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Alpha male status and availability of conceptive females are associated with high glucocorticoid concentrations in high-ranking male rhesus macaques (*Macaca mulatta*) during the mating season

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**Highlights**

- Glucocorticoids of high-ranking male rhesus macaques are positively associated with the number of conceptive females present.

- Alpha males have higher glucocorticoid (GC) concentrations than other high-ranking males.

- Male age and number of adult males were not significantly associated with GC concentrations during the mating season.

- Hierarchy stability was not significantly associated with GC concentrations in high-ranking males during the mating season.
Abstract
The relationship between male mating opportunities, stress, and glucocorticoid concentrations is complicated by the fact that physiological stress and glucocorticoid concentrations can be influenced by dominance rank, group size, and the stability of the male dominance hierarchy, along with ecological factors. We studied the three highest-ranking males in nine different social groups within the same free-ranging population of rhesus macaques on Cayo Santiago, Puerto Rico, during the mating season, to examine variation in glucocorticoid concentrations in relation to number of females that conceived each month, alpha status, number of adult males in a group, and male rank hierarchy stability. We found that glucocorticoid concentrations were highest in the early mating season period when more females conceived in each group and declined linearly as the mating season progressed and the number of conceptive females decreased. Alpha males had significantly higher mean monthly glucocorticoid concentrations than other high-ranking males throughout the study period. Male age, number of adult males in a group, and hierarchy stability were not significantly associated with glucocorticoid concentrations. Our findings suggest that alpha males may experience significantly higher levels of physiological stress than their immediate subordinates and that this stress coincides with the period of the mating season when most conceptions occur.

1. Introduction
Male mammals often engage in intense competition for access to mates and can incur significant physical, physiological, and health-related costs (Corlatti et al., 2012, Emery Thompson and Georgiev, 2014, Kappeler and van Schaik, 2004, Kershaw and Hall, 2016, Key and Ross, 1999, Sands and Creel, 2004). These costs include physical injury (Arlet et al., 2009, Smuts, 1987), stress (Bergman et al., 2005, Corlatti et al., 2012, Higham et al., 2013, Sands and Creel, 2004, Setchell et al., 2010), reduced immune function (Corlatti et al., 2012, Prall and Muehlenbein, 2014), reduced energy balance (Georgiev, 2012), and a variety of health impairments (e.g. Nunn and Altizer, 2004). While it has often been assumed that the potential costs of male competition would be borne most heavily by the losers (i.e. males with low competitive success), a variety of evidence has accumulated to suggest that, at least under some conditions, physiological stress can be skewed towards the highest ranking individuals (Abbott et al., 2003, Cavigelli and Caruso, 2015, Creel, 2001, Goymann and Wingfield, 2004).

Physiological stress can be assessed by measuring concentrations of glucocorticoids (GCs), a group of steroid hormones secreted from the adrenal glands that are involved in regulating metabolism and are important for mobilizing energy as part of the “fight-or-flight” response (Sapolsky, 1992, Sapolsky et al., 2000). GCs capture the generalized systemic response to a variety of stressors, both physical and psychological, which can, at chronically high levels, negatively affect health and survival (Mendoza et al., 2000). The relationship between glucocorticoids and male competition has received particular attention in studies of non-human primates, among which many species form stable, multi-male, multi-female groups. Among primates, there are some species in which subordinate males had elevated GC concentrations compared to dominant males, including squirrel monkeys (Coe et al., 1979, Manogue, 1975) and olive baboons (Sapolsky et al., 1997, Virgin and Sapolsky, 1997). High-ranking males had higher GC concentrations than low-ranking males in bearded capuchin monkeys (Sapajus libidinosus: Mendonca-Furtado et al., 2014), white-faced capuchin monkeys (Cebus
capucinus: Schoof et al., 2016), chimpanzees (Pan troglodytes: Muller and Wrangham, 2004), and Japanese macaques (Macaca fuscata: Barrett et al., 2002). A number of explanations have been offered for the positive correlations between glucocorticoid concentrations and rank in male primates. Given that high-ranking males of most species have an advantage in securing mating opportunities, one hypothesis is that high glucocorticoid concentrations reflect the high physiological costs associated with mating effort, including mate guarding and direct aggressive competition for mates. In support of this, males in several seasonally breeding species have elevated GC concentrations during the mating season (e.g., Cebus apella: Lynch et al., 2002; Eulemur fulvus: Ostner et al., 2008b; Brachyteles arachnoides: Strier et al., 1999). In seasonally-breeding mandrills and in non-seasonally breeding bonobos, a positive association between rank and glucocorticoid concentrations was found only during mating periods (Setchell et al., 2010, Surbeck et al., 2012). However, in male Assamese macaques (Macaca assamensis), GC concentrations were higher in the mating season (Ostner et al., 2008a), but did not correlate with investment in mate-guarding (Schülke et al., 2014). Instead, GC concentrations were predicted by the amount of aggression received, resulting in higher concentrations for low-ranking males (Ostner et al., 2008a).

Alternatively, or additionally, high-ranking males may suffer increased physiological stress in their efforts to maintain high dominance rank against challenges from other males. These effects could contribute to the overall physiological costs of mating effort, but should also be expected to persist in non-mating periods when the dominance hierarchy is unstable. In male rhesus macaques and baboons, high-ranking males had higher GC concentrations than other males only during periods of social instability (Higham et al., 2013, Sapolsky, 1983, Sapolsky, 1992). Other studies report higher GCs among males in larger groups (Goymann et al., 2003, Raouf et al., 2006). Under natural conditions, cliff swallows living in larger breeding colonies had higher GC concentrations (Raouf et al., 2006). Similarly, in male spotted hyenas, GC concentrations were unrelated to social status, but were highest for those males living in the largest social group (Goymann et al., 2003). Male spotted hyenas are similar to male rhesus macaques in that they queue for dominance (Goymann et al., 2003); thus, we can predict that rhesus macaques may show a similar pattern in which the number of adult males present in the group may be more influential in predicting GC concentrations than a male's individual dominance status.

For logistical reasons, most relevant studies of wild primates have examined variation in GCs within a single group of individuals. In such studies, inferences about causality from correlations between dominance rank and GCs are difficult to evaluate because dominance rank covaries with many behavioral factors. Furthermore, to the extent that many of these studies rely on the presence or absence of a significant correlation, they fail to reveal whether these relationships are driven by the proximate forces affecting high-ranking males or those affecting low-ranking males, which may be different. Factors that vary primarily between groups, such as number of other males or the overall availability of mating opportunities, may be expected to exert the biggest impact on the stress experienced by high-ranking individuals. Thus, in this study, we examined factors predicting GC
variation among top-ranking males in nine different social groups of free-living rhesus macaques. We examined whether absolute rank (alpha vs. non-alpha top-ranking males), the number of females that conceived in each group during a given month, the number of males in each group, or male hierarchy stability influenced the absolute GC concentrations experienced by top-ranking males in different groups. Because the groups were all members of the same provisioned population with low climatic variation over the course of this study, we could assume that the influence of ecological parameters (e.g. temperature and rainfall) on physiological stress was equivalent across all groups.

Rhesus macaques are highly social cercopithecid monkeys that usually range in south, southeast, and central Asia. They live in groups with matriarchal structure and linear dominance hierarchies for both males and females (Maestripieri, 2003, Manson, 1998, Missakian, 1972). Males disperse from their natal groups and join new groups at puberty. In the free-ranging rhesus macaque population on the island of Cayo Santiago, PR, migrating males generally enter a new group at the bottom of the hierarchy and gain dominance with seniority (Berard, 1999, Bercovitch, 1997, Manson, 1998). However, exceptions to this pattern, in which an immigrant adult male aggressively takes over the alpha position in a new group, have been reported (Georgiev et al., 2016). Rhesus macaques are seasonal breeders. On Cayo Santiago, mating occurs during the spring and summer and is followed by births during autumn and winter (Hernández-Pacheco et al., 2016). Female rhesus macaques on Cayo Santiago have a high degree of reproductive synchrony, such that the majority of females come into estrus at the same time and the number of pregnant females increases over the course of the mating season (Dubuc et al., 2011, Ostner et al., 2008c). Estrous females engage in consortships in which the males mate-guard the females (Dubuc et al., 2012). In the Cayo Santiago rhesus population, high-ranking males have a small reproductive advantage over low-ranking males (Berard et al., 1994, Dubuc et al., 2011, Widdig et al., 2004). A previous study showed that during the mating season, top-ranking males participated in more consortships with estrous females and had the highest levels of copulatory activity (Higham et al., 2011). Individual male copulation rates were negatively correlated with concentrations of urinary C-peptide of insulin (a validated biomarker of energy balance in rhesus macaques and other primates; Girard-Buttoz et al., 2011, Emery Thompson, 2017) and body fat, indicating that top-ranking males made the greatest mating effort and were in the worst energetic condition at the end of the mating season (Bercovitch, 1997, Higham et al., 2011). Associations between GC concentrations and rank in rhesus macaques have been inconsistent, with some studies finding no relationship between GC concentrations and dominance rank (Bercovitch and Clarke, 1995), while others reporting that high-ranking individuals have higher GC concentrations only during periods of instability (Higham et al., 2013; see also McFarland and MacLarnon, 2013).

In this study we analyzed data on GC concentrations of the alpha males and the two next highest-ranking males from each of nine social groups of free-ranging rhesus macaques on Cayo Santiago, during the mating season. Given that males in most rhesus macaque groups do not typically contest dominance rank, we hypothesized that male GC concentrations would be mainly driven by mating
effort, operationalized here by the number of females that conceived in a given month and the number of males within each group that competed over those females. We tested the following four predictions:

1) GC concentrations vary in relation to the number of females that conceive within a group: If GC concentrations of top-ranking males are driven by the potential costs associated with mating effort, we expect that GC concentrations will be highest during the months when the most females conceive and to diminish with the number of mates available. The alternative to this prediction is that GC concentrations increase over the course of the mating season, indicating an accumulation of physiological stress and worsening physical condition, rather than being more closely linked to immediate investment in mating effort.

2) GC concentrations are higher in alpha males than among other top-ranking males: We predict that alpha males, in particular, will have significantly higher GC concentrations than other top-ranking males. Alpha males are predicted to have priority of mating access, but prior studies suggest that alpha males have limited control over paternity in this population of rhesus macaques (Widdig et al., 2004), indicating that they must expend considerable effort on mate-guarding to achieve high reproductive success (Dubuc et al., 2011).

3) GC concentrations increase with the number of adult males in a group: If the GC concentrations of top-ranking males are driven by the physiological costs of mating effort, we predict that males in groups with more adult males will have higher GCs. Alternatively, males in larger groups (which have more males) may have lower GC concentrations than those in smaller groups because of their competitive advantage in inter-group competition (on Cayo, larger groups outrank smaller groups: Balasubramaniam et al., 2014).

4) GC concentrations are impacted by male hierarchy stability: If the GC concentrations of top-ranking males are driven by general aggressive competition rather than mating effort, we predicted that top-ranking males in unstable groups (where the top-ranking males changed dominance status during the study) would have higher GC concentrations than those in stable groups.

It is important to note that we are not explicitly testing the relationship between individual levels of mating effort and GC concentrations, but rather the effect of periods of increased mating opportunities within the group on top-ranking male GC concentrations. Previous studies have shown that high-ranking males have higher reproductive success (Berard et al., 1994, Dubuc et al., 2011, Widdig et al., 2004), participate in more consortships with estrous females (Higham et al., 2011), and have higher levels of copulatory activity (Higham et al., 2011) than low-ranking males. Thus, the number of conceptions per month indicates the mating opportunities available to males during that month, and regardless of mating success, we expect that top-ranking males would at least be aware of these opportunities and would adjust mating effort appropriately. We are therefore testing if top-ranking males have variation in their GC concentrations in relation to the temporal variation in mating opportunities, which we have measured with the number of conceptions per group each month.
2. Methods

2.1. Study site and subjects

Cayo Santiago is a 15.2 ha island located 1 km off the coast of Puerto Rico. A colony of approximately 400 free-ranging rhesus macaques was established on the island in 1938 with wild-caught animals from India (Rawlins and Kessler, 1985). By 2013, the population had grown to over 1200 individuals. The animals have divided themselves into 9 social groups. They are provisioned with water and monkey chow and are able to forage on the vegetation growing on the island. Thorough records of all births and deaths have been maintained since 1956; thus, the ages of all individuals are known and the size of each group is regularly updated (Rawlins and Kessler, 1986). These birth records were used to back-calculate (based on an average gestation length in this species of ca.167 days, Silk et al., 1993) the number of infants conceived during each month in each group during the study period.

2.2. Data collection

The study was conducted during the mating season of 2013. The mating season occurred between February and July 2013; however, data collection did not begin until March, due to the trapping season, which prevents behavioral data collection on Cayo Santiago. Study subjects were 21 adult males belonging to 9 different groups (see Table 1 for details). At the beginning of the study period, these subjects occupied the three top-ranking positions in each group (i.e., the alpha male, the beta male, and the gamma male). Dominance rank was determined at the beginning of the study based on outcome of fights, access to food, displacement events, fear grins, and avoidance behaviors (Maestripieri and Wallen, 1997). The three highest-ranking males were identified in each group and their dominance rank was subsequently reassessed throughout the study through ad libitum observations. Five of the nine groups were considered unstable because changes in the dominance ranks of the top-ranking males in these groups occurred during the study. Two of the groups were small, recently formed groups that stayed on the periphery of the larger group from which they had split-off. During parts of the study, these groups were not considered independent from the larger group. For this reason, only 3 males were studied from these two groups combined and they were classified as top-ranking (because of their high rank within the newly formed groups), but none of the individuals were identified as alpha because they were submissive to the higher-ranking individuals in the larger group to which they were peripheral. The other three unstable groups had major changes in the rank of the highest ranking males throughout the study. In one group, the alpha male was severely injured during an intergroup interaction and subsequently was no longer part of his original group. In another group, the highest-ranking positions were taken over by outside males that displaced the top-ranking males when they entered the group. In the final unstable group, the alpha male was displaced by lower ranking males. Four of the groups had stable composition and dominance hierarchies throughout the study period. It was not possible to study all the males in each group because that would have required studying over 500 males.
Table 1. The age, rank, and alpha status of study subjects, the number of adult males, size, name, stability status, and number of conceptions of the subject's group, and the average number of samples collect per month for each subject.

<table>
<thead>
<tr>
<th>Male</th>
<th>Age</th>
<th>Rank</th>
<th>Alpha</th>
<th>M</th>
<th>Group size</th>
<th>Group</th>
<th>N/month</th>
<th>Stability</th>
<th>Conceptions</th>
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<td>60</td>
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<td>3.33</td>
<td>S</td>
<td>60</td>
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<td>F</td>
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<td>S</td>
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<td>U</td>
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<td>26</td>
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<td>3</td>
<td>0</td>
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<td>S</td>
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</table>

Fecal samples were collected opportunistically 5 days per week from approximately 7:00 to 14:30 h. They were placed on ice until the end of the day when they were frozen at −20 °C. All samples were extracted with 80% methanol following Palme (2005). The fresh weight of the fecal pellet used for extraction was recorded (0.5 ± 0.03 g) and used to calculate the final concentration of hormone in the sample. The samples from three males (occupying one group) were extracted in a different lab than all other samples. Mean values of GCs from sample set 1 (N = 74 monthly male means from 20 males) were 12.46 ng/g wet weight (range: 2.7–28.92) and mean values from dataset 2 (N = 12
monthly male means from 3 males from one of the groups) were 9.64 ng/g wet weight (range: 5.5–13.47). While there was some evidence that extraction procedure differences might have affected final GC values in control samples, we accounted for this effect by including 'sample set' as a random effect in statistical models. Furthermore, we re-ran our analyses by only using the larger dataset (20 males): results were not affected in any significant way. All samples were assayed in the Hominoid Reproductive Ecology Laboratory at the University of New Mexico.

2.3. Assay procedures

We analyzed 240 fecal samples, collected from the 23 study males between 1 March and 8 July 2013. Prior to assay, extracted samples were dried down at 37 °C under a nitrogen stream and reconstituted in assay buffer. Samples were assayed for corticosterone using the ImmunoChem double-antibody radioimmunoassay kit, following protocols from Beehner and McCann (2008). This antibody cross-reacts with several major fecal metabolites of cortisol and has been found to reliably detect fecal glucocorticoid elevations following ACTH administration in a variety of mammals, including macaques (Wasser et al., 2000). We compared this assay to a group-specific assay for 3α,11β-Dihydroxy cortisol metabolites (Ganswindt et al., 2003, Heistermann et al., 2004) that has been used previously for rhesus macaques (e.g., Brent et al., 2011, Higham et al., 2013). Results from macaque fecal samples were significantly correlated ($r = 0.419, N = 497, P < 0.001$), but the corticosterone assay performed more consistently. Assay accuracy, assessed by the recovery of standards added in duplicate to an extracted fecal sample, was 102.6 (± 11.7 SD)%$. Serial dilution of an extracted sample produced curves parallel to the standard curve. Inter-assay CVs were 11.0% and 11.8% for low and high fecal pools, respectively. Intra-assay CV, calculated as the mean CV of duplicate determinations, was 5.6%.

2.4. Statistical analysis

We excluded 21 samples with high intra-assay CVs ($\geq 15\%$) from our statistical analyses. The final dataset contained 219 samples (mean number of samples per male per month = 2.6; median = 2; min = 1; max = 8). We calculated the mean monthly GC concentration for each male for each month, resulting in 86 mean male-month values, which we used in further mixed-effects model analysis.

To examine factors affecting male GC concentrations, we applied linear mixed-effects model analysis in R (R Core Team, 2014). The dependent variable was the monthly male mean of GC concentration (ng/g wet weight), which was normally distributed following a log-10 transformation (Shapiro-Wilk W test: $W = 0.98, N = 86, P = 0.26$). To test the predictions outlined above, we included several fixed effects in our model: (1) male rank (whether the subject was alpha or not); (2) number of females that conceived per month in each group (based on an estimated gestation length of 167 days (Silk et al., 1993) and birth records of offspring conceived that year), (3) number of adult males ($\geq 5$ years old) per group, (4) male age, and (5) male dominance hierarchy stability (1/0). We included three random effects as follows: sample set (two different sample sets, with three males occupying one group being
extracted in a different lab than the rest of the samples), study group (nine different groups) and male ID (23 different individuals). We specified male ID as nested within group ID. Specifying male ID as a random effect, in addition to averaging multiple samples from the same male to first obtain a mean monthly value ensured that we accounted for repeated observations of the same males during the study period and minimized pseudoreplication.

Initially, we fitted a model containing two-way interactions between alpha status and other fixed effects and tested it against a null model (containing only the random effect terms). To obtain a more parsimonious model, we then excluded any non-significant interactions. Finally, we visually confirmed that the data met model assumptions by examining the plots of fitted values vs. residuals and quantile-quantile plots.

3. Results

The average glucocorticoid concentration for all males was 12.07 ± 4.82 ng/g wet weight (range: 2.7–28.93, N = 86 male-monthly means, calculated from 219 fecal samples). For all males, glucocorticoid concentrations were highest at the beginning of the study period (Table 2) and declined linearly as the mating season progressed (Fig. 1), from an average of 15.56 ng/g wet weight in March to 8.10 ng/g wet weight in July. Thus, the fecal glucocorticoid concentrations of top-ranking males dropped, on average, by almost half from the early mating season to the end of the mating season. The peak in GC concentrations is at the beginning of the mating period, which coincides with the peak in conceptions for this mating season (Fig. 1).
Table 2. Linear mixed model summary of factors affecting variation in GC concentrations (log-transformed values) of high-ranking male rhesus macaques during the mating season on Cayo Santiago, Puerto Rico.

<table>
<thead>
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<th>Factor</th>
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<th>SE</th>
<th>LRT</th>
<th>P-value</th>
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<td>Intercept</td>
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<td>0.091</td>
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<td>Alpha status</td>
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<td>0.039</td>
<td>6.159</td>
<td>0.013</td>
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<td>Monthly number of conceptions</td>
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<td>0.002</td>
<td>8.581</td>
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<td>Male age</td>
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<td>0.006</td>
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<td>0.844</td>
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<td>Number of adult males in group</td>
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<td>Male hierarchy stability</td>
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<td>0.049</td>
<td>2.737</td>
<td>0.098</td>
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Fig. 1. Female conceptions per month (Jan – Jul) and male glucocorticoid (GC) concentrations among alpha and non-alpha high-ranking (HR) males across nine social groups on Cayo Santiago (Mar – Jul). Bars indicate means of male monthly means. Error bars show SE. Number of female conceptions estimated from birth records by subtracting 167 days (average gestation length in rhesus macaques; See Silk et al., 1993).

The model containing the two-way interactions between alpha male status and the number of males, number of females that conceived, and male hierarchy stability, as well as the independent effect of age, was significant when tested against the null model, containing only the random effects (log likelihood ratio test, LRT, against the null: $\chi^2 = 21.59$, $P = 0.006$). However, none of the interactions considered were significant (all $P > 0.30$) and the independent effect of male age was similarly not significant (LRT = 0.03, $P = 0.87$). To examine the effects of our predictors independently from their non-significant interactions with alpha status we removed all interaction terms to obtain a more parsimonious model ($\chi^2 = 17.555$, $P < 0.004$, Table 2). Alpha males had significantly higher average
monthly glucocorticoid concentrations than other top-ranking males (Table 2) throughout the study period (Fig. 2). The number of conceptions in a group was positively associated with GC concentrations (Table 2) of top-ranking males (Fig. 3). Male age and number of males in a group were not significantly associated with male GC concentrations. Males in unstable groups had slightly higher GC concentrations ($12.42 \pm 2.34$ ng/g wet weight) than males in stable groups ($11.74 \pm 3.45$ ng/g wet weight), but the difference was not significant ($P = 0.098$; Table 2).

Fig. 2. Boxplots of mean glucocorticoid concentrations of non-alpha, high-ranking males vs. alpha males across the entire study period. The empty circles show outliers; black circles show the individual data points in the dataset, which have been jittered to avoid over-plotting. In the case of outliers (hollow circles) the black circles replicate those values. Alpha males had higher GCs than other high-ranking males. For results of LMM see Table 2 and text.
4. Discussion

High glucocorticoid concentrations among high-ranking male primates have been attributed alternatively to the high physiological costs of mating effort itself and/or those of aggressive competition over dominance, but correlative studies can rarely disentangle these effects. We examined predictors of GC concentrations among male rhesus macaques in order to test the hypothesis that, in this species that engages relatively rarely in dominance contests, variation in mating effort would be the key predictor of the GC concentrations of high-ranking males. In contrast to previous studies, we focused on top-ranking males to specifically understand the proximate sources of variation in their physiological stress levels, independently of any difference between high and low ranking males. In support of this hypothesis, we found that top-ranking males exhibited the highest GCs during the month of March, when the number of female conceptions was the highest of the entire mating season. Alpha males had higher GC concentrations than other top-ranking males throughout the mating season. The number of adult males and the hierarchy instability in the group did not influence GC concentrations, suggesting that the effect of dominance competition on the physiology of the top-most males in the hierarchy, beyond the effect of being the alpha male, was limited, at least in this population. We also did not find any significant effects of age on GC concentrations of these top-ranking males (see also Rakhovskaya, 2011).

Prior studies examining the overall correlation between dominance rank and GCs in male rhesus macaques have produced mixed results (Bercovitch and Clarke, 1995, Higham et al., 2013), but neither a significant correlation nor a lack of correlation across all ranks can inform us as to whether alpha males are exceptional. Our study indicates that across multiple groups, alpha males are specifically more susceptible to physiological stress compared to those adjacent in rank. This accords with similar findings in long-tailed macaques (Macaca fascicularis: Girard-Buttoz et al., 2014) and savannah baboons (Papio cynocephalus), where only the alpha male had significantly higher GC concentrations compared to other males (Gesquiere et al., 2011). Our results uniquely extend the current understanding of rank-related variation in GC concentrations by demonstrating that during the mating season alpha males consistently have higher GCs than other top-ranking males, even across widely varying sociodemographic conditions. While rank and group tenure length are correlated in this species and also somewhat related to age (Bercovitch, 1997, Manson, 1998, Berard, 1999), our analyses suggest that alpha status, rather than age, was a better predictor of increased GC production. Given a limited role for aggressive competition within groups during the mating season, our finding suggests that alpha males can experience elevated stress from causes other than aggressive challenges. Prior studies suggest that high rank confers only a minor reproductive advantage in this population (Berard et al., 1994, Dubuc et al., 2011, Widdig et al., 2004), and that the
high energetic costs associated with long consortships may be the factor that limits any one male's ability to monopolize breeding opportunities (Higham et al., 2011, Higham et al., 2013). Our study suggests that alpha males may suffer these costs disproportionately, perhaps because priority of access might give them more opportunities to mate guard (but see Dubuc et al., 2011), or because their physical condition permitted them to invest more heavily (Higham et al., 2011, Higham et al., 2013). However, in our efforts to sample from multiple groups, we were unable to probe deeper into how mate guarding may specifically have affected the GCs of alphas versus other top-ranking males. Our results do suggest that scramble mating competition may be a more important factor in driving GC production than contest mating competition among the rhesus macaques of Cayo Santiago. Top-ranking male GC concentrations were positively associated with the number of conceptions per month in each group, suggesting that males may experience physiological stress during a period when many females are in estrus and males are trying to consort with as many females as possible. Conversely, the number of sexually mature males in a group was not associated with GC concentrations, which suggests that males are not engaging in significant levels of contest competition for mates. Furthermore, if males were primarily engaging in contest competition, then we would expect GC concentrations to be higher later in the mating season when far fewer females are in estrus and males would need to compete directly for access to those females. Instead, we saw that as the number of males in each group stays relatively constant over the mating season and the number of estrus females declined, male GC concentrations declined also, rather than increased, as would be expected if contest competition was occurring.

Our finding that GC concentrations were higher at the beginning of the mating season than at the end, corresponding to the time of the mating season when most females conceived, supports previous findings of GC variation in male rhesus macaques (Rakhovskaya, 2011) and extends findings of general mating season GC increases in a large number of primate and non-primate taxa (Macaca fascicularis: Girard-Buttoz et al., 2014; Papio ursinus: Kalbitzer et al., 2015; Cebus apella nigritus: Lynch et al., 2002; Eulemur fulvus rufus: Ostner et al., 2008b; Macaca assamensis: Ostner et al., 2008a; Cervus elaphus: Pavitt et al., 2015; Rupicapra rupicapra: Corlatti et al., 2012). In Alpine chamois, for example, it is specifically mating effort that is associated with increased cortisol concentrations – territorial males have elevated cortisol concentrations during the rut and non-territorial males do not (Corlatti et al., 2012). In many of these species, the mating season is associated with greatly increased rates of male aggression or with costly physiological investments. In rhesus macaques, aggressive competition over mates is relatively rare, and while males do undergo seasonal increases in body mass, this appears to be an increase in fat reserves to support endurance rivalries during the mating season rather than investment in muscle mass that could be of advantage in direct male-male combat (Bercovitch, 1997).

Across a variety of contexts, GC concentrations increase with energetic stress, consistent with a metabolic role of GCs in mobilizing energy reserves for immediate use (Behie et al., 2010, Busch and Hayward, 2009, Gesquiere et al., 2008, Jaimez et al., 2012, Martinez-Mota et al., 2007, Rangel-
Negrin et al., 2009). Based on their study of chimpanzees, Muller and Wrangham (2004) introduced the idea that high GC concentrations in high ranking males may be indicative of high energetic costs, a prediction that was later support by direct observations of reduced feeding and lower energy balance in males on mating days (Georgiev et al., 2013, Georgiev et al., 2014). Previous research has emphasized the energetic costs associated with male mate-guarding effort in rhesus macaques (Higham et al., 2011, Higham et al., 2013). These energetic demands are high enough that male condition declines substantially over the mating season (Bercovitch, 1997, Higham et al., 2011). This change in body condition through the mating season is positively associated with reproductive success in rhesus macaques, such that males who sire offspring have significantly more fat at the beginning of the mating season than those who do not sire offspring, but have comparable amounts of fat at the end of the mating season (Bercovitch and Nürnberg, 1996). Our findings are consistent with the interpretation that GC concentrations are high early in the mating season when males are exerting more effort to secure mating opportunities and when more conceptions occur, and decline as the physiological challenges associated with mating effort subside. This indicates that elevated GCs are associated with the immediate need to mobilize energy for mating effort, but not necessarily with the progressive loss of condition that takes place over the duration of the mating season. It is possible that the long-term effects are mediated by variation in physical preparedness for the mating season. High-ranking rhesus males are significantly fatter at the beginning of the mating season than middle- and low-ranking males (Bercovitch, 1997). They also have higher levels of innate immune system functionality and lower levels of oxidative damage before the start of mating season than lower-ranking individuals (Georgiev et al., 2015) suggesting overall better health. In mandrills, high-ranking males had the lowest oxidative damage during the non-breeding season and were the only males to show an increase in oxidative damage during the mating season (Beaulieu et al., 2014). High-ranking males starting the mating season at such an advantage over lower-ranking individuals, may therefore have a greater ‘margin’ for tolerating the physiological costs associated with securing sexual access. Previous studies have indicated that male hierarchy stability can impact stress levels (Higham et al., 2013, Sapolsky, 1983, Sapolsky, 1992, Setchell et al., 2010). Higham et al. (2013) found that high-ranking males on Cayo Santiago had significantly higher GC concentrations only during periods of instability. During these periods, males in the population have similar GC patterns to species that are characterized by direct contest competition over dominance, such as mandrills and chacma baboons (Higham and Maestripieri, 2014). In chacma baboons and mandrills, subordinate males have higher GC concentrations in stable conditions, but high-ranking males have higher GC concentrations during periods of instability (Bergman et al., 2005, Setchell et al., 2010). If the costs of male status competition explain high GCs among high ranking males, we expected rank hierarchy instability to increase GC concentrations. We found that males in unstable groups had slightly higher GC concentrations than males in stable groups, but the difference was not significant. This suggests that stability of the dominance hierarchy affects within-group differences in GC concentrations between the top and the bottom of the hierarchy more so than it does differences among the top of the
hierarchies of different groups. However, having longitudinal data on hierarchy stability both inside and outside the mating season, and its associations with shifts in GCs, would improve the power of such analyses and should be important targets for future research.

The results of this study contribute to our understanding of the physiological stress associated with alpha male status and breeding seasonality. However, there were several limitations to this research. First, data were collected only during the mating season. Thus, our results can only describe the factors that influence physiological stress during the mating season. In the absence of mating effort, we might not only expect changes in the absolute level of physiological stress, but changes in the proximate factors that predict it. Second, we studied only the three highest-ranking males from each group. Therefore, our study does not provide information about the factors affecting GC concentrations among lower-ranking males or how low-ranking males might differ in their physiological response to increased mating effort or competition for status from the high-ranking individuals that we studied. Although we were able to compare stable and unstable groups, when major changes in the dominance hierarchies occurred in unstable groups, we were not able to compare GC concentrations before and after takeovers for all males involved (e.g. new alpha males or immigrant males). Thus, additional research is needed to better understand how mating competition and changes in dominance hierarchies affect all individuals within a group. Additionally, we used the number of conceptions in each group per month as a proxy for estimating the potential mating opportunities for males in our study. We use this approach combined with known data on estrus synchrony and dominant male mating success to discuss potential mating effort by males; however, we do not compare GC concentrations to behavioral data on mating effort. Thus, our results do not indicate how GC concentrations vary in relation to individual mating behaviors, but rather how GC concentrations vary in relation to periods of increased overall mating activity and the availability of potential mating partners.

As an additional limitation to our study, GC concentrations provide an overall assessment of ‘physiological stress’, but it is difficult to ascertain the exact source of this stress. A key function of GCs is the mobilization of energy reserves for immediate use, leading to the prediction, supported by empirical data from a variety of primates, that high GC concentrations are indicative of energetic stress (Emery Thompson, 2017). Nevertheless, psycho-social stressors also affect the functioning of the hypothalamic-pituitary-adrenal axis (Creel et al., 2012, Kohn et al., 2016, Wittig et al., 2015) and can confound the use of GC concentrations purely as indicators of energetic shortfalls (Emery Thompson, 2017). In this study, two sets of factors are likely to contribute to the higher GC concentrations among alpha males relative to other high-ranking males. First, the specific energetic costs of mating effort, assessed via urinary C-peptide levels, a marker of energy balance, are higher among higher-ranking males compared to lower-ranking males due to their more intensive engagement in endurance rivalries (Higham et al., 2011). Alpha males should thus incur the highest energetic costs of all. Second, levels of psycho-social stress are also expected to be higher among the top-ranked animals during the mating season, given the need for increased vigilance in monitoring
multiple females in estrus (especially at the peak of mating), further exacerbated by the greater frequency of extra-group male incursions during this season (Lindburg, 1969). Although previous work has not identified a clear relationship between GC excretion and dominance status in single, stable groups during the mating season (Higham et al., 2013), our ability to target the top-ranking males across multiple groups provides additional insight into the physiological demands of high status among rhesus macaque males.

Overall, our findings add to a growing body of literature linking male mating effort with physiological stress, and more generally showing that mating and reproduction are costly in rhesus macaques (Higham and Maestripieri, 2014, Hoffman et al., 2008, Maestripieri and Georgiev, 2016). Male mating behaviors are positively associated with GC concentrations in a variety of species, including long-tailed macaques (Girard-Buttoz et al., 2014), chacma baboons (Bergman et al., 2005, Kalbitzer et al., 2015), and red deer (Pavitt et al., 2015). This stress can have health implications (Cavigelli and Caruso, 2015). Based on this evidence, it is important that future models of male reproductive strategies consider both the benefits and the costs of rank acquisition and mating effort to lifetime reproductive success.

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