Physiological stress hormone levels and mating behaviour are negatively correlated in male rhesus macaques (*Macaca mulatta*)

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Abstract

In order to further understanding of the factors that cause and mediate stress in social animals, many studies have examined differences in male physiological stress levels between mating and non-mating seasons, and related these differences to levels of male-male competition. Very few have explicitly tested whether variation in stress levels is related to mating behaviour itself. We provide preliminary evidence of a negative relationship between faecal levels of glucocorticoid metabolites and mounting frequency in free-ranging male rhesus macaques (*Macaca mulatta*). This finding may help explain the previous observation that male rhesus macaques show no difference in their levels of faecal glucocortocoids between the breeding and non-breeding season, despite the former being associated with elevated levels of aggression. Our study highlights the importance of directly quantifying mating behaviour when investigating the potential impact of reproductive competition and seasonality on stress physiology.

Keywords: aggression; glucocorticoids; mating; stress

Introduction

Identifying the causes and consequences of stress, and the means by which it is mediated among gregarious animals, represents a fundamental goal in the study of social behaviour (Sapolsky, 2005). One topic of particular interest has been the exploration of physiological stress levels among males, and how these relate to the intensity of reproductive competition. Elevated testosterone levels among males in the breeding season promote aggression, particularly related to male-male competition and mate-guarding (Wingfield et al. 1990). The psychological (Sapolsky, 2005) and metabolic (Muller and Wrangham, 2004) costs of such aggression would be predicted to increase males' physiological stress hormone levels during this season.

Such a relationship has been observed in a number of mammal species. For example in Assamese macaques (Ostner et al. 2008) and bison (Mooring et al. 2006), higher physiological stress levels, assessed through quantification of glucocorticoid (GC) levels, were found in males in the breeding season compared with the non-breeding season. Such differences are typically attributed to increased levels of male-male competition and aggression during the period when mating occurs. In some species however, for example ring-tailed lemurs (Gould et al. 2005) and rhesus macaques (Higham et al. 2013), no clear differences in male GC levels linked to the breeding season were found, despite heightened levels of aggression during this period. In yet others, such as yellow-pine chipmunks (Place and Kenagy, 2005), males' GC levels were actually lowest during the breeding season. The causes of these differences in findings are unclear, but one potentially important factor is the impact of sexual behaviour on stress physiology.

A very few studies have explored the potential link between mating behaviour itself (rather than mating season) and male stress hormone levels. For bison bulls a positive relationship between number of copulations and GC concentrations was observed (Mooring et al. 2006). Similarly, a subset of male rock hyraxes that sing, a territorial and sexual behaviour observed primarily in the mating season, were observed to copulate more frequently and have higher cortisol levels than those males that don’t sing (Koren et al. 2008). In contrast to these two studies, among wild male olive baboons, males with higher ‘copulatory success’ (combining copulation and consortship) were observed to have lower plasma cortisol levels shortly after anaesthetisation (Sapolsky, 1982).

Here we explore the relationship between GC levels and mounting frequency in free-ranging male rhesus macaques (*Macaca mulatta*) during the mating season. We also explore the link between GC levels and three behaviours previously linked to variation in such levels among Old World monkeys: the receipt of aggression, which has been found to be positively related to GC levels (Ostner et al. 2008), and the giving (Shutt et al. 2007) and receipt (Gust et al. 1993) of grooming, which have been found to be negatively related to these levels. Due to cortisol’s important metabolic role (e.g. Muller and Wrangham, 2004) we also explore the link between GC levels and the amount of time spent moving.

To our knowledge, this is the first study to explore simultaneously the relationships among this set of variables in a non-human primate species. We predicted that due to the stress associated with gaining access to, and mate-guarding females, male GC levels would be positively associated with mounting frequency, the amount of aggression received, and the amount of time spent moving. Due to the associated stress relieving benefits of grooming we predicted that GC levels would be negatively associated with the frequency of both giving and receiving grooming.

Materials and methods

Study animals

This study was carried out on the free-living population of rhesus macaques inhabiting the island of Cayo Santiago, Puerto Rico. Rhesus macaques live in multi-male – multi-female groups (Teas et al. 1980) and are seasonal breeders (Herndon 1983). Cayo Santiago is home to six groups in total, and males migrate between groups. Animals are provisioned daily with monkey chow and water, and also forage freely on natural vegetation (Rawlings and Kessler, 1986). Data collection took place between April and June 2007, which covers part of the mating season of our study group (full mating season: April to September). Data were collected on eight individually recognisable adult males living in a single group (group V: total group size 92 animals, including 18 adult males and 21 adult females). The eight study animals represent all of the non-natal males in group V that were present for the entire study period. Natal males were excluded from our study to avoid the influence of kinship on our findings; kinship can have a significant impact on the distribution of grooming and agonistic behaviour in rhesus macaque groups (e.g. Bernstein and Ehardt 1985; Call et al. 1996).

The project was approved by the Institutional Animal Care and Use Committee of the University of Puerto Rico, Medical Science Campus (reference number: A4350107).

Behavioural data collection

Behavioural data were collected by RM each day from 1000hrs until the field sited closed at 1400hrs, following the collection of faecal samples from 0700-1000hrs (see below). Data were collected in 15min continuous focal observations (Altmann 1974) using a Psion Workabout with Observer v.5.0 software. The order of focal watches was randomized each day to avoid biases in the times at which individual males were followed. During each focal watch, data were collected on the occurrence of heterosexual mounts. It is important to note that although we were unable to distinguish between ejaculatory and non-ejaculatory mounts, the frequency of mounting and the occurrence of ejaculation are highly positively correlated in this species (Mehlman et al. 1997). A mount was scored when pelvic thrusting by the male was seen, which was taken to infer that intromission had occurred. All aggression received by the subject from adults in the group was recorded, along with information on whether the aggression was of low intensity (displace, visual or vocal threat, short charge) or high intensity (chase, physical contact or escalated attack). The duration of all bouts of grooming (i.e. one animal picking through the fur of another) given to or received from other adults, and the duration of all bouts of moving (i.e. travelling when not foraging), were also recorded. All animals were sampled once every one or two days of data collection. In total 4548 minutes of focal observations were recorded, between 550-582min for each subject. Additional data on agonistic behaviour were collected *ad libitum* and combined with focal data for determination of the dominance hierarchy. Based on the analysis of 558 dyadic interactions, MatMan revealed that the dominance hierarchy was significantly linear (Landau's linearity index=1.0; p<0.001).

Faecal sample collection and glucocorticoid analysis

Faecal samples (uncontaminated by urine) were collected over the study period between 0700 and 1000hrs, following methods described by Hodges and Heistermann (2003). To minimise hormone degradation, faecal samples were collected immediately following defecation, carried in a cool-bag containing ice-packs, and stored at -18°C after each day in the field. In total we collected 12-16 faecal samples per male (average = 7 subjects sampled/week) over the study period. Samples were imported to the UK under DEFRA licence no. AHZ/2537/2007/3. GC analyses were carried out at Roehampton University using standard protocols.

Faecal samples were analysed within four months of collection in the field; faecal hormone levels have been shown to be stable over a number of years when frozen (Palme 2005). Faecal samples were homogenized and extracted twice according to the method described by Ziegler et al. (2000). Extraction efficiency, assessed by recovery of radiolabelled oestradiol, was 98% +/-12%. Faecal extracts were analysed for concentrations of 11ß-hydroxy-etiocholanolone (3α,11ß-dihydroxy-CM), a major metabolite of cortisol, using a group-specific immunoassay. This measurement has been validated for assessing adrenocortical activity in various primates (Heistermann et al. 2006), including rhesus macaques (Hoffman et al. 2011). Serial dilutions of faecal extracts from different males gave displacement curves parallel to those obtained for the standard solution. Sensitivity of the assay at 90% binding was 1.2pg/well. Intra- and inter-assay coefficients of variation of high and low value quality controls were 9.2% (n=17) and 8.3% (n=10; high), and 13.6% (n=15) and 18.7% (n=10; low), respectively. All assay results were standardized for differences in extraction volume and faecal weight, and are presented as ng hormone/g dry faecal weight.

Statistical analysis

Associations between GC levels and behaviour have previously been explored by looking at fine-grained temporal variation in these variables (e.g. Crockford et al. 2008; Arlet et al. 2009), by taking mean levels calculated over the study period (e.g. Gust et al. 1993; Ostner et al. 2008), or in both of these ways (e.g. Higham et al. 2009). Data were analysed here using both approaches, allowing comparison of our results with the broadest range of other published work in this area.

Firstly, weekly means of faecal GC (FGC) levels were calculated for each male. The lag for excreted cortisol metabolites to appear in faeces is approximately 30-46 hours in macaques, including the rhesus macaque (Heistermann et al. 2006; Hoffman et al. 2011), and excreted material accumulates over an extended period. With this in mind, it is not possible to collect faecal samples matched with specific, short and infrequent events (e.g. mounting). Following standard practice in studies of non-invasive primate endocrinology (e.g. Engelhardt et al. 2005; Higham et al. 2009), weekly FGC values were calculated using the faecal collection date minus two days, to reflect the lag in excretion time. For each male weekly means were also calculated for all other variables. Generalised linear mixed models

(GLMMs) were performed in STATA v10.1 software (StataCorp 2007) to explore the relationships between FGC levels and our behavioural variables (nesting week inside individual as random factors). Our dependent variable, weekly FGC level, was transformed using a square-root transformation to improve normality (Zar 1999). We entered mounting (rate/min), high and low intensity aggression received (rates/min), grooming given and received (s/min), moving (s/min) and rank as our independent variables.

We used model selection (Symonds & Moussalli 2011) to determine which combination of behavioural variables (including rank) best explained the variability in subject weekly FGC levels across the mating season. The strength of each model was calculated according to its Akaike Information Criterion (*AIC*: Akaike 1974). The model with the lowest *AIC* was selected as the one with the best fit. Using the forward method (Pinheiro & Bates 2000) to select the 'best model', we first ran a series of GLMMs on the dependent variable (i.e. weekly FGC level) with each predictor variable (i.e. rate of mounting, grooming received, grooming given, high intensity aggression received, low intensity aggression received, moving and rank) independently, and calculated their respective AICs (table 1). A composite model was then built by progressively entering the predictor variables from the above models in order of low to high AIC (giving six different models; table 1). Based on the *AIC* difference criteria (Burnham & Anderson 2002; differences in *AIC* between 0-2 = substantially equivalent, 4-7 = equivalent, >10 = non-equivalent), predictor variables were removed from the 'best model' if their inclusion did not improve (i.e. lower) the AIC of the model by > 4. Likelihood ratio tests, using critical χ^2 values, were used to compare the relative strength of the 'best model' compared to the other five models. GLMMs were performed in StataCorp (2007).

Secondly, mean values over the total study period were calculated for each male for: FGC levels, rate of mounting, rates of receiving low and high intensity aggression, percentage time moving and percentage time giving and receiving grooming. We used bivariate correlations to investigate (1) the relationships between FGC levels and each of the behavioural variables, (2) the relationships between FGC levels and rank, and (3) the inter-relationships of any variables found to be correlated with FGC levels. These analyses were performed using IBM SPSS Statistics v20. As samples sizes were small and some analyses used ranked data, Spearman's rank correlations were employed.

Results

We found that a model with the inclusion of mounting and high and low intensity aggression rates was the best fit model for weekly FGC levels (Model 2; *AIC* = 410.39, *d.f* = 4, table 1 and 2). Using likelihood ratio tests, based on critical χ^2 values, we found model 2 to be a significantly better model than model 1 ($\chi^2 = 8.27$, $P < 0.05$) and not significantly different from models 3 ($\chi^2 = 0.15$, ns), 4 ($\chi^2 = 0.12$, ns), 5 ($\chi^2 = 2.33$, ns) and 6 ($\chi^2 = 2.942$, ns). Model 2 had fewer explanatory variables than models 3 to 6 and was thus considered the best fit model.

Bivariate correlations using means calculated for the whole study period showed that FGC levels were significantly negatively related to rate of mounting ($r_s = -0.850$, $P = 0.007$, fig. 1), low intensity aggression ($r_s = -0.970$, $P < 0.001$) and moving ($r_s = -0.786$, $P = 0.02$). These three behavioural correlates of mean FGC levels were themselves positively correlated (mounting and low intensity aggression: $r_s = 0.813$, $P = 0.01$; mounting and moving: $r_s = 0.874$, $P = 0.01$; low intensity aggression and moving: $r_s = 0.802$, $P = 0.02$).

Mean FGC levels were unrelated to the mean rate of receiving high intensity aggression (r_s = 0.098, P = 0.82), mean percentage time giving (r_s=0.143, *P* = 0.74) or receiving grooming (r_s = 0.214, *P* = 0.61), or to rank (r_s = -0.381, *P* = 0.35). There was no significant correlation between high intensity aggression and either low level aggression (r_s = -0.110, *P* = 0.80) or mounting (r_s = -0.06, *P* = 0.89).

Discussion

Our results provide evidence of a negative relationship between mounting frequency and physiological stress levels among free-ranging male rhesus macaques during the breeding season. Before exploring these findings further, we feel it is important to flag up two important caveats. Firstly, we were unable to collect behavioural data throughout the whole day, and thus cannot exclude the possibility that individual males might vary in the proportion of their mounts that occur during the data collection period (1000-1400 daily) compared to outside of this time. Secondly, we did not distinguish between ejaculatory and non-ejaculatory mounts (although frequency of mounting and occurrence of ejaculation are highly positively correlated in this species; Mehlman et al. 1997). We feel, therefore, that our results should be seen as preliminary, and hope that they will promote further, more detailed investigation of the associations between mating behaviour and stress physiology. Nevertheless, with these caveats in mind, the potential mechanisms underlying the negative relationship found between mounting frequency and physiological stress levels merit discussion.

One possibility is that males that are more stressed are less likely to achieve mounts. Indeed, studies of rodents indicate that stress can impact negatively on aspects of male sexual behaviour including motivation and performance (e.g. Retana-Marquez et al. 1996). Alternatively, more frequent mounting may reduce males’ stress levels and/or their reactivity to subsequent stressful events. There is evidence that oxytocin levels increase during sexual arousal and following ejaculation (Bancroft 2005). This hormone may suppress cortisol levels (Heinrichs et al. 2003); such stress-attenuating effects may underlie the marked reduction in anxiety (a subset of stress) seen after mating in rats (Waldherr and Neumann 2007) and lower blood pressure reactivity to stressful events which is seen following recent intercourse in humans (Brody 2006).

Such stress-reducing effects of sexual activity may explain the unexpected negative association in our study between mean levels (over the whole study period) of FGC and rates of receiving low intensity aggression. As it seems biologically improbable that receiving aggression directly reduces FGC levels, this association may simply be a by-product of the positive correlation (over the whole study period) between rates of mounting and receiving low intensity aggression. Similarly, the negative association between FGC levels and the amount of time spent moving may also be explained by the positive correlation between moving and mounting frequency. Our results suggest that males that mount frequently may experience buffering against the associated costs (e.g. metabolic) of increased aggression and movement (e.g. Muller and Wrangham, 2004), as well as achieving high mating success. This may help explain why male rhesus macaques don’t show higher stress levels in the breeding season, compared to the non-breeding season

(Higham et al. 2013), despite it commonly being associated with high rates of aggression (e.g. Wilson and Boelkins 1970; Mehlman et al. 1997).

Our study adds to the growing number exploring the relationships between male reproductive behaviour and physiological stress levels in mammals, and is one of the very few to quantify male mating behaviour directly. Amongst these, some find a positive association between mating behaviour (or an index thereof) and GC levels (Barrett et al. 2002; Mooring et al. 2006; Koren et al. 2008), while in others (Sapolsky 1982), including ours, the relationship is negative.

This disparity among studies may result from variation between species in factors such as the intensity of the breeding season, the degree of reproductive skew among males or the nature of male-female and male-male social relationships. Understanding how such factors influence stress levels among males is a key area for further research. Our study highlights the importance of investigating mating behaviour itself in future studies of the interactions between reproductive competition and stress physiology.

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Table 1. AIC values of individual and composite models of GLMMs on weekly faecal glucocorticoid levels

Variables included	**AIC**	**D.F**
Individual models		
High intensity aggression	428.12	
Low intensity aggression	428.92	
Mounting	430.16	
Moving	437.48	
Rank	438.17	
Grooming given	440.73	
Grooming received	441.48	
Composite models		
(1) High intensity aggression; low intensity aggression	418.66	1
(2)* High intensity aggression; low intensity aggression; mounting	410.39	2
(3) High intensity aggression; low intensity aggression; mounting; moving	410.54	3
(4) High intensity aggression; low intensity aggression; mounting; rank	410.51	3
(5) High intensity aggression; low intensity aggression; mounting; grooming given	412.72	3
(6) High intensity aggression; low intensity aggression; mounting; grooming received	413.32	3

*denotes best fit model

Table 2. Results of the ‘best model’ GLMM for the relationship between faecal glucocorticoid levels and behavioural variables (N = 62)

	β ± SE	***Z***	***P***	**95% CIs**
High intensity aggression	70.75 ± 181.29	0.39	0.696	-284.57 to 426.07
Low intensity aggression	-59.52 ± 94.86	-0.63	0.530	245.44 to 126.39
Mounting	-19.33 ± 9.72	-1.99	0.047	38.39 to 0.28
Random effects				
Subject ID nested inside week: estimated variance = 7.81				

Figure Legends

Figure 1. The relationship between mean faecal glucocorticoid concentration and mean mounting frequency