns*), grimace (JT $r = -0.028$, $N = 12$, *ns*, BL $r = -0.444$, $N = 12$, *ns*), and sexual inspection (JT $r = -0.331$, $N = 12$, *ns*, BL $r = -0.366$, $N = 12$, *ns*) were not significantly correlated with fecal progesterone levels.

Discussion

Progesterone treatment has been shown to decrease testosterone levels in laboratory monkeys (Zumpe et al. 2001), but it is as yet unknown the degree to which progesterone regulates testosterone for monkeys living in a more complex social and ecological environment. Research on wild or free-ranging subjects allows us to examine how progesterone interacts with testosterone-dependent processes and how it physiologically affects sexual behavior in the natural environment. These results may yield insights into the evolution of progesterone function in male non-human primates. In our study, although analysis at the individual level failed to show consistent results across all monkeys for some predictions, the pooled results supported P1, P2, P3, and P4 in the current study. It is noteworthy that the tests we ran for each monkey were significant for the three top-ranked males and were non-significant, but in the predicted direction, for the two lower-ranked males (GBZ was the highest-ranked male, followed by CT, YX, JT, and BL, see Xia et al. 2015).

Progesterone and testosterone in the natural environment

Previous studies have suggested that high levels of testosterone are energetically expensive (Barrett et al. 2002; Wingfield et al. 1990), and extensive evidence shows a primary role for testosterone in spermatogenesis (Brown and Follett 1977). Testosterone decreases in the non-mating season may therefore be adaptive, and our study suggests that progesterone could be one contributing factor in its decrease. However, it is unclear why this relationship should be stronger for some monkeys than for others. Furthermore, the two lower-ranking monkeys had levels of both hormones well above those of YX, a higher-ranking but much older monkey. A “basement effect” is therefore unlikely.

In our data set, the dramatic rise in progesterone shown by high-ranking males in the non-mating season was one notable difference between the high- and low-ranking males. As shown in Fig. 1, progesterone levels were otherwise quite similar across all five monkeys in the mating season. If progesterone partially or wholly regulates testosterone, this seasonal difference in progesterone levels could be driving the correlations we found for the higher-ranking monkeys, whereas the lower variation seen in lower-ranking monkeys’ progesterone levels, coupled with the fact that we only had two low-ranked individuals, may have made a correlation more difficult to detect for them. It remains to be determined what

triggers seasonal changes in progesterone and whether there is adaptive significance to these changes. Progesterone levels could be a contributing factor to behaviors that are potentially important for reproductive success, such as infant tolerance and infant holding. In Tibetan macaques, adult males handle infants as social “bridges” to regulate their interactions (Ogawa 1995), so infant tolerance is an important aspect of a male’s position in the dominance hierarchy. Subordinate males more often approach to bridge with more dominant individuals (Bauer et al. 2013; Ogawa 1995), and high-ranking males hold infants more often than do lower-ranked males (DPX, personal observation). Barrett et al. (2002) reported that non-contact aggression correlated with testosterone levels in Japanese macaques (*Macaca fuscata*). If progesterone decreases testosterone levels, we might also expect a lower level of aggression directed toward infants in our study population. The opposite result has been reported in male mice (Schneider et al. 2003): progesterone was shown to increase infant-directed aggression, including infanticidal behavior. However, the long-term association between males and females in a multi-male, multi-female primate group, combined with relatively promiscuous mating, makes infanticide an ineffective reproductive strategy, whereas it may be adaptive in mice. We expect a species such as Tibetan macaques to evolve a strategy based more on infant protection than on infant-directed aggression. It is possible that in our population, high-ranking males hold infants more often than low-ranking ones (DPX, personal observation) because high-ranking males have higher progesterone levels.

Progesterone and sexual behaviors in the natural environment

Many studies have indicated that treatment with progesterone (Andersen and Tufik 2006; Zumpe et al. 2001; Zumpe et al. 1997) and systemic progesterone (Lbanez et al. 2004; Michael et al. 1991; Michael and Zumpe 1993a; Michael and Zumpe 1993b) significantly reduces ejaculatory frequencies in male mammals. Whether progesterone regulates sexual behavior in males living in natural environments has not been investigated. In the present study, we found that male Tibetan macaques’ copulations were negatively correlated with progesterone levels, although only significantly for the three highest-ranking males. Copulations during the non-mating season when progesterone is high and testosterone is low have been reported for this species (Li et al. 2007), perhaps because progesterone modifies copulatory behavior to be non-ejaculatory in the non-mating season.

In the present study, we also found that adult male Tibetan macaques’ sexually motivated behaviors were significantly negatively correlated with progesterone levels in high-ranking monkeys, but not in low-ranking ones. These results suggest that progesterone may affect sexual motivation of

high-ranking, non-human primate males. While the frequency of copulations and perhaps sexual inspections are likely to be affected by female sexually motivated behaviors such as presenting, other male behaviors such as sexual chase and grimace may occur independent of female behaviors. Grimaces by males are often ignored by females (DPX, personal observation), even during the mating season, and sexual chases, by definition, involve the female fleeing from the pursuing male. Thus, seasonal changes in these behaviors likely reflect a true difference in male sexual motivation, rather than merely a male’s response to a female’s interest in mating. Similarly, progesterone treatment has been shown to decrease sexual urges in male human and non-human primates (see Andersen and Tufik 2006 for a review), although most human research has focused on sexual offenders and not on males from the general population.

Taken together, our results suggest that progesterone’s role in regulating sexual motivation is mediated through reduction in testosterone secretion. However, Zumpe et al. (2001) reported a dampening effect of progesterone on male cynomolgus monkeys (*Macaca fascicularis*), which led them to conclude that progesterone and testosterone levels are unrelated. It is thus possible that progesterone might affect male sexual behaviors in the current study, which shows that progesterone was independent of, and possibly additive to, testosterone’s effects. Future researchers should seek ways to tease apart the causal relationship, if any, between these two hormones and sexual behaviors in for animals living in natural settings.

Our study provides evidence supportive of a relationship between progesterone, testosterone, and sexual behaviors in free-ranging, non-human primate males. We also provide insight into mechanisms to use progesterone to reduce sexual urges in human sexual offenders. However, we caution that, because of our small sample size ($n = 5$ males) and the difficulty in manipulating variables in animals living in their natural environments (for example, altering progesterone dose by manual control for wild animals), we are unable to draw conclusions about the causation of progesterone acting on testosterone, and of testosterone acting on sexual behaviors. Whether, and how, progesterone increases an animal’s reproductive success also remains to be determined.

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