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Article

Androgen correlates of male reproductive effort in wild male long-tailed macaques (*Macaca fascicularis*): A multi-level test of the challenge hypothesis

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HIGHLIGHTS

- We studied social and ecological correlates of male androgen levels in wild macaques.
- Androgen levels rose with seasonal and social short-term reproductive challenges.
- Parity status and dominance rank of guarded females influenced male androgen levels.
- Albeit seasonal variation, high-ranking males had higher androgen levels year round.
- Androgen rise serves to enhance female monopolisation and male dominance tenure.

ARTICLE INFO

ABSTRACT

The challenge hypothesis (Wingfield et al., 1990) has been broadly utilised as a conceptual framework to study male androgen correlates of reproductive challenges in mammals. These studies mainly assessed male androgen responsiveness to a general degree of challenge over extended periods of time. Short term co-variation between the socio-sexual challenging context and androgen levels remains, however, largely understudied. We thus aim at providing a multi-level test of the challenge hypothesis by investigating the inter- and intra-individual variations in faecal androgen excretion associated to 1) breeding seasonality, 2) dominance rank, 3) mate-guarding activity and 4) value of the guarded female. We studied long-tailed macaques, a species in which males engage in highly challenging monopolisation of females over discreet periods of time. This particularity allows testing specifically the predicted increase from *level B* to *level C* in the challenge hypothesis. The study was carried out during two reproductive seasons on three groups of wild long-tailed macaques. We combined behavioural observations and non-invasive measurements of faecal androgen metabolite (fAM) levels. We found that, as predicted by the challenge hypothesis, male long-tailed macaques respond not only to seasonal but also to short term reproductive challenges by adapting their androgen levels. First, males exhibited a seasonal rise in fAM levels during the mating period which may be triggered by fruit availability as shown by our phenological data. Second, males had increased androgen levels when mate-guarding females and, across mate-guarding periods, males had higher fAM levels when monopolising high-ranking parous females than when monopolising low-ranking ones. Finally, high-ranking males had higher fAM levels than low-ranking males year round. Our study confirms that, in species with a high degree of female monopolisability, androgen may be an important physiological fitness enhancing tool for males by increasing female monopolisation efficiency (in particular with highly valuable females) and helping males to respond to rank take-over challenges.

1. Introduction

In vertebrates, androgens play an important role in priming males for the challenge of male–male competition over access to

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receptive females (reviewed in [1–3]). Androgens mediate muscle mass growth [4,5], enhance muscular performances [6] and allows for immunoredistribution of leucocytes to the periphery to prepare for potential injuries [7]. They also promote spermatogenesis (i.e. activating sperm production and enhancing sperm concentration, [4,8,9]) and are often associated with the expression of male sexual and aggressive behaviours (reviewed in [10,11]). Androgens thus affect a variety of traits that determine male–male competitive ability for successful reproduction and, by influencing male sexual behaviour and the outcome of male–male competition, greatly affects individuals' fitness [12,13]. Therefore, assessing the link between temporal fluctuation in androgen levels and male reproductive behaviours is important to understand the physiological mechanisms regulating male reproductive performance.

The "challenge hypothesis" [10] was proposed to conceptualise the relationship between males' socio-sexual behaviours and their androgen levels. This hypothesis postulates the existence of three levels of circulating androgens [10,14]: *level A*, which is the non-breeding androgen baseline, *level B*, which is the breeding baseline, sufficient for spermatogenesis to proceed and for the expression of sexual behaviours, and *level C*, which is the physiological maximum androgen level that is reached in response to specific social challenges encountered during the breeding period (e.g. male–male aggression, territory defence, female monopolisation).

Initially proposed to explain the seasonal and proximate variations of androgen levels in birds, the challenge hypothesis has meanwhile been tested in a broad range of vertebrate taxa (reviewed in [11]). These studies have largely confirmed the observation made in birds and show that the seasonal increase in androgen levels from *level A* to *level B* occurs in a diverse range of seasonally breeding vertebrates and that this elevation is often associated with an increase in aggression rate (mammals, [15–17], reptiles, [18], for a review on birds see [10, 19]). Furthermore, studies on several fish and bird species confirmed the proposed proximate male androgen responsiveness to specific social challenges (i.e. increase in androgen levels from *level B* to *level C*) using simulated territorial intrusion experiments in the presence of a fertile female [14,20]. In mammals, and in particular in primates, the challenge hypothesis has been utilised as a theoretical framework to study the relationship between male socio-sexual behaviours and androgen levels [16,17,21–23]. From these studies it is known, for example, that in a breeding context (i.e. in the presence of fertile females) primate male androgen levels correlate positively with the rate of inter-group encounters (e.g. white-faced capuchins, *Cebus capucinus* [24]), male–male aggression rates (e.g. long-tailed, *Macaca fascicularis* and Assamese macaques, *M. assamensis* [16,17]), and the occurrence of mate-guarding behaviour (e.g. savannah baboons, *Papio cynocephalus* [23]). Basing their analysis on behavioural and physiological parameters averaged per individual and/or over extended period of time (e.g. 1 month blocks), all of these studies examined male androgen responsiveness to a general degree of challenge. The pattern of intra-individual co-variation between the socio-sexual challenging context and androgen levels on a shorter term, for example daily, basis (i.e. the rise in androgens from *level B* to *level C* according to the challenge hypothesis), however, is so far largely unstudied in mammals.

The timely restricted mate-guarding behaviour seen in many mammalian species may be a very suitable behaviour for testing the androgen rise from *level B* to *level C* predicted by the challenge hypothesis. In primates, mate-guarding is commonly defined as a "close, persistent following of a female by a male that involves exclusion of other males from access to the female" (cf. [25]). It is a highly challenging and costly behaviour for male primates since during mate-guarding, males face a decrease in feeding time [25–29] and an elevation in physiological stress levels [30,31], aggression rates [30,32] and vigilance time [30]. These costs are outweighed by the fact that mate-guarding significantly increases male reproductive success (rhesus macaques, *Macaca mulatta* [33,34], long-tailed macaques [35,36]; Japanese macaques, *M. fuscata* [28]; mandrills, *Mandrillus sphinx* [37]), and a recent revision of the

"challenge hypothesis" predicts a stronger androgen response to challenges associated with high fitness benefits [14]. Since males do not monopolise females continuously during the entire breeding season but mate-guard over discreet periods of a few hours up to a month (reviewed in [38]), this behaviour specifically allows to test the prediction of androgen responsiveness to specific social challenges (i.e. rise from *level B* to *level C*) as postulated by the challenge hypothesis.

Beyond the general difference between mate-guarding and non-mate-guarding periods, the level of challenge can also vary across different mate-guarding periods depending on the value of the mate-guarded female. In primates, the reproductive value of females often varies with dominance and parity status. High-ranking and/or parous females often produce more offspring and offspring of better quality (i.e. more likely to survive until adulthood) than low ranking and/or nulliparous females [39–43]. In turn, males preferentially mate-guard and/or mate with high-ranking parous females than with low-ranking nulliparous ones (e.g. Barbary macaques, *Macaca sylvanus* [44]; long-tailed macaques [35], chimpanzees [45]; mandrills [46]; savannah baboons [47]). In some species, males also exhibit mating preferences towards females with whom they have strong social bonds, independent of female rank, parity or fertility status (e.g. rhesus macaques [48]; Japanese macaques [49]; savannah baboons [47]). This overall mate-choice towards females with higher reproductive and social value can also be expressed at the level of the investment and thoroughness with which males mate-guard the females. In long-tailed macaques, males are more aggressive towards other males when mate-guarding high-ranking females and/or females with whom they have strong social bonds than when mate-guarding other females [50]. This latter example highlights how the level of challenge faced by the male during mate-guarding depends on the value of the female. This thus may add an additional dimension of analysis to the "challenge hypothesis", i.e. androgen responsiveness in relation to differences in the quality of the females being mate-guarded, that remains to be investigated.

In our study we aimed to provide a multi-level test of the challenge hypothesis. Specifically we aimed to investigate the inter- and intra-individual variations in faecal androgen metabolite (fAM) excretion associated to 1) breeding seasonality, 2) male dominance rank, 3) mate-guarding effort and 4) value of the female monopolised. Inter-individual variations refer to variations in fAM levels across individuals, which can be attributed to consistent inter-individual differences in other parameters (e.g. dominance rank). Intra-individual variations refer to variation in fAM levels within the same individual across time in response to specific seasonal and/or social challenges (e.g. mating period, mate-guarding effort). We used long-tailed macaques as a model species. In original primary rainforests, this species lives in medium sized (10–50 individuals) multi-male multi-female groups, which contain around 2–8 adult males and 4–18 adult females [39]. Males leave their natal group as sub-adults to reach maturity in a new group [40]. Overall, males migrate several times during their lifetime, whereas females remain life-long in their natal group where they form matrilines. Long-tailed macaques are non-strictly seasonal breeders, i.e. females can conceive year round [51]. There are nevertheless periods of 4–7 months during which the number of receptive females and subsequent births (in the wild on average 163 days after conception [36]) is increased, the timing of which depends on inter-annual variation in seasonality of fruit abundance [51]. Females undergo 1–3 ovarian cycles before conceiving and are sexually active almost throughout the whole ovarian cycle and additionally during the first and second trimester of pregnancy [36,52]. During both periods of sexual activity, females mate on average once per hour during peak times (i.e. the fertile phase of the ovarian cycle an days 48 to 52 after conception) and with a variety of males, but are also intensively and for a number of subsequent days mate-guarded by specific males [36,52]. During ovarian cycles, only high ranking males (alpha- and beta-males) invest extensively into mate-guarding, and particularly the alpha-male mate-guards females throughout the fertile phase [35,36]. Consequently,

male reproductive success is highly skewed towards the alpha-male in long-tailed macaques [35,36] and males fight with each other over rank [39,40]. Alpha-male tenure lasts on average 25 months and the alpha-male is challenged by outside as well as resident males, whereby resident males are usually more successful [39,40]. Obviously, mate-guarding is highly beneficial for male long-tailed macaques [36]. Yet, this behaviour is also challenging since males experience higher physiological stress levels and higher rates of male–male aggression during mate-guarding [30]. In long-tailed macaques, males exhibit a seasonal rise in androgen levels during the reproductive period [16], but little is known about the factors driving inter- and intra-individual temporal differences in androgen levels in response to specific male reproductive challenges.

In a previous study [16], we analysed seasonal pattern in faecal androgen excretion in male long-tailed macaques over a single reproductive season. In the current study we first aimed at examining this pattern over an extended time period (i.e. two mating periods). Based on our previous finding, we predict that male androgen levels will be higher during the mating periods than during the non-mating periods (rise from *level A* to *level B* sensu the challenge hypothesis), (Prediction *P1*). Secondly, we tested for androgen correlates of male dominance rank between high-ranking males (alpha- and beta-males, i.e. mate-guarding males) and low ranking males (other males from the same groups, i.e. non-mate-guarding males). We predict that high ranking males have higher androgen levels than low ranking males during reproductive periods (when mate-guarding behaviours are expressed) but not during non-reproductive periods (Prediction *P2*). Thirdly, we analysed whether mate-guarding effort is associated with an intra-individual increase in androgen levels. Here, we specifically tested for the predicted rise from *level B* to *level C* sensu the challenge hypothesis using mate-guarding as an example of a challenging behaviour. We predict that androgen levels are higher on days when males mate-guard females than on other days (Prediction *P3*). Finally, we investigated whether the magnitude of the rise in faecal androgen metabolites from *level B* to *level C* (if any) is associated with the degree of the challenge. To this aim, we assessed the effect of the guarded females' reproductive (i.e. female dominance rank and parity status) and social (i.e. the strength of male–female social bonds) values on male fAM levels during mate-guarding. We predict that males experience a higher rise in fAM levels when mate-guarding high-ranking, parous and/or strongly socially bonded females than when monopolising other females (Prediction *P4*).

2. Methods

2.1. Animals and study site

The study was carried out on three groups of wild long-tailed macaques (*M. fascicularis*) living in the primary lowland rainforest surrounding the Ketambe Research Station (3°41′N, 97°39′E), Gunung Leuser National Park, North-Sumatra, Indonesia. The forest structure and phenological composition have been described in detail by Rijksen [53] and van Schaik and Mirmanto [54]. The long-tailed macaques in the area have been studied since 1979 [35,51,55]. For our study, we collected data between March 2010 and April 2011 focusing on three groups: Camp (C), Ketambe Bawa (KB) and Ketambe Atas (KA). Faecal samples were collected regularly during the entire study period and behavioural data during two consecutive mating periods (see below). All adult individuals were individually known and well habituated to human observers. The total size of a social group varied from 22 to 58 individuals (see Table 1 for details on group compositions). The study was conducted completely non-invasively and under the permission of the authorities of Indonesia. We adhered to the Guidelines of the Use of Animals in Research, the legal requirements of Indonesia and the guidelines of the involved institutes.

Table 1
Composition and mating periods of the study groups.

Group	N. females	N. males	N. total	Mating period 1	Mating period 2
Camp	14–15	6–9	54–58	01.03.10–06.06.10	17.02.11–14.04.11
Ketambe Bawa	9–10	4–8	31–36	01.03.10–14.07.10	18.01.11–14.04.11
Ketambe Atas	7	4–7	22–25		18.12.10–14.04.11

2.2. Behavioural data collection

Behavioural data were collected by C.G-B and six experienced Indonesian and international field assistants. All assistants were trained by C.G-B for two months and inter-observer reliability was assessed repeatedly based on behavioural observations collected simultaneously by two observers on the same focal animal (measurement of agreement kappa >0.8 for each assistant). The observations covered two mating periods. Mating period was defined for each group separately as the period between the first mate-guarding day and the last mate-guarding day ever observed by any male of the respective group (see Table 1 for details about the mating periods in the different study groups). From March to July 2010, four observers followed groups C and KB every day (two observers in each group) and from December 2010 until April 2011, all three groups were generally followed every other day and frequency of observation increased to every day when alpha- and/or beta-males were observed mate-guarding. The group KA was not studied between March and July 2010 because the habituation of this group was being finalised during that time.

Each day, groups were followed from dawn to dusk. We collected focal behavioural observations solely on alpha- and beta-males because they are the only males to mate-guard females extensively in long-tailed macaques [36]. All behavioural data were recorded using a hand-held computer Psion Workabout Pro (Teklogix®). Every evening, the identity of the males to observe the next day was determined between all the alpha- and beta-males of each study group based on the mate-guarding activity of each male and on whether they were followed or not that day. Priority was given to mate-guarding males (i.e. males who spent more than 50% of the time following a female). Which male was mate-guarding on any given day was determined every evening directly in the field based on the data uploaded from the handheld into a laptop supplemented by ad libitum observation of non-focal male mate-guarding behaviour. Males were then followed half or full day depending on the number of observers available and on the number of males to follow (e.g. when four observers were in the forest and more than four males were mate-guarding females the previous day, at least one observer had to switch males after 6 h of observations in order to cover all the mate-guarding males that day). On each observation day, one observer was solely responsible for the entire focal protocol of one male.

The activity of the focal animal was recorded every minute using instantaneous sampling [56] and comprised the following categories: resting, being vigilant, feeding, drinking, travelling, aggressing (receiving and/or giving aggressions), affiliating, allogrooming and self-grooming (for a full description of the behavioural categories see [30]). The mate-guarding behaviour of the focal male and the distance between him and the mate-guarded female were also recorded every minute. Whether a male was mate-guarding or not a female on a given minute was coded a posteriori. A male was considered as "mate-guarding" when he followed a sexually active female for more than 5 consecutive minutes and maintained a distance of less than or equal to 10 m between him and the female. A female was considered sexually active if she was observed copulating at least once on a given day. When the female moved away from the male and the male did not follow her for more than 2 min, the mate-guarding activity was considered to have ended. Daily mate-guarding time was quantified as overall time spent

mate-guarding any female regardless of whether the male mate-guarded the same female for the entire day or mate-guarded different females consecutively. In addition, all copulations and aggressions (including submissive expressions) between any adult individuals were recorded using all occurrences sampling for the focal male and ad libitum for all the other individuals. Aggressions comprised threatening, chasing, hitting and biting. Finally, the identities of all males within 10 m of the focal individual were recorded every 5 min.

2.3. Faecal sample collection and hormone analysis

Faecal samples were generally collected once a week from four males in each group: alpha- and beta-males and two low-ranking males (rank 3 and bellow) to determine the general effect of male dominance rank on male faecal androgen metabolite (fAM) levels (i.e. to test prediction *P2*: see Table 2 for number of faecal samples collected per male). For low-ranking males, we selected the two non-alpha-, non-beta-males which were the ones most often present in the group during the 6 months preceding the data collection period. In group C, a male left the group in December 2010 and we then started collecting faecal samples from a fifth male in the group in order to have always 2 low ranking males as comparative basis (see Table 2 for details on sample collection period for each male). In addition, during mate-guarding periods, we collected one faecal sample every third day from the mate-guarding male. Samples were collected at different times of the day (in the morning and in the afternoon); however, the time of sample collection was shown to have no significant influence on fAM levels in male long-tailed macaques [16]. Right after defecation, samples were homogenised and 2–3 g of faeces were collected and stored in a polypropylene vial and placed on ice in a thermos bottle. At the end of each fieldwork day, the samples were frozen at −20 °C in a freezer. In July 2011, all samples were transported, on ice, to the hormone laboratory of the Bogor Agricultural University (IPB) and then freeze-dried and pulverised before transportation to the Endocrinology Laboratory of the German Primate Centre for analysis.

For hormone analysis, an aliquot (50–70 mg) of the faecal powder was extracted within 3 ml of 80% methanol by vortexing for 10 min [57,58]. For monitoring changes in androgen levels, faecal extracts were analysed for immunoreactive epiandrosterone [59], a major metabolite of testosterone in macaques [17,60]. The assay has been previously validated and successfully used to reliably assess androgen levels from faeces in several species of macaques (e.g. [17,61]) including long-tailed macaques [16].

Hormone measurements were carried out by microtiter plate enzyme immunoassay according to methods previously described [16, 62]. Intra- and inter-assay coefficients of variation of high- and low-value quality controls were 8.9% and 13.7% (high) and 6.3% and 15.8% (low), respectively. All faecal hormone levels reported are expressed as ng/g dry faecal weight.

2.4. Determination of dominance hierarchy and female parity status

During the focal samples, we recorded ad libitum [56] any agonistic interaction and the occurrence of 'bared-teeth-face', a unidirectional submissive display [63] between any adult members of the groups. We built the dominance hierarchy in each group for males and females separately using aggressive or submissive interactions in which a clear winner and loser could be identified. The aggressive interactions used in this analysis were chase and displacement. We then entered winner and loser into a sociometric matrix and compiled dominance ranks with Matman 1.1.4 using the I&SI method with 10,000 randomisations [64]. We used I&SI since this method performs better than the David's score when interactions between some dyads are missing (see [65]). Prior to statistical analysis (see below), we standardised female ordinal rank to a mean of 0 and a standard deviation of 1 in each group to obtain a range of values comparable between the three groups containing different numbers of females (Table 1). Following standardisation, high ranking females received scores that were <0 and low ranking females received scores that were >0.

Since the long-tailed macaques in the Ketambe research area have not been studied between 2001 and the start of our study in 2010, we did not know the reproductive history of the study females. Consequently, we assessed the parity status of the female visually based on the size of the nipples. In long-tailed macaques, nulliparous female nipples are similar to male nipples and distinctively shorter than parous female nipples.

2.5. Determination of fruit availability

We included fruit availability into our analysis as a control variable since it has recently been suggested that male androgen levels may respond to food availability [14]. For this, we selected 120 trees (360 trees in total) from species amongst the tree species producing fruit eaten by *M. fascicularis* [66] in each of the three study group home ranges. For details about the tree selection procedure see [29]. Each tree was surveyed monthly, within the last 3 days of every month, by a field assistant experienced in phenology and fruit abundance was recorded using a logarithmic scale (0: absence, 1: 1–10 items, 2:11–100, 3:101–1000, 4:1001–10000, 5: >10000). The average monthly score of fruit abundance in each territory was highly correlated with the percentage of trees fruiting. We therefore used percentage of trees fruiting as an index of fruit availability for the analyses.

2.6. Statistical analyses

In order to investigate our different predictions (*P1–P4*), we used a set of three different generalised linear mixed models (GLMMs [67]) with Gaussian error structure. In each model, fAM levels were the

Table 2
Dominance rank, number of faecal samples collected, period of faecal sample collection and behavioural observation time for each of the study males.

Male ID	Group	Rank	N. faecal samples	Period of faecal sample collection	Focal observation time (hours)	Observation days
QJ	C	1	93	March 2010–April 2011	668	147
RJ	C	2	85	March 2010–April 2011	455	114
GJ	C	3	87	March 2010–April 2011		
HJ	C	4	19	December 2010–April 2011		
YJ	C	5	35	March–November 2010		
CJ	KB	1	94	March 2010–April 2011	388	122
SJ	KB	2	82	March 2010–April 2011	323	85
MJ	KB	3	54	March 2010–April 2011		
IJ	KB	4	48	March 2010–February 2011		
LJ	KA	1	42	October 2010–April 2011	185	68
ZJ	KA	2	30	October 2010–April 2011	111	48
KJ	KA	3	11	October 2010–March 2011		
DJ	KA	4	10	October 2010–March 2011		

response variable and values were log-transformed to achieve a symmetric distribution.

2.6.1. Influence of mating period and rank on male fAM levels

To test whether male reproductive effort influences inter-individual variation in fAM levels, we analysed the effect of dominance rank (as a proxy for male reproductive effort) and of period (mating vs. non-mating periods) on fAM levels (Predictions *P1* and *P2*). We used a GLMM with fAM levels as the response. Each line of the model (i.e. each data point) consisted of the fAM level of a single sample. In this model, we included male dominance rank (two categories: high-ranking for alpha- and beta-males, and low-ranking for two other males in the same group), period and the interaction between the two as fixed factors. In this analysis, we also included fruit availability as fixed effect and animal ID and group as nested random factors. Fruit availability on a given day was approximated using the fruit availability measured on the closest monthly record. For example, the percentage of tree fruiting recorded on the 31st of January was used as the fruit availability score for all the days between the 16th of January and the 15th of February. Since the interaction between dominance rank and period was not significant in this model (LRT, P > 0.8), we reran the model without the interaction.

2.6.2. Influence of mate-guarding, copulation and aggression on male fAM levels

For analysing the influence of male behaviour, and in particular of mate-guarding, on intra-individual variation in fAM levels, we considered only those days of observation for which at least 1 h of focal data was recorded. The final data set comprised 2088 h of focal observations over 600 days (see [29] for detail about observation and mate-guarding time).

For each day, we calculated the percentage of observation time spent mate-guarding by each focal male. We also calculated for every observation day the copulation rate (i.e. number of copulations between the focal male and any adult female per hour), the rate of male–male aggressions (i.e. the number of aggressions given and received by the focal male from/towards any other adult male per observation hour) and the number of males in proximity (defined as the average number of adult males within 10 m per 5 min scan). We also calculated the number of sexually active females in each group on each observation day.

We tested whether the percentage of time spent mate-guarding on a given day affected fAM levels (Prediction *P3*) using a GLMM (model 2). Since the time-lag for excretion of testosterone metabolites into the faeces is between 24 and 56 h in long-tailed macaques [60], we matched daily mate-guarding time, aggression and copulation rates (see above) with fAM levels measured in samples collected at either day +1 or day +2 after the observations. When samples were available at both days, we used the mean fAM levels of the two samples. In this model, we also added the daily rate of copulations and of aggressions with other males to test if this factor influenced fAM output. Each data point in our model was thus an observation day. In addition, as for model 1, we also included fruit availability and male dominance rank as factors in model 2. Finally, to account for the degree of male–male competition for access to receptive females and the potential for male–male interaction we also included as control factors the number of sexually active females and the number of males in proximity in this model. As for the other model we also included animal ID and group as nested random factors.

Since mate-guarding may have an influence on the fAM levels only in a context of high male–male competition (i.e. high aggression rate and/or many males in proximity), in model 2, we tested for the significance of the interactions between mate-guarding time and 1) aggression rate, and 2) number of males in proximity, as well as between all three variables (mate-guarding, aggression rate and number of males in proximity). Since none of

these interactions were significant (LRT, all P > 0.15) we reran the model without any interaction.

2.6.3. Influence of female quality on male fAM during mate-guarding

To test the influence of female quality on male fAM during mate-guarding, we first measured dyadic male–female social bond strength using an approach inspired from the calculation of the "composite index of sociality" [68]. However, since we did not collect focal behavioural observations on all the males present in each of the groups, we could not compute a "composite index of sociality". Instead, we used the number of approaches and grooming time to calculate a "male-centred" association index (hereafter AI) between males and females [50]. We computed the AI for each male–female dyad as follows: $AI = [(G_{ij}/G_{ix}) + (A_{ij}/A_{ix})] / 2$ where G_{ij}/G_{ix} is the grooming time of male i with female j (G_{ij}) relative to the total grooming time of male i with all females in the group (G_{ix}). Similarly, A_{ij}/A_{ix} is the number of time male i approached or was approached by female j (A_{ij}), relative to the total number of time male i was approached or approached all females in the group (A_{ix}). To obtain a measure independent of male mate-guarding activity, we used only data collected on days during which the male did not mate-guard females at all. The mean focal observation time on non-mate-guarding days was 160.2 h per male (range: 39.6–265.4 h).

To analyse the effect of female quality on male fAM levels during mate-guarding, we considered only those days of observation for which at least 1 h of focal data was recorded and the male mate-guarded a female for at least 25% of observation time. We discarded from the analyses the days during which the male did not mate-guard the same female for at least 70% of his mate-guarding time, and days during which females where mate-guarded after conception (see [50] for details), because interest in pregnant females is reduced [50] and hence the costs for males and the hormonal pattern associated to mate-guarding might differ between periods before and after conception.

We used a GLMM to test whether the rank of the guarded female, her parity status and male–female AI had an effect on a male's fAM level (Prediction *P4*, Model 3). In this model, we also added male mate-guarding time, male rank, male–male aggression rate, male copulation rate, number of sexually active females in the group, number of males in proximity of the focal male and fruit availability as control factors and animal ID and group as nested random factors. As for model 2, each data point in this model was one observation day and each parameter was computed daily. We matched male daily behavioural pattern and male fAM levels using the same procedure as in model 2. Male primates often prefer to mate with and/or mate-guard parous than nulliparous females regardless of their rank [45–47]. The effect of female rank on fAM output may therefore be present only for parous females. To test for this eventuality, we assessed the significance of the interaction between parity status and rank in Model 3.

2.6.4. Autocorrelation term and assumptions' checking

Each model was fitted in R 2.15.0 (R Development Core Team 2010) using the function lmer of the R-package lme4 [69]. In all models, the response variable (i.e. log fAM levels) was likely to show temporal autocorrelation unexplained by the fixed effects included, potentially leading to violation of the assumption of independent residuals. Therefore, we included a temporal autocorrelation term into these models using an approach developed by Roger Mundry (see [70]).

In each model, we checked that the assumptions of normally distributed and homogeneous residuals were fulfilled by visually inspecting a qqplot and the residuals plotted against fitted values. We checked for model stability by excluding data points one by one from the data and comparing the estimates derived with those obtained for the full model. Variance inflation factors [71] were derived using the function vif of the R-package car [72] applied to a standard linear model excluding the random effects. VIFs which are less than 5 indicate that

covariation between the predictors is not a problem [73]. In all our models VIFs were less than 2.3. The other diagnostics also did not indicate obvious violations of the assumptions.

For each model, we first determined the significance of the full model as compared to the corresponding null model using a likelihood ratio test (R function 'anova' with argument test set to "Chisq"). The null models included all the control factors as well as the random factors, i.e. in Model 1: all factors except "male dominance rank" and "period"; in Model 2: all factors except "mate-guarding time", "male–male aggression rate" and "male copulation rate"; and in Model 3: all factors except "male–female AI", "female dominance rank" and the interaction between these two factors. To achieve a more reliable P-value, we fitted the models using Maximum Likelihood rather than Restricted Maximum Likelihood [74]. Only if the likelihood ratio test revealed significance did we consider the significance of the individual predictors. P-values for the individual effects were based on Markov Chain Monte Carlo sampling (MCMC) [67] and derived using the functions 'pvals.fnc' and 'aovlmer.fnc' of the R package languageR [75].

3. Results

In each model, the full model was significantly different from the null model (LRT, all P < 0.05, Tables 3–5). We therefore calculated the significance of the single effects in each model (Tables 3–5, see also below).

3.1. Seasonal variation in fAM and fruit availability

In order to illustrate the seasonal pattern of variation in fAM levels in male long-tailed macaques and its relationship to the variation in fruit availability we computed a figure depicting the monthly mean in both parameters (Fig. 1). Yet note that all our statistical analyses were based on daily behavioural rates and hormonal levels. Monthly levels of fAM (calculated across all males sampled, i.e. high and low ranking males) throughout the 14 month study period followed a distinct seasonal pattern (Fig. 1). The lowest fAM concentration occurred at the beginning of the non-mating period (in July and August 2010, Fig. 1). Subsequently, male fAM levels started rising by September 2010, three months prior to the start of the mating period, to reach a peak in February 2011, at the onset of the mating period when males were copulating and mate-guarding females in all of the three study groups (Fig. 1). Overall, fAM levels were negatively associated with fruit availability and males had the highest fAM levels on months when fruit availability was the lowest (March 2010 and February 2011). In line with the monthly pattern, we found a strong negative effect of fruit availability on fAM levels in all of the three models (all P_{MCMC} < 0.001, Tables 3–5). This indicates that, overall, male fAM levels increased with decreasing fruit availability.

Table 3
Results of the likelihood-ratio-tests (LRT) run to compare full versus null models, estimates ± SE, t-value and MCMC p-values for the GLMM run to test the influence of dominance rank and period (mating or non-mating) on fAM levels (Model 1). "AC term" refers to the autocorrelation term. P values <0.05 are indicated in bold.

Model 1 Response: fAM levels Rank: high (α + β) vs. low (others)			
Null vs. full model	df	χ^2	P
	2	160.74	<0.001
	Estimate ± SE	t	P_{MCMC}
Intercept	3.16 ± 0.05	58.17	**0.001**
Rank (low)	−0.20 ± 0.07	−2.74	**0.005**
Period (mating)	0.21 ± 0.02	13.20	**<0.001**
% tree fruiting	−0.10 ± 0.01	−12.83	**<0.001**
AC term	0.07 ± 0.01	9.75	**<0.001**

Table 4
Results of the likelihood-ratio-tests (LRT) run to compare full versus null models, estimates ± SE, t-value and MCMC p-values for the GLMM run to test the influence of mate-guarding, male–male aggression and copulation on fAM levels. "MG" refers to mate-guarding, "N. males" refers to the number of males in proximity, "N. sex. act. fem." refers to the number of sexually active females and "AC term" refers to the autocorrelation term. P values <0.05 are indicated in bold.

Model 2 Response: log fAM levels Rank: alpha vs. beta			
N. obs. days	273		
Null vs. full model	df	χ^2	P
	3	27.41	<0.001
	Estimate ± SE	t	P_{MCMC}
Intercept	7.68 ± 0.12	64.59	**<0.001**
Male rank (beta)	−0.20 ± 0.06	−3.43	0.052
MG time	0.14 ± 0.03	4.50	**<0.001**
Aggression rate	−0.01 ± 0.03	−0.36	0.757
Copulation rate	0.05 ± 0.03	1.56	0.122
% tree fruiting	−0.27 ± 0.04	−7.04	**<0.001**
N. male	−0.06 ± 0.03	−1.94	0.063
N. sex. act. fem.	0.07 ± 0.03	2.16	**0.033**
AC term	−0.08 ± 0.04	−2.24	0.052

3.2. Influence of mating period and rank on male fAM levels

As expected from the monthly pattern, males had significantly higher fAM levels during the mating than during the non-mating periods (Model 1, N = 690 samples, P_{MCMC} < 0.001, mean ± SE mating periods: 2325 ± 249 ng/g faeces, mean ± SE non-mating period: 1645 ± 228 ng/g faeces, Table 3, Fig. 2a). There was also a strong effect of dominance rank on fAM levels with high-ranking males (alpha and beta) having significantly higher fAM levels than low-ranking males (Model 1, P_{MCMC} = 0.005, mean ± SE high-ranking males: 2659 ± 365 ng/g faeces, mean ± SE low-ranking males: 1618 ± 228 ng/g faeces, Table 3, Fig. 2b).

Table 5
Results of the likelihood-ratio-tests (LRT) run to compare full versus null models, estimates ± SE, t and MCMC p-values for the GLMM run to test the influence (during mate-guarding) of male–female AI, female rank and female parity status on male's fAM levels (Model 3). P values <0.05 are indicated in bold.

| Model 3 Response: log fAM levels Rank: alpha vs. beta			
N. obs. days	128		
Null vs. full model	Df	χ^2	P
	4	9.93	0.042
	Estimate ± SE	t	P_{MCMC}
Intercept	8.11 ± 0.14	57.38	**<0.001**
Male rank (beta)	−0.18 ± 0.07	−2.70	**0.037**
Male–female AI	−0.08 ± 0.67	−1.28	0.750
Fem. rank	In an interaction		
Fem. parity	In an interaction		
Fem. rank*fem. parity	−0.44 ± 0.15	−2.95	**0.004**
Aggression rate	−0.03 ± 0.04	−0.89	0.695
Copulation rate	−0.02 ± 0.03	−0.74	0.615
N. sex. act. fem.	0.01 ± 0.04	0.20	0.862
N. females	−0.03 ± 0.10	−0.34	0.575
N. males	−0.01 ± 0.04	−0.28	0.688
% tree fruiting	−0.30 ± 0.05	−6.13	**<0.001**
MG time	0.01 ± 0.04	0.29	0.852
AC term	−0.11 ± 0.04	−2.88	**0.007**
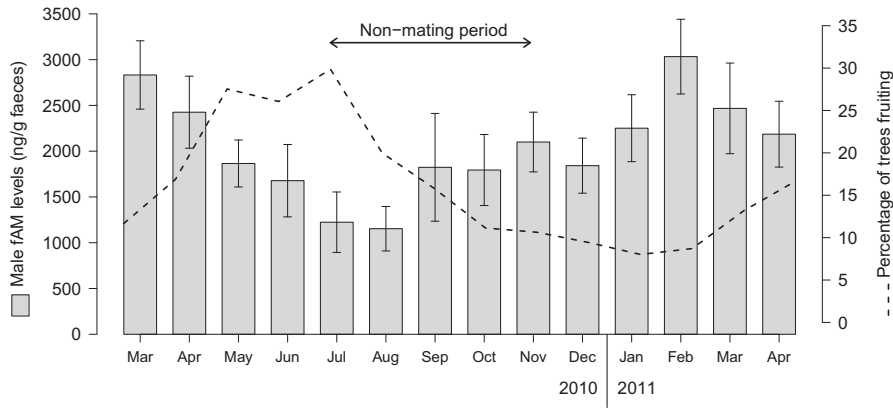
Fig. 1. Monthly variation in male faecal androgen metabolite levels (fAM, mean ± SE, grey bars) and fruit availability (mean percentage of tree fruiting over the three study group territories, dotted line).

3.3. Influence of mate-guarding, copulation and aggression on male fAM levels

The amount of time a male spent mate-guarding had a significant positive effect on fAM levels (Model 2, N = 273 observation days, $P_{MCMC} < 0.001$, Table 4, Fig. 2c). The more time a male spent mate-guarding the higher were his fAM levels. Independent of whether and how much a male spent time on mate-guarding, increasing numbers of sexually active females increased male fAM levels ($P_{MCMC} = 0.033$, Table 4). In addition, in this model, male dominance rank (alpha vs. beta) and the number of males in proximity tended to influence fAM levels but the effect was not statistically significant ($P_{MCMC} = 0.052$ and 0.063 for dominance rank and number of male in proximity respectively, Table 4). Alpha-males tended to have higher fAM levels than beta males and males in general tended to have lower fAM levels when more males were in proximity to them. Finally, aggression and copulation rates had no significant effect on male fAM levels (all P > 0.1, Table 4).

3.4. Influence of female quality on male fAM during mate-guarding

The AI between the male and the guarded female did not influence a male's fAM levels (Model 3, N = 128 days, $P_{MCMC} = 0.750$, Table 5). In contrast, the interaction between "female rank" and "female parity status" was significant ($P_{MCMC} = 0.004$, Table 5) indicating that the relationship between guarded-female dominance rank and male fAM levels was contingent on the female's parity status. During monopolisation of parous females, males had higher fAM levels when mate-guarding high-ranking females than when guarding low-ranking ones (Fig. 3). However, this pattern was reversed when males were monopolising nulliparous females. In this context, males had higher fAM levels when mate-guarding low-ranking females than when guarding high-ranking ones (Fig. 3). In this model, male dominance rank also had a significant effect on fAM levels. Beta males had lower fAM levels than alpha-males ($P_{MCMC} = 0.037$, Table 5). In contrast, male–male aggression rate, copulation rate, the number of sexually active females, the number of females in the group, the number of males in proximity and mate-guarding time did not have a significant effect on fAM levels in this model (all $P_{MCMC} > 0.5$, Table 5).

4. Discussion

Our results show that seasonal (i.e. mating period) as well as specific behavioural reproductive challenges (i.e. mate-guarding) were associated with a rise in androgen levels in male long-tailed macaques. This generally confirms the predictions of the challenge hypothesis (i.e. a seasonal rise from *level A* to *level B* and an additional rise from *level B*
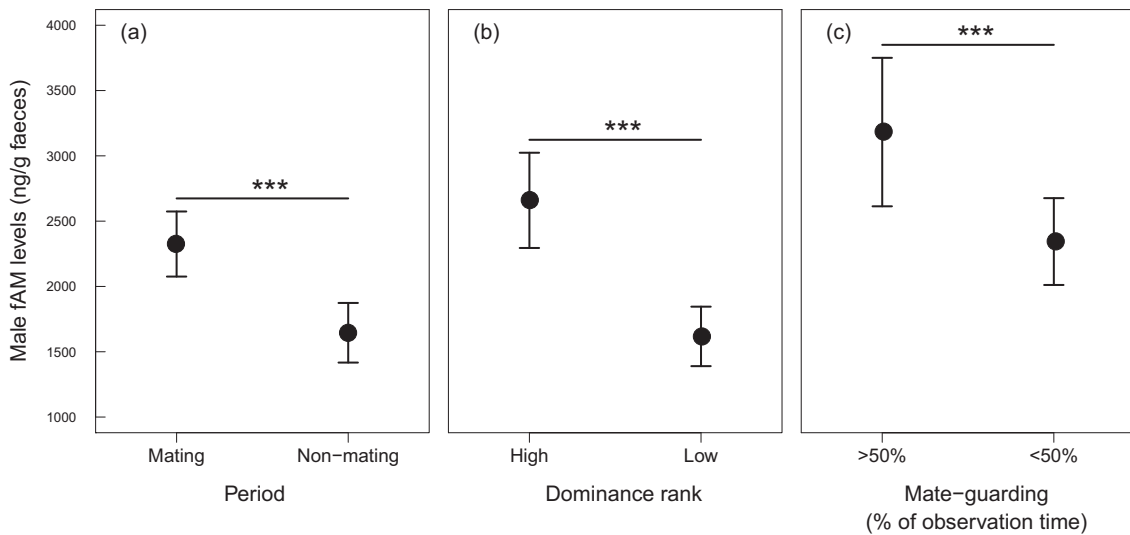

Fig. 2. Influence of period (a), dominance rank (b) and mate-guarding intensity (c) on male fAM levels. The mean ± SE fAM levels over all males in each of the periods (a), over males in each rank category (b) and over all high-ranking males while mate-guarding less and more than 50% of observation time (c) are depicted. ***P < 0.001. Please note that while in graph (c) mate-guarding intensity has been split into two categories (bellow and above 50% of observation time), in model 2 the factor "mate-guarding time" was a continuous variable.
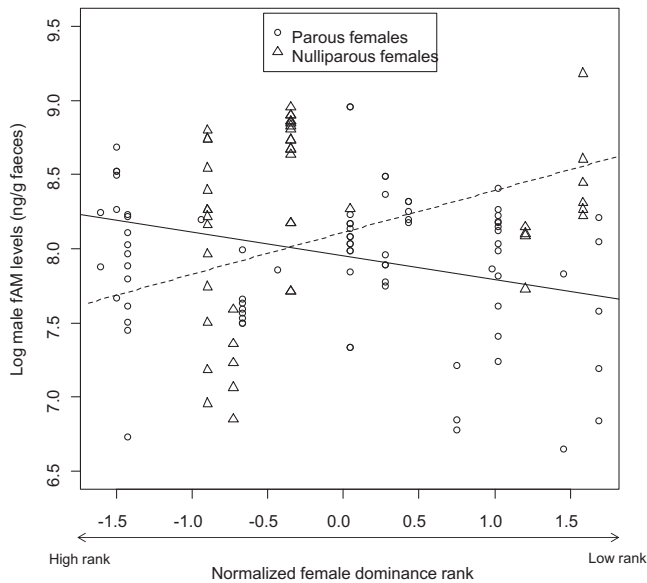
Fig. 3. Effect of the guarded female's dominance rank on male fAM levels for nulliparous females (triangles) and parous females (circles). The lines depict the linear relationship predicted by model 3 for nulliparous (dashed line) and parous females (solid line) and the triangles and circles depict the raw data points.

to *level C* in response to specific challenges: [10,14]). Additionally, we found inter-individual differences in androgen levels in that high-ranking males had higher fAM levels than low-ranking males. Yet, contrarily to what we predicted, this effect was also present during the non-mating period indicating that other factors than mate-guarding intensity alone influence inter-individual differences in androgen output. Finally, in line with our predictions we found that, across mate-guarding periods, males' fAM levels varied depending on the reproductive value of the guarded females (i.e. female dominance rank and parity status). During monopolisation of parous females, males had higher androgen levels when mate-guarding high-ranking females than when guarding low-ranking ones whereas the effect of female dominance rank on male androgens was reversed during monopolisation of nulliparous females.

As predicted (Prediction *P1*), the studied male long-tailed macaques exhibited a distinct seasonal pattern of androgen excretion with a marked elevation during the mating period when male–male competition is the highest. This corroborates our previous finding on the same population [16], strengthening the assumption that seasonal variation in androgen levels is a general characteristic of wild male long-tailed macaques. This general pattern resembles the seasonal hormonal profile reported in a broad diversity of vertebrate taxa (mammals [9,17, 76]; birds [77], see [10,19] for a review; and reptiles [18,78]) and thus seems to be a quite common phenomenon. More intriguing is our finding that the rise in androgen levels already occurred several months before the onset of the reproductive season, a phenomenon which has so far been described in primate species with a much stronger degree of reproductive seasonality (e.g. sifakas, *Propithecus verreauxi* [79]; squirrel monkeys, *Saimiri boliviensis* [5]; rhesus macaques [80]; and Japanese macaques [81]). In strictly seasonally reproducing species (i.e. income breeders), the timing of the period during which females are fertile is highly predictable and males can rely on exogenous cues such as photoperiod length to assess this timing and to physiologically prepare for male–male reproductive competition [82]. In contrast, in non-strictly seasonally reproducing species (capital breeders) such as long-tailed macaques, the timing of female fertile phases may occur year-round and is thus unpredictable, often depending on food availability ([51], reviewed in [82]). Yet male long-tailed macaques seem to initiate preparatory physiological changes well in advance of the

forthcoming mating period, i.e. during times when no female is yet sexually active and reproductive challenges are absent ([16], this study). It might be that, in this species, environmental cues, such as food availability, trigger these physiological changes. Our finding that food availability had a strong negative effect on male androgen levels corroborates this idea. This latter result however contrasts with other studies on non-seasonal breeders like savannah and olive baboons in which males have low androgen levels when food availability is the lowest [83,84]. Maintaining high levels of androgen is energetically costly [1,85], and, during the dry season when food resources are scarce male baboons may face an energetic trade-off between energy allocated to self-maintenance and energy allocated to reproduction [84]. The reason for this difference between long-tailed macaques and baboons may thus be related to differences in the environment in which the two species live. The environment baboons live in seems to be more seasonal and extreme in terms of aridity [83] so that food during the dry season may be limited to a degree to which males cannot afford keeping up high androgen levels since these are energetically costly [1,85]. Also, in yellow baboons reproduction opportunities are quite evenly spread over the year so that low androgen levels during certain periods of the year may not drastically impact male reproductive success. In contrast, in long-tailed macaques, it may be maladaptive for males to have low androgen levels when food availability is lowest given that this occurs at the onset of the reproductive season (in February and March in our study, see also [51]) when males need to be physiologically prepared for intense male–male competition over reproductive opportunities. It appears thus that the seasonal patterns of male androgen levels in primate species with low reproductive seasonality differ depending on the species-specific trade-off between adapting to the environment and to reproductive challenges.

Even though in our study all males experienced a similar seasonal rise in androgen levels during the mating season (i.e. the interaction between dominance rank and period were not significant), we found consistent inter-individual differences in androgen output with high-ranking males having higher androgen levels than low-ranking males. In contrast to our prediction (*P2*) however, the rank-related difference in androgen levels was not restricted to the mating period but remained year round. Rank-related differences extending beyond the period during which females are fertile have been reported in non-seasonal (chimpanzees [21]; savannah baboons [86]) but also in strictly seasonal (mandrills [87]) breeding primates. In chimpanzees, savannah baboons and mandrills but also in long-tailed macaques, high-ranking males are able to monopolise females efficiently and achieve a substantially higher reproductive success than low-ranking males [35–37,88–90]. As a consequence, males contest aggressively for high-rank and high-ranking males face the threat of rank challenges year round [21,86,87, 91]. In this context, higher androgen levels may physiologically prepare males to better respond to these challenges (discussed in [92]). In contrast, no relationship has been found between dominance rank and androgen levels in species with lower female monopolisability, i.e. in species in which being a high-ranking male does not provide a substantial reproductive advantage and in which, as a consequence, males fight and challenge less often other males to achieve high ranks (e.g. Assamese macaques [17] and bonobos [22]). This illustrates that, in line with the prediction of the challenge hypothesis, individual males raise their androgen levels above those of their conspecifics only when the benefit of doing so outweighs the potential costs of elevated androgen concentrations (e.g. immunosuppression, energetic costs and increased predation risk; discussed and reviewed in [85]).

Within the mating period, males may further optimise their competitive ability by raising their androgen levels to a physiological maximum (*level C* sensu the challenge hypothesis). Given the potential costs of elevated androgen levels (see above), this is only expected to occur during short-term, particularly challenging periods with potential high reproductive benefits [10]. Long-tailed macaques appear to reach *level C*

when mate-guarding females since, in line with our prediction (*P3*), males had higher androgen levels when mate-guarding females than when not. Similarly in savannah baboons and spotted hyenas (*Crocuta crocuta*), androgen levels are higher in males who mate-guard females than in males who do not [23,93].

There are several explanations for why elevation of androgen levels during mate-guarding may be beneficial. First of all, mate-guarding enhances male reproductive success to a substantial degree [36,94–96]. At the same time, it is often associated with heightened degree of challenge like increased male–male aggressions [97–99] and this was the case also in our study population [30]. High androgen levels may thus help priming males for potential aggressive encounters with other males. In fact, androgen activates vasopressin receptors in the hypothalamus, which facilitates the expression of aggressive behaviours [100], and enhances muscle performance [6]. During mate-guarding male long-tailed macaques also exhibit increased stress hormone levels [30]. Increases in both androgen and stress hormone levels might be part of an overall physiological mechanism through which male long-tailed macaques prepare for the potential challenge of mate-guarding. Yet this physiological response appears to be more anticipatory (rise of both hormones in a situation with a higher risk of aggression, i.e. during mate-guarding) rather than a response to the aggression themselves since we did not find a significant effect of male–male aggression rates on males' androgen (this study) or stress hormone levels [30]. This contrasts with a previous study on long-tailed macaques [16] in which male–male aggression rates were correlated to males' androgen levels during the mating period. Different time frames in the analysis (monthly means used in Girard-Buttoz et al. 2009, daily values used in the current study) might explain these discrepancies. Beyond priming males for potential aggressive interactions, elevated androgen levels during mate-guarding might also enhance males' abilities to monitor females. Male long-tailed macaques are more vigilant when mate-guarding females than when not mate-guarding [30] and elevated androgen levels help in sustaining directed attention (reviewed in [101]). Finally, androgen promotes sperm production [3] which in turn may enhance during mate-guarding the chance for the male to fertilise the ova of the guarded female.

Elevated androgen levels during mate-guarding are thus most likely adaptive since they possibly enhance the efficiency of female monopolisation hereby increasing the fitness benefits derived from this specific behaviour. Yet these benefits might also depend on the female reproductive value [39–43]. In our study, we found that during monopolisation of parous females males had higher androgen levels when mate-guarding high-ranking than when guarding low-ranking females (Prediction *P4*). In line with the predictions of the challenge hypothesis [10], male long-tailed macaque androgen levels thus appear to rise to a physiological maximum in periods when the fitness payoff of heightened androgen levels is the highest. In fact, elevated androgen levels might help males to efficiently monopolise females (see above) and high-ranking female long-tailed macaques (in particular parous ones) produce higher quality offspring than low-ranking females (i.e. most likely to survive until adulthood and to achieve high rank in the future [39,40]). A previous study suggests that male long-tailed macaques aim at better securing paternity with the most valuable females since they are more vigilant and more aggressive towards other males when mate-guarding high-ranking females than when guarding other females [50]. In turn, the expression of aggressive behaviour and the efficiency of vigilance might be mediated by androgens ([6,100,101], see also above). Yet this explanation holds only for the monopolisation of parous females. During monopolisation of nulliparous females, our study males had lower androgen levels when mate-guarding high-ranking females than when mate-guarding low ranking ones. This is somewhat surprising but in this context, female spatial positioning and centrality in the group, rather than the reproductive value of the guarded female, may explain the effect of female dominance rank on male androgen levels. In long-tailed macaques, low ranking females spend more time at the periphery of the group than high-ranking females [40]. As a by-product, males may occupy a less central position in the group when mate-guarding low-ranking nulliparous females than when guarding high-ranking ones. In turn, not being at the centre of the group likely represents a challenge for the male which may lead to an increase in androgen levels. At the periphery of the group, high-ranking males may have less males from their own group in proximity and hence fewer potential allies to support them and form coalition against extra-group males attempting to take over mate-guarding or rank [40]. In our study, two extra group males attempted several times to challenge the alpha-males of two of our study groups and to break their mate-guarding activity. Yet mate-guarding takeovers were only successful when no other resident male was present within 20 m (unpublished data). It might thus be crucial for male long-tailed macaques to have high androgen levels when guarding low-ranking nulliparous females not because these females are of high reproductive value but because they need to be physiologically ready to respond to a higher risk of extra-group male challenges. The threat of this challenge might also explain why male long-tailed macaques are less physiologically stressed when they have more males in proximity and why they are more physiologically stressed when they mate-guard low-ranking nulliparous than when guarding high-ranking nulliparous females [50]. The effect of female rank on centrality in the group and the associated challenge it represents for the guarding male might be less pronounced in parous females who are older than nulliparous ones and more integrated socially into the group. Furthermore this effect might be overridden by the fact that high ranking parous females are more attractive to males than any other females whereas nulliparous females are unattractive regardless of their rank [39,40]. A rise in male androgen levels linked to the high reproductive value of the females might thus be triggered only when mate-guarding high-ranking parous females. Altogether this might explain why we found reversed relationships between the rank of the female guarded and male androgen levels for parous and nulliparous females.

Collectively our study shows that, as predicted by the challenge hypothesis [10,14], male long-tailed macaques respond not only to seasonal but also to short term reproductive challenges (i.e. mate-guarding in our study) by adapting their androgen levels. While ecological factors (e.g. food availability), may be the primary drivers of long-tailed macaques' seasonal androgen changes, socio-demographic factors may regulate more proximate variations in androgen output [85]. In species with a high degree of female monopolisability (such as long-tailed macaques), a multilevel fine-tuned androgen management may help males to better respond to the challenges of rank take-over attempts and mate-guarding. This in turn may increase the efficiency of female monopolisation and enhance dominance tenure length. Future studies should investigate short-term variations in male androgens in species in which males mate-guard females but which exhibit a low degree of female monopolisability and in which sociality is more important for dominance rank acquisition than male fighting abilities (e.g. Assamese macaques [102,103]). By complementing the present study, such data would facilitate our understanding of the mechanisms underlying long- and short-term male physiological responses to reproductive challenges. In our study, we also found an effect of dominance rank on male androgen levels, which confirms that androgens may promote the ability of high-ranking males to maintain their rank in species in which rank is achieved via contest competition. Finally, considering the strong influence of fruit availability on androgen output in our study males, we encourage future studies examining the challenge hypothesis in wild animals to include more systematic assessments of food availability (see also [14]).

Acknowledgements

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