Title:

Correlates of androgens in wild male Barbary macaques: testing the challenge hypothesis

Short title:

Androgens in male Barbary macaques

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Abstract

Investigating causes and consequences of variation in hormonal expression is a key focus in behavioural ecology. Many studies have explored patterns of secretion of the androgen testosterone in male vertebrates, using the challenge hypothesis (Wingfield et al., 1990) as a theoretical framework. Rather than the classic association of testosterone with male sexual behaviour, this hypothesis predicts that high levels of testosterone are associated with male-male reproductive competition but also inhibit paternal care. The hypothesis was originally developed for birds, and subsequently tested in other vertebrate taxa, including primates. Such studies have explored the link between testosterone and reproductive aggression as well as other measures of mating competition, or between testosterone and aspects of male behaviour related to the presence of infants. Very few studies have simultaneously investigated the links between testosterone and male aggression, other aspects of mating competition and infant-related behaviour. We tested predictions derived from the challenge hypothesis in wild male Barbary macaques (*Macaca sylvanus*), a species with marked breeding seasonality and high levels of male-infant affiliation, providing a powerful test of this theoretical framework. Over 11 months, 251 hours of behavioural observations and 296 fecal samples were collected from seven adult males in the Middle Atlas Mountains, Morocco. Faecal androgen levels rose before the onset of the mating season, during a period of rank instability, and were positively related to group mating activity across the mating season. Androgen levels were unrelated to rates of male-male aggression in any period, but higher ranked males had higher levels in both the mating season and in the period of rank instability. Lower androgen levels were associated with increased rates of male-infant grooming during the mating and unstable periods. Our results generally support the challenge hypothesis and highlight the importance of considering individual species' behavioural ecology when testing this framework.

Keywords:

Androgens; Barbary macaques; challenge hypothesis; rank instability; male-infant affiliation

Introduction

The challenge hypothesis (Wingfield, Hegner, Dufty, & Ball, 1990), originally proposed to explain temporal variation in testosterone levels in seasonally breeding birds, has served as a powerful framework for investigating the patterns of androgen secretion in male vertebrates. This hypothesis posits that during the breeding season, testosterone levels increase from a pre-breeding baseline to a breeding baseline, sufficient for sperm production and the expression of sexual behavior. Beyond the breeding baseline, the highest levels are expected during periods of acute social challenges associated with reproductive competition, such as when males are mate guarding or establishing dominance relationships (Wingfield et al., 1990). As such, high testosterone levels should more closely follow measures of reproductive competition, such as aggression, rather than sexual activity itself (Wingfield et al., 1990). High testosterone levels prime males for competition (Oliveira, 2004), but maintaining such levels is costly, as testosterone increases energy use and mortality, suppresses the immune system, and interferes with paternal care (Wingfield, Lynn, & Soma, 2001). Therefore, high levels of testosterone are only expected when the adaptive benefits exceed these costs, and low levels should occur at other times.

In support of the challenge hypothesis, elevated testosterone - or more generally androgen - levels during the breeding season, in accordance with increased reproductive competition during this period, have been found in many seasonally breeding primates, e.g. Verreaux's sifaka (*Propithecus verreauxi*: Brockman, Whitten, Richard, & Benander, 2001), golden lion tamarins (*Leontopithecus rosalia*: Bales, French, McWilliams, Lake, & Dietz, 2006), ring-tailed lemurs (*Lemur catta*: Gould & Ziegler, 2007), Assamese macaques (*Macaca assamensis*: Ostner, Heistermann, & Schulke, 2011), and long-tailed macaques (*Macaca fascicularis*: Girard-Buttoz et al., 2015). On a shorter time scale, elevated androgen levels have been found to be associated with specific measures of reproductive competition. For example, increased levels of androgens are associated with being in the presence of parous estrous females among chimpanzees (*Pan troglodytes*: Sobolewski, Brown, & Mitani, 2013), with time spent mate guarding in long-tailed macaques (Girard-Buttoz et al., 2015) and with consortship in yellow baboons (*Papio cynocephalus*: Onyango, Gesquiere, Altmann, & Alberts, 2013). By contrast, in male muriquis (*Brachyteles arachnoides*), androgen levels do not differ significantly between mating and non-mating periods, possibly reflecting the low levels of overt aggression over access to mates in this species (Strier, Ziegler, & Wittwer, 1999).

In addition to seasonal changes, androgens have also been positively associated with overall rates of male aggression in ursine colobus monkeys (*Colobus vellerosus*: Teichroeb & Sicotte, 2008), Assamese macaques (Ostner et al., 2011) and olive baboons (*Papio anubis*: Sapolsky, 1983), and also with aggression in specific contexts such as during replacement of resident males in siamangs (*Symphalangus syndactylus*: Morino, 2015), and during incursions of new males into the group among ursine colobus monkeys (Teichroeb & Sicotte, 2008). In other species, a lack of relationship has been found between androgens and overall rates of aggression, for example in chacma and Guinea baboons (*Papio papio*: Kalbitzer, Heistermann, Cheney, Seyfarth, & Fischer, 2015), and long-tailed macaques (Girard-Buttoz et al., 2015) or between androgens and aggression in specific contexts such as intergroup encounters, for example in siamangs (Morino, 2015) and moustached tamarins (*Saguinus mystax*: Huck, Löttker, Heymann, & Heistermann, 2005).

The challenge hypothesis specifically predicts that during periods of social instability linked to reproductive competition, such as when dominance relationships are being established or challenged, or group composition is changing, androgen levels will be elevated in dominant individuals relative to subordinates (Wingfield et al., 1990). This prediction has been supported in a wide variety of primates (olive baboons: Sapolsky, 1983; Verreaux's sifaka: Brockman et al., 2001; bonobos, *Pan paniscus*: Marshall & Hohmann, 2005; rhesus macaques, *Macaca mulatta*: Higham, Heistermann, & Maestripieri, 2013; geladas, *Theropithecus gelada*: Pappano & Beehner, 2014). A relationship between androgens and rank has also been found during stable periods in some species where reproductive success is highly skewed towards high ranking males, (e.g. mandrills, *Mandrillus sphinx*: Setchell, Smith, Wickings, & Knapp, 2008; yellow baboons: Gesquiere et al., 2011; long-tailed macaques: Girard-Buttoz et al., 2015), which may reflect the higher ongoing costs of maintaining high rank, namely responding to aggressive challenges and asserting dominance. By contrast, in many other species no relationship between rank and androgens exists during stable periods (e.g. ursine colobus monkeys: Teichroeb & Sicotte, 2008; Assamese macaques: Ostner et al., 2011; rhesus macaques: Higham et al., 2013; siamangs: Morino, 2015).

A further prediction of the challenge hypothesis is that high androgen levels are inhibitory to paternal care, and such an effect has been documented through both observational association and experimentation, predominantly in studies of bird species (Wingfield et al., 1990; Wingfield et al., 2001). In line with this prediction, androgen levels have been found to be negatively associated with intensity of paternal care (infant carrying) in siamangs (Morino, 2015), and a decrease in androgens in the birth season has been found in black tufted-ear marmosets (*Callithrix kuhlii*), a species in which males show high levels of infant carrying (Nunes, Fite, & French, 2000). By contrast, in Geoffroy's marmosets (*Callithrix geoffroyi*) no such relationship has been found between androgen levels and paternal care (Cavanaugh & French, 2013), and in Verreaux's sifaka the presence of infants was not related to variation in androgen levels (Brockman et al., 2001). Furthermore, it has been suggested that an increase in androgens may reflect the need for aggressive protection against infanticide, for example in the birth season in red-fronted lemurs (*Eulemur fulvus rufus*: Ostner, Kappeler, & Heistermann, 2008), or during extra-group male incursions in ursine colobus monkeys (Teichroeb & Sicotte, 2008).

Here, we test predictions of the challenge hypothesis in wild male Barbary macaques, a species in which this framework has not previously been explored, and which offers an unusual combination of multi-male, multi-female social system, strong breeding seasonality and high levels of male affiliative behavior towards infants. Barbary macaques are considered a relatively "tolerant" species (Thierry, 2000) with a relaxed dominance style (Preuschoft, Paul, & Kuester, 1998) and generally low levels of aggression (Thierry & Aureli, 2006). Females mate promiscuously during the marked breeding season (Small, 1990), limiting the ability of high ranking males to monopolize mating access (Kuester & Paul, 1992), although mating success is still somewhat skewed towards high ranking individuals (Bissonnette, Bischofberger, & van Schaik, 2011; Young, Hähndel, Majolo, Schülke, & Ostner, 2013). The mating season represents a time of high male reproductive competition, as evidenced by increased rates of both aggression (Berghänel, Ostner, Schröder, & Schülke, 2011) and male-male inflicted injuries (Kuester & Paul, 1992) during this period. Unusually for a species with a polygynandrous social system, male Barbary macaques frequently interact with infants, carrying, grooming and huddling with them (Whitten, 1987). These affiliative behaviors are not considered true paternal care, since males do not preferentially interact with their own offspring (Ménard et al., 2001; Paul, Kuester, & Arnemann, 1996); however, they may benefit males by increasing the likelihood of mating with the mother of the infant in the subsequent breeding season (Ménard et al., 2001).

We explore diverse aspects of the challenge hypothesis, using data on individual males' rates of aggression and affiliative behavior with infants, and on group levels of mating activity, collected across breeding and non-breeding seasons (including a period of rank instability). Specifically, following the challenge hypothesis (Wingfield et al., 1990), we predict that androgen levels will be (1) higher during the mating season than the non-mating season; (2) higher during the unstable than the stable period; (3) higher in more dominant individuals during the unstable but not stable periods (i.e. higher when male rank is overtly under challenge); (4) positively correlated with rates of aggression as a measure of male reproductive competition; (5) positively correlated with group-level mating activity as an alternative measure of male reproductive competition; (6) negatively correlated with rates of affiliative behavior with infants.

Methods

This study was conducted non-invasively and adhered to standards as defined by the European Union Council Directive172 86/609/EEC, and the Ethics Committee of the University of Roehampton (LSC 15/ 124). Research permission was provided by the Haut- Commissariat aux Eaux et Forêts et à la Lutte Contre la Désertification of Morocco. This research adhered to the American Society of Primatologists principles for the ethical treatment of primates.

Study Site and Subjects

This study was conducted on a wild group of Barbary macaques, at a site in Ifrane National Park (33°25.0N; 005°10.0W) in the Middle Atlas Mountains of Morocco. The study group (termed the “tourist group”) experiences daily tourist-macaque interactions, including feeding and taking of photographs (Maréchal et al., 2011). At the start of the study this group consisted of 40 individuals, including 12 adult males and 12 adult females, two sub-adult males, one sub-adult female, six juveniles and seven infants. Age classes were categorized after Fa (1984). This group was well habituated to the presence of observers and all adults and sub-adults could be individually identified. Behavioral and fecal data were collected between February and December 2012. This period encompassed the end of one mating season (MS1: 1 – 28 February 2012), followed by a six and a half month non-mating season (NMS: 01 March – 15 September 2012) and part of another mating season (MS2: 16 September – 27 December 2012). Mating season was defined as the time between the first and last ejaculatory copulations, although we excluded from this classification one apparently complete copulation that occurred 19 days after all other mating activity had ceased, and was therefore considered anomalous.

Overall, the present study utilized behavioral data and fecal samples collected from seven adult males of the tourist group (Table 1). Of the 12 adult males in the group, three young adults were excluded from data collection due to time constraints, one male died on 05 April 2012, another male was not seen again after sustaining a severe injury on 25 October 2012 and was presumed dead. To maintain a complete dataset across seasons, data from these latter two males were excluded from analyses. Two males included in analyses were not seen for some weeks during the mating season: CH was last seen on 24 October and reappeared on 20 November 2012; DO was last seen on 28 October and reappeared on 11 November 2012.

Behavioral Data Collection

Behavioral data were collected using continuous focal sampling (Altmann, 1974). Data were collected five days per week, from approximately 8 am to 5 pm on each study day. One continuous focal sample lasting from 10 to 20 minutes was taken per individual per day and the order of focal samples was randomized and spread throughout the day. Focal samples varied in length, as data were being collected as part of a post event-matched control study related to tourist-macaque interactions (Maréchal, Semple, Majolo, & MacLarnon, 2016), but this variation in focal sample length was not related to variables included in the present analysis. Aggressive behaviors recorded during focal samples were 'charge' and 'physical aggression'. A charge was defined as one monkey moving quickly and forcefully towards another for less than five meters. Physical aggression was defined as one monkey chasing another at high speed for over five meters and/or biting or slapping another monkey. The male-infant care behavior recorded during focal samples was male-infant grooming; both directions of grooming were included, as infants groom males while they are being tended by and/or are huddling with them. The number of unique mating dyads seen per day was recorded. Aggressive and displacement interactions between two males, where there was a clear winner and loser, were recorded *ad libitum* and used solely to determine dominance relationships. A displacement was defined as one monkey moving away from another approaching monkey, without the involvement of aggression. For aggressive events, a clear winner and loser were defined when one monkey displayed one or more submissive behaviors (present submission, make room, give ground or flee) in response to an aggression and without giving a counter aggression.

Fecal Sample Collection and Hormone Analysis

Fecal samples were collected using methods described by Hodges and Heistermann (2003). Briefly, when a monkey was seen defecating without urine or blood contamination, the fecal sample was collected and homogenized using a latex glove or stick, and solid objects (e.g. seeds) were removed. Approximately 2-3g was transferred to a collection tube (Azlon tubes 30 ML HDPE; cat. BWH0030PN). The tube was then stored in a padded box with ice blocks in the field before being stored in a freezer at – 20 °C at the end of the day. Samples were kept frozen while transported to the UK, and were then stored at – 20 °C at the University of Roehampton. Fecal samples were collected in such a way that, ideally, two samples were collected from each male in the first half of a month, and two samples in the second half (see Table 1 for total number of samples per male). Fecal samples were collected at any time during the day. Individual mean fecal androgen levels from samples collected in the morning (before 12pm) were not significantly different from those collected in the afternoon (after 12pm), suggesting that time of day had no effect on fecal androgen levels (morning: 1616 ± SD 807 ng/g dry fecal weight; afternoon: 1709 ± SD 932 ng/g dry fecal weight; Wilcoxon signed rank test: N=7, Z = -0.845, P = 0.398).

Fecal samples were extracted with 80% methanol using methods previously described (Heistermann, Finke, & Hodges, 1995). Extraction efficiency, determined by the recovery of tritiated estradiol added to the samples before extraction, was 85.1 ± 5.2% (Maréchal et al., 2011). Extracted fecal samples were assayed for epiandrosterone (fEA), a major metabolite of testosterone in macaque fecal samples (Möhle, Heistermann, Palme, & Hodges, 2002). Hormone levels were measured using microtiter enzyme immunoassays in accordance with protocols previously described (Girard-Buttoz, Heistermann, Krummel, & Engelhardt, 2009; Möhle et al., 2002). The assay was validated for use in Barbary macaques as a measure of androgen levels in fecal samples by comparing the levels of juvenile (fecal samples from another study at the same site) and adult males (samples from present study) (juveniles: N=6, mean=651.7ng EA/g dry fecal weight; adults: N=8, mean=3365.2ng EA/g dry fecal weight; Kruskal-Wallis test, p<0.01). This assay has previously been used successfully to measure androgen levels in other macaque species (Girard-Buttoz et al., 2009; Higham et al., 2013; Ostner et al., 2011). The intra- and inter- assay coefficients of variation for high and low value quality controls were 8.2% and 6.2% (high) and 7.7% and 15.5% (low), respectively. Serial dilutions of test samples had displacement curves parallel to the standard curve. The assay sensitivity at 90% binding was 0.65pg/50μl.

Table 1 here

Data Analysis

To compare seasonal differences in androgen levels, we combined hormone data from MS1 and MS2 in all analyses. We quantified rates of aggression (charge and physical aggression) per hour given or received by the focal male toward or from another male during focal observations as a measure of the level of male reproductive competition. As an alternative measure of male reproductive competition, we recorded the number of mating dyads in the group per day. To measure the level of male-infant care, we calculated the percentage of focal observation time that focal males spent in grooming interactions with infants.

Calculating dominance rank and defining stable/unstable periods in the male hierarchy

We constructed the male dominance hierarchy, based on 334 dyadic winner-loser interactions from nine adult males of the study group, by using an elo-rating system (Neumann et al., 2011). Data from the two males who died during the study period (see above) were used in the calculation of elo-ratings but not in further analyses. A plot of the elo-ratings for each adult male in the group over time is shown in Figure 1. As suggested by Neumann et al. (2011), elo-ratings before all individuals had at least nine interactions were considered provisional since they may not reflect an accurate measure of relative rank. For this reason, elo-ratings before May were not considered when determining rank. Males with a high rating were considered dominant over those with a lower rating. Elo-ratings were calculated in R 3.3.2 (R Core Team 2016) using functions adapted from the supplementary material from Neumann et al. (2011), and used to determine rank at the end of each season, as outlined below.

Figure 1 here

Rankings from May 2012 to the end of July 2012 were stable, with only relatively few temporary rank reversal interactions involving low ranking males - ranks 5-7, out of 8 (as well as the seven males in the main analyses, male NU was included here as he was alive for most of the study period). By contrast, beginning in early August, the three highest ranking males were involved in rank-reversal interactions. In particular, the male who was ranked alpha in the beginning of August (NU), rapidly lost rank over the next three months, before sustaining a severe injury on 25 October 2012 and subsequently disappearing. Then, beginning at the start of the mating season the rankings of three mid-ranking males also became unstable. By the end of the study period, it became apparent that four males had permanently changed ranks during the mating season (MS2): the males ranked second (KI) and third (TW) swapped ranks, and the males ranked fourth (FI) and fifth (DO) also swapped ranks. Therefore, study males were given two ordinal (1-7) rankings: one determined from Elo-ratings in mid-September, at the end of the non-mating season, was used for analyses up until this date (i.e. 06 February – 15 September), and one determined at the end of the study in late December, was used for analyses after this date (i.e. 16 September – 27 December). These two male dominance rankings were therefore used in further analyses (Table 1). Note that rank varies for some individuals (KI, TW, FI and DO) across the unstable period, which bridges the two rank periods.

We considered a period of hierarchy instability to have begun on 09 August 2012, when the top ranked male, NU (who was not included in main analyses due to his death during the study period) started losing rank (Fig. 1). The hierarchy remained unstable, meaning there was a relatively high proportion of rank-reversal interactions as well as the disappearance of NU, until the end of the study period (27 December 2012). Therefore, we defined the “unstable period” as being from 09 August – 27 December 2012. By contrast, the preceding five months of the non-mating season had relatively low number of rank reversal interactions. Therefore, we defined the “stable period” as being from 01 March – 08 August 2012.

Statistics

All data were found to be non-normally distributed using Kolmogorov-Smirnov tests. We used a set of Generalized Linear Mixed Models (GLMM) to test the influence of different variables on fEA levels. fEA values were log-transformed for analyses so that residuals from each model met assumptions of normality. All models were fitted with a Gaussian error structure. Each line in the dataset included log fEA levels from a single fecal sample. Because of a time lag of 24 – 36 hours to peak excretion of testosterone metabolites in macaque fecal samples (Möhle et al., 2002), we matched all samples with behavioral data and number of mating dyads from one and two days prior to the day that a fecal sample was collected. If data were available for both days prior to the collection of a fecal sample, then we used the mean of these two days, otherwise only the one available day of data was used. If there were no behavioral data available for either of the previous two days, then data from that fecal sample were not included in the GLMM model. In all models, male ID was included as a random factor.

We ran GLMMs in R 3.3.2 (R Core Team 2016) using the function lmer from the R package lme4 (Bates, Mächler, Bolker, & Walker, 2015). For each model, we checked assumptions of normality and homogeneity of residuals by visually inspecting q-q plot of residuals and a plot of residuals against their fitted values respectively. In models 2-7 we checked for collinearity of predictor variables by calculating Variance Inflation Factors (VIF: Field, 2005) of a standard linear model, excluding the random effect, using the vif function of R package car (Fox & Weisberg, 2011). Assumptions of normality were met and VIFs in all models were below 2.31. To test for the significance of the full models, we compared each full model to the corresponding null model which only included the random effect, using the R function ANOVA with argument set to “Chisq”. We determined the significance of the individual predictor variables by a likelihood ratio test by comparing the full with the respective reduced models (Barr, Levy, Scheepers, & Tily, 2013) using the R function drop1. We carried out Kruskal-Wallis and Wilcoxon signed-rank test tests using IBM SPSS Statistics version 21.

Relationship of male fEA levels with mating season and with rank stability

We constructed GLMMs to test for an association between log fEA levels and season (model 1), between log fEA levels and period (model 2), and between log fEA levels and both season and period (model 3). Note that since MS1 was considered neither a stable nor unstable period, we were only able to include MS2 in model 3. We used Wilcoxon signed-ranked test to assess whether there was a difference in mean fEA levels between consecutive months, i.e. to test for significant temporal changes in androgen levels.

Relationship of male fEA levels with rank and behavior

We constructed four GLMMs (models 4-7) to test for the relationship between log fEA levels and rank, male-male aggression, and male-infant grooming time. Models 4 and 6 explored these relationships during the mating season and unstable period, respectively, and also tested for a link between log fEA levels and number of mating dyads. Models 5 and 7 tested for relationships during the non-mating season and stable period, respectively.

Results

Androgen levels varied markedly over the study period (Fig. 2); levels were consistent from February to July, with then significant increases from July to August (Wilcoxon signed-rank test: N=7, Z = -2.366, $P < 0.05$), and August to September (N=7, Z = -2.366, $P < 0.05$); levels in September were not significantly different from October (N=7, Z = -0.338, P = 0.735) but dropped significantly from October to November (N=7, Z = -2.366, $P < 0.05$) and stayed low through December. Strong seasonal variation was also seen in levels of male-male aggression, with levels markedly higher during the mating season (Fig. 3a). Male-infant grooming time (Fig. 3b) and number of mating dyads (Fig. 3c), also peaked during the mating season, between October and December.

Figure 2 here

Figure 3 here

<u>Prediction 1. Androgen levels will be higher during the mating season than the non-mating season</u>

GLMM model 1, testing for the relationship between fEA and season, was significantly different from the null model ($\chi^2 = 4.18$, df=1, $P < 0.05$). Overall, male fEA levels were significantly higher during the mating season than during the non-mating season (Model 1: N=296, $P < 0.05$, mean fEA non-mating season=1464 ± SE 88 ng/g dry fecal weight, mean fEA mating season=1976 ± SE 186 ng/g dry fecal weight; increase from non-mating to mating season = 35%; Table 2).

Prediction 2. Androgen levels will be higher during the unstable than the stable period

GLMM model 2, testing for the relationship between fEA and rank stability period, was significantly different from the null model ($\chi^2 = 56.27$, df=1, $P < 0.001$). GLMM model 3, testing for the relationship between fEA and season and rank stability period together, was also significantly different from the null model ($\chi^2 = 64.40$, df=2, $P < 0.001$). fEA levels were significantly higher during the unstable period than the stable period (Model 2: N=270, $P < 0.001$, mean fEA stable period=1156 ± SE 56 ng/g dry fecal weight, mean fEA unstable period=2357 ± SE 184 ng/g dry fecal weight; increase from stable to unstable period = 104%; Table 2).

When included in the same model (model 3) mating seasonality and rank stability are correlated with each other (r=0.752), meaning that the results of testing predictions 1 and 2 presented above, demonstrating significant relationships between mating seasonality and fEA levels and between rank stability and fEA levels, may not be independent. fEA levels were more strongly related (significance and effect size) with rank stability than with mating seasonality (Model 3: Table 2).

Table 2 here

Prediction 3. Androgen levels will be higher in more dominant individuals during the unstable but not stable period

GLMM models 4 and 6, testing for the relationship between fEA and male-male aggression, male-infant grooming, rank stability, and group-level mating activity during the mating season and unstable period respectively, were significantly different from the null model (Model 4: $\chi^2 = 21.17$, df=4, $P < 0.001$; Model 6: $\chi^2 = 12.70$, df=4, $P < 0.05$). However, GLMM models 5 and 7 testing for the relationship between fEA and male-male aggression, male-infant grooming and rank stability during the non-mating season and stable period respectively were not significantly different from the null model (Model 5: $\chi^2 = 2.08$, df=3, $P = 0.556$; Model 7: $\chi^2 = 6.21$, df=3, $P = 0.102$).

Male rank had a significant negative association with fEA levels during the mating season and trended towards significance during the unstable period (Models 4 and 6, Table 3a and 3c). Male rank was not significantly associated with fEA levels during the non-mating season and stable period (Models 5 and 7, Table 3b and 3d). In other words, during the mating season and unstable period, males with high rank generally had higher fEA levels than lower ranked males.

Prediction 4. Androgen levels will be positively correlated with rates of aggression as a measure of male reproductive competition

fEA levels were not related to rates of male aggression during the mating season, non-mating season, unstable period, or stable period (Models 4-7, Table 3).

Prediction 5. Androgen levels will be positively correlated with group-level mating activity as a measure of male reproductive competition

The number of mating dyads per day was significantly and positively associated with fEA levels during the mating season, but not during the unstable period (Models 4 and 6, Table 3a and 3c).

Prediction 6. Androgen levels will be negatively correlated with rates of infant care

Male-infant grooming time had a significant negative association with fEA levels during the mating season and unstable period; in other words, when fEA levels were higher in these periods, male-infant grooming time was lower (Models 4 and 6, Table 3a and 3c). A positive association was found between male-infant grooming time and fEA levels in the stable period, such that higher fEA levels in this period were linked with higher levels of male-infant grooming (Model 7, Table 3d). However, since the full model for the stable period was not significantly different from the null model, this result must be interpreted with caution. No link was found between male-infant grooming time and fEA levels during the non-mating season (Model 5, Table 3b).

Table 3 here

Discussion

The unusual biology of the Barbary macaque, in terms of its combination of social factors, reproductive seasonality, and male-infant interactions, allowed us to test simultaneously predictions of the challenge hypothesis relating to reproductive competition, rank stability and male affiliative behavior with infants. Overall, our findings provide general support for this hypothesis, and provide new insights into the factors affecting androgen levels among male primates.

Among our study animals, androgen levels began to rise in August, well before the mating season, peaking six weeks later, at the start of this season, and then declining for the remainder of its duration. The August rise in androgen levels was coincident with the beginning of the five-month period of rank instability, with peak levels seen six weeks into this time. Overall, androgen levels were higher in the mating season and unstable period compared to the non-mating and stable periods respectively, with the difference being greater between the stable v. unstable periods than between the non-mating v. mating seasons. There are two possible explanations for this temporal pattern of change in androgen levels. Firstly, the coincident increases in androgen levels and instability in the hierarchy indicate these may be causally related – the challenges of attempting to move up in rank may lead to increases in androgen levels, increases in androgen levels may lead to males attempting to make such a move, or both effects may be at work (Wingfield et al., 1990). Secondly, a rise in androgens preceding the onset of mating behavior indicates males may undergo physiological preparation for the mating season, as has been suggested to explain similar pre-mating season rises in androgen levels in long-tailed macaques (Girard-Buttoz et al., 2015) and Assamese macaques (Ostner et al., 2011). It is possible that both processes are important, even though the direct temporal association was stronger between variation in androgen levels and rank stability than between androgen levels and mating seasonality. Rank instability is relatively rare in the study population, but when it occurs it is usually associated with the mating season (Majolo, pers. obs.), as in our study year. In male Barbary macaques, rank instability during the mating season may be due to an increase in coalition formation during this period (Berghänel, Ostner, Schröder et al., 2011), especially by older and lower ranking post-prime males against younger and higher ranking prime males, which de-stabilize dyadic dominance relationships (Berghänel, Ostner, & Schülke, 2011; Bissonnette et al., 2011).

It is notable that androgen levels peaked at the beginning of the mating season, in the middle of September, and then dropped markedly throughout the rest of this season, during which time levels of mating activity and aggression were at their highest. This drop in androgen levels may be linked to changes in body condition (Pérez-Rodríguez, Blas, Viñuela, Marchant, & Bortolotti, 2006), such that at the start of the mating season males are in optimal body condition and able to sustain high androgen levels, but as physical condition deteriorates over the course of this season androgen levels fall accordingly. In rhesus and Assamese macaques, body condition declines during the mating season (Higham et al., 2013; Ostner et al., 2011) and the same may be true of Barbary macaques, although crude measures of condition - body size or coat quality - did not decline across the mating season in our study animals (Maréchal et al., 2016).

High ranking males generally had higher androgen levels than subordinates during the mating season and the unstable period, but not outside of these times. These results are in line with studies which found elevated androgen levels in dominant individuals during periods of social instability in seasonally breeding (Brockman et al., 2001; Higham et al., 2013) and non-seasonally breeding primate species (Marshall & Hohmann, 2005; Sapolsky, 1983). The lack of an association between androgen levels and rank during the period of hierarchy stability and outside the mating season contrasts with the pattern seen in yellow baboons, where higher ranked males have higher androgen levels in both stable and unstable periods (Gesquiere et al., 2011), and in mandrills where androgen levels are related to rank, independent of rank stability and mating period (Setchell et al., 2008). This difference may be explained by the more relaxed dominance style and relatively egalitarian social system of male Barbary macaques (Preuschoft et al., 1998; Thierry & Aureli, 2006), under which maintenance of rank does not present a year-round challenge.

Contrary to the prediction of the challenge hypothesis, we found no link between androgen levels and rates of aggression in our study species. From the studies that have tested such a relationship previously in primates, no clear pattern has emerged, with no obvious factor such as breeding seasonality or mating system differentiating those species in which the prediction is supported (e.g. Ostner et al., 2011; Sapolsky, 1983; Teichroeb & Sicotte, 2008) from those in which it is not (e.g. Girard-Buttoz et al., 2015; Kalbitzer et al., 2015). In Barbary macaques, despite a marked rise in rates of aggression in the mating season, these rates are still relatively low; overall, overt aggression may not provide the most appropriate measure of the levels of male-male competition experienced in our study species, although it is also possible that the estimated excretion lag time between serum and fecal androgen levels used in analyses here is too long, or too short. Disentangling rates of aggression, rank instability, and other measures of competition (including for example coalitions and group take-overs) across different species may provide a clearer understanding of how androgen levels respond to - or are shaped by - male-male reproductive competition. Our finding of a positive relationship during the mating season between androgen levels and mating competition, as determined by group-level mating activity, suggests that the latter provides a more meaningful index of reproductive competition among male Barbary macaques than do rates of aggression. Together, these findings highlight the importance of considering the behavioral ecology of the species in question when seeking the best means to test predictions of the challenge hypothesis in different contexts.

We found a negative relationship between androgen levels and rates of male affiliative behavior towards infants in Barbary macaques in both the mating season and the unstable period, in line with the prediction of the challenge hypothesis that testosterone inhibits male care of infants. In contrast, we found a positive relationship between these variables in the stable period although full model was not significantly different from the null model. While we must be cautious with the interpretation, a number of factors may explain this latter result, each of which is linked to the fact that this period includes the birth season and first few months of infants' lives. Firstly, this is a time when infanticide risk is likely to be higher as infants are still dependent on their mothers, and these females are not yet cycling (Hrdy, 1979); during this period, male affiliative behavior may serve the function of infant guarding, with androgen levels elevated in response to the potential challenge. In support of this idea, it has been found that male Barbary macaques that spent more time carrying infants had higher physiological stress levels (Henkel, Heistermann, & Fischer, 2010). Alternatively, as male handling may increase access to females in the subsequent mating season (Ménard et al., 2001), males may be competing for access to infants as a form of reproductive competition; this again highlights the importance of considering for each species the different ways in which males compete among themselves.

The results of the current study add to our understanding of the complex array of specific behavioral features that underpin temporal variation in androgen levels - both within and between individuals - among different primate species. Most predictions derived from the challenge hypothesis were met. For those that were not, the specific behaviors explored (e.g. aggression) may not most accurately reflect in this species the nature of male-male challenge and male-infant care - the fundamental principles on which this hypothesis is based. Careful consideration of species' behavioral and reproductive ecology is important to generate the most biologically relevant tests of the challenge hypothesis.

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Figure legends:

Fig. 1: Elo-ratings for nine adult males in the study group. Each symbol on a line represents when a new dyadic dominance interaction was recorded. A high elo-rating indicates high rank. Rankings before May were considered provisional and not an accurate reflection of relative rank. Note that one male (MI) died on 05 April and another male (NU) disappeared on the 25 October and therefore there are no rating interactions for these males after these dates respectively. MS1 (06 Feb – 28 Feb) and MS2 (16 Sep – 27 Dec) = mating season 1 and 2, NMS (01 Mar – 15 Sep) = non-mating season. Stable period: 01 Mar – 08 Aug, unstable period: 09 Aug – 27 Dec.

Fig. 2: Bi-weekly variation in fEA levels of seven adult males across the study period. Depicted are means ± SE. MS1 (06 Feb – 28 Feb) and MS2 (16 Sep – 27 Dec) = mating season 1 and 2, NMS (01 Mar – 15 Sep) = non-mating season. Stable period: 01 Mar – 08 Aug, unstable period: 09 Aug – 27 Dec.

Fig. 3: Bi-weekly variation in: (a) male-male aggression per hour; (b) male-infant grooming time; (c) daily mating dyads. Depicted are means ± SE. MS1 (06 Feb – 28 Feb) and MS2 (16 Sep – 27 Dec) = mating season 1 and 2, NMS (01 Mar – 15 Sep) = non-mating season. Stable period: 01 Mar – 08 Aug, unstable period: 09 Aug – 27 Dec.

TABLE 1: A summary of focal hours and hormonal data collected per study subject (excluding NU who disappeared part way through the second period (P2). Some males had different ranks for different parts of the study period (1 = most dominant, 7 = most subordinate): P1 = 6 Feb - 15 Sep 2012. P2 = 16 Sep - 27 Dec 2012.

Male ID	Rank P1 (P2)	Observation days	Fecal samples	Mean fEA (ng/ g dry fecal weight)	Observation time (hours)
GA	1 (1)	200	44	1337	38.5
KI	2 (3)	193	42	1764	36.1
TW	3 (2)	201	43	3497	37.7
FI	4 (5)	201	41	1659	37.2
DO	5 (4)	193	41	1249	36.2
CH	6 (6)	180	41	1346	33.8
PE	7 (7)	198	44	829	31.5
Total			296		251.0

TABLE 2: Summary of GLMM results for the relationship between fEA with (a) season, (b) period and (c) both season and period. In all models: response = log fEA, Number of males = 7, random effect = male ID

	Estimate	**SE**	**χ^2**	**df**	**P-value**
(a) Model 1: Season (N=296)					
Intercept	3.138	0.068	-	-	-
Season (non-mating, mating)	-0.062	0.030	4.18	1	0.041
(b) Model 2: Period (N=270)					
Intercept	3.010	0.064	-	-	-
Period (unstable, stable)	0.222	0.028	56.27	1	< 0.001
(c) Model 3: Season and period (N=270)					
Intercept	2.884	0.077	-	-	-
Season (non-mating, mating)	0.127	0.044	8.12	1	0.004
Period (unstable, stable)	0.313	0.042	50.49	1	< 0.001

TABLE 3: Summary of GLMM results for (a) the mating season, (b) the non-mating season, (c) the unstable period and (d) the stable period. In all models: response = log fEA, Number of males = 7, random effect = male ID

	Estimate	SE	χ^2	df	P-value
(a) Model 4: Mating season (N=99)					
Intercept	3.402	0.115	-	-	-
Aggression/ hour	0.037	0.031	1.32	1	0.251
Male-infant grooming (% time)	-0.483	0.227	4.40	1	0.036
Mating dyads/ day	0.033	0.011	7.56	1	0.006
Rank	-0.084	0.025	8.12	1	0.004
(b) Model 5: Non-mating season (N=162)					
Intercept	3.211	0.121	-	-	-
Aggression/ hour	0.005	0.011	0.19	1	0.664
Male-infant grooming (% time)	0.243	0.317	0.58	1	0.445
Rank	-0.033	0.027	1.39	1	0.239
(c) Model 6: Unstable period (N=118)					
Intercept	3.506	0.125	-	-	-
Aggression/ hour	0.004	0.014	0.10	1	0.754
Male-infant grooming (% time)	-0.614	0.217	7.63	1	0.006
Mating dyads/ day	-0.004	0.010	3.82	1	0.694
Rank	-0.060	0.027	0.15	1	0.051
(d) Model 7: Stable period (N=125)					
Intercept	3.123	0.123	-	-	-
Aggression/ hour	0.015	0.013	1.30	1	0.255
Male-infant grooming (% time)	0.464	0.230	3.99	1	0.046
Rank	-0.031	0.027	1.17	1	0.278