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## **Stress, social behaviour, and secondary sexual traits in a male primate**

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**Running head:** Behaviour, stress and ornaments in mandrills

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## ABSTRACT

We examined variation in glucocorticoid levels in the mandrill, a brightly coloured primate species, to identify major social influences on stress hormones, and investigate relationships among glucocorticoid levels, testosterone and secondary sexual ornamentation. We collected a total of 317 fecal samples for 16 adult male mandrills over 13 months, including mating and non-mating periods and periods of both dominance rank stability and instability, and compared fecal glucocorticoid levels with dominance rank, rank stability, presence of receptive females, gastro-intestinal parasite infection, fecal testosterone and facial red coloration. Glucocorticoid levels did not vary systematically with dominance rank, but increased when the dominance hierarchy was unstable, and increased in the presence of receptive females. The relationship between dominance rank and glucocorticoid levels changed direction according to the stability of the dominance hierarchy: glucocorticoid levels were higher in subordinate males under stable conditions, but under conditions of instability higher ranking males had higher glucocorticoid levels. The influence of dominance rank also interacted with the presence of receptive females: glucocorticoids were higher in dominant males than in subordinates, but only during mating periods, suggesting that dominant males are more stressed than subordinates during such periods. These findings support previous studies showing that the relationship between glucocorticoids and dominance rank in male baboons is dependent on the social environment. We also found that males with higher glucocorticoids suffered a higher diversity of gastrointestinal parasite infection, in line with evidence that glucocorticoids suppress the immune system in other species. However, we found no support for the

stress-mediated immunocompetence handicap hypothesis for the evolution of condition-dependent ornaments: glucocorticoid and testosterone levels were positively related, rather than the negative relationship predicted by the hypothesis, and we found no relationship between red colour and glucocorticoid levels, suggesting that glucocorticoids do not play a role in translating social conditions or physical health into ornament expression in this species.

**KEYWORDS:** sexual selection; sexual signal; immunocompetence handicap hypothesis; badge-of-status; condition-dependent ornaments

## INTRODUCTION

Vertebrates respond to stress by activating the hypothalamus-pituitary-adrenal axis and releasing glucocorticoids into the bloodstream. These stress hormones mobilize energy by stimulating the release of glucose into the bloodstream and lipolysis in adipose tissue, and enhance delivery of glucose, fatty acids and triglycerides to skeletal muscle and the brain (Sapolsky, 1994; Wingfield et al., 1998). Glucocorticoids also divert energy from various costly physiological processes that are not required for immediate survival, including digestion, energy storage, growth, immunity and reproduction (reviews in Sapolsky, 2000; Sapolsky, 2002). Thus, while adaptive in the short-term, chronic glucocorticoid elevation has serious negative effects on the organism, including reproductive failure and decreased resistance to disease (Sapolsky, 2002). Social interactions can be important sources of stress, and attention has focussed on the relationship between glucocorticoid levels and dominance status in group-living species, such as primates. In addition, the negative influence that stress exerts on the expression of condition-dependent traits has led to hypotheses suggesting that sexually selected traits honestly reflect individual quality by advertising the degree to which the bearer can tolerate the immunosuppressive effects of glucocorticoids.

In this study we combined fecal measures of glucocorticoid levels for 16 adult male mandrills with data concerning male dominance rank, rank stability, the presence of receptive females, male sociality, gastro-intestinal parasite infection and red coloration to investigate two main sets of questions: First, do social factors influence stress levels in this

group-living primate? Second, do stress hormones influence the expression of secondary sexual traits? Here we provide background on relevant previous findings, introduce our hypotheses, and make specific predictions related to each of these questions. We summarise our hypotheses and predictions in Table 1.

### **Social influences on stress in male primates**

First, we test the hypothesis that dominance rank influences glucocorticoid levels (Hypothesis 1). In some group-living species, including olive baboons (*Papio hamadryas anubis*) (Sapolsky, 1992), chacma baboons (*Papio hamadryas ursinus*) (Bergman et al., 2005), and Assamese macaques (*Macaca assamensis*) (Ostner et al., 2008a), subordinate males have higher glucocorticoids than dominant males. This is thought to be due to the costs of attempting to achieve higher rank and harassment from higher-ranking males (the subordinate stress paradigm, Creel, 2001). Conversely, however, in other species, including Japanese macaques (*Macaca fuscata*) (Barrett et al., 2002), chimpanzees (*Pan troglodytes*) (Muller and Wrangham, 2003), Verreaux's sifakas (*Propithecus verreauxi*) (Fichtel et al., 2007), and gray-cheeked mangabeys (*Lophocebus albigena*) (Arlet et al., 2009), it is dominant males that have higher glucocorticoids, likely due to the stress associated with sexual activity and mate-guarding, and repeated challenges from other males (Creel, 2001; Creel et al., 1996; Morell, 1996). Finally, still other studies have found that dominance rank and glucocorticoids are independent of one another, for example in rhesus macaques (*Macaca mulatta*) (Bercovitch and Clarke, 1995), tufted capuchins (*Cebus*

*apella*) (Lynch et al., 2002), redfronted lemurs (*Eulemur fulvus*) (Ostner et al., 2008b), and mountain gorillas (*Gorilla gorilla*) (Robbins and Czekala, 1997).

Mandrills live in multi-male, multi-female groups, with a strong male hierarchy. Males attain alpha rank in two ways: via physical aggression or by succession, if the two highest-ranking males fight and both are injured, leaving a third as the new alpha male (Setchell, 2003; Setchell et al., 2005). Occupying the top rank position is extremely valuable to males in terms of reproductive success: alpha males in our study population account for 77-100% of peri-ovulatory mate-guarding and sire 33-100% of offspring in any one breeding season (Setchell et al., 2005). If the subordinate stress paradigm (Creel, 2001) holds for male mandrills, then we predicted that subordinate males would have higher glucocorticoids than dominant males (Prediction 1A). Alternatively, if stress is higher in dominant males, due to increased sexual activity and mate-guarding, and repeated challenges from other males (Creel, 2001; Creel et al., 1996; Morell, 1996), then dominant males should have higher levels of glucocorticoids than their subordinates (Prediction 1B).

Second, we tested the hypothesis that rank instability influences glucocorticoid levels (Hypothesis 2). Studies of olive baboons provide insight into the diversity of findings relating glucocorticoids to dominance rank, by showing that the relationship is contingent on the stability of the dominance hierarchy, and thus, presumably, on the predictability of social interaction (Sapolsky, 1992a, , 1993; Sapolsky et al., 1997). When male hierarchies are stable, subordinate status and social isolation are both associated with higher basal levels of glucocorticoid (Sapolsky et al., 1997). However, this is no longer the case when the

hierarchy is unstable, when dominant males show higher glucocorticoid levels (Sapolsky, 1992a, 1993; Sapolsky et al., 1997). Similar findings have since been reported for chacma baboons (Bergman et al., 2005) and meta-analyses of data available for primates (Abbott et al., 2003), and for group-living animals in general (Goymann and Wingfield, 2004), have also concluded that the ability to predict and control the social environment is important in the relationship between glucocorticoids and rank, and that the physiological costs associated with different social ranks are determined by the way in which rank is achieved and maintained, rather than by the rank position itself. Goymann and Wingfield (2004) have formalized this relationship between social status and stress hormones in the 'allostatic load' model. 'Allostatic load' refers to the cumulative physiological costs of maintaining homeostasis in the face of both predictable and unpredictable events (McEwen and Wingfield, 2003). An increase in allostatic load is typically accompanied by a rise in glucocorticoid levels, and the relative allostatic load of social status predicts whether dominants or subordinates express higher or lower GC concentrations (Goymann and Wingfield, 2004).

Hypothesis 2 predicts that allostatic load, and therefore glucocorticoid levels, increase in all males when dominance ranks are unstable and interactions are unpredictable (Prediction 2A). Further, If rank stability mediates the relationship between dominance rank and glucocorticoid levels, as in other male primates, then we predicted that we would find an interaction between the influence of dominance rank per se, and rank stability, such that dominant males show higher glucocorticoid levels when the hierarchy is unstable, while



subordinate status should be linked with higher levels of glucocorticoids when male hierarchies are stable (Sapolsky et al., 1997) (Prediction 2B).

In addition to the influence of dominance rank and rank stability on glucocorticoid levels in male primates, the presence of receptive females is also linked with elevated cortisol levels in long-tailed macaques (*Macaca fascicularis*) (Glick, 1984), Japanese macaques (Barrett et al., 2002), tufted capuchins (Lynch et al., 2002), Assamese macaques (Ostner et al., 2008a), and redfronted lemurs (Ostner et al., 2008b). This likely reflects increased male-male competition for access to receptive females, and the energetic burden of mate-guarding, which constrains a male's foraging activity (Alberts et al., 1996; Bercovitch, 1983). Thus, our third hypothesis was that the presence of receptive females would influence glucocorticoid levels (Hypothesis 3). If direct competition over access to females results in increased stress levels in male mandrills, then glucocorticoid levels should be higher when receptive females are present than when they are not (Prediction 3A). Further, this effect should be stronger in dominant males, who mate-guard females, than in subordinates, and thus we predict that there will be a significant interaction between the influence of dominance rank and the presence of receptive females on male glucocorticoid levels (Prediction 3B).

Finally, we hypothesized that sociality would influence on male glucocorticoid levels (Hypothesis 4). Alpha male mandrills are the most sociable males while lower-ranking males range from group associated to solitary (Setchell and Dixson, 2001c; Wickings and Dixson, 1992), suggesting that subordinates may ameliorate social stress by avoiding other

males. Studies of other species have shown that glucocorticoid levels are positively associated with aggression received (e.g. Ostner et al., 2008a), leading us to predict that if subordinate male mandrills ameliorate social stress by avoiding other males, then peripheral and solitary males should experience lower glucocorticoid levels than social, group-living males, because the former are able to avoid social interactions and thus potential conflict (Prediction 4).

### **Stress hormones and secondary sexual traits**

Male mandrills possess a suite of secondary sexual coloration, including bright red coloration on the face, rump and genitalia that is expressed more in dominant males (Setchell and Dixson, 2001c), reflects testosterone levels (Setchell et al., 2008b), and attracts females independently of dominance rank (Setchell, 2005). Males with suppressed ornamentation are able to develop maximal expression (i.e. to express their physiological potential) if 'released' from the social enforcement of low rank (Setchell and Dixson, 2001a). Handicap theories of mate choice propose that such exaggerated secondary sexual traits are condition dependent, and that only individuals of superior quality will be able to express costly ornamentation (e.g. Zahavi, 1975). For example, the parasite-mediated sexual selection hypothesis suggests that secondary sexual traits reliably reflect the ability of an individual to resist parasites (Hamilton and Zuk, 1982; Moller and Saino, 1994). Members of the opposite sex will be selected to choose the most ornamented mate because such mates provide fitness benefits, either directly, in terms of parasite transmission avoidance and increased investment in offspring or both, or indirectly, in the form of 'good

genes' for vigour and health that will be passed on to offspring (Able, 1996; Andersson, 1994; Hamilton and Zuk, 1982; Zahavi, 1975). An extension of this hypothesis, the immunocompetence handicap hypothesis (ICHH), holds that testosterone-dependent ornaments signal the ability to cope with the immunosuppressive effects of testosterone (Folstad and Karter, 1992). However, the relationship between testosterone and immune function has been subject to much controversy, and clear-cut evidence that natural physiological levels of testosterone consistently suppress the immune response is lacking (Braude et al., 1999; Hasselquist et al., 1999; Olsen and Kovacs, 1996).

An alternative model suggests that the ICHH functions via a trade-off between glucocorticoid levels and the immune system (Braude et al., 1999; Evans et al., 2000; Hillgarth and Wingfield, 1997; Møller, 1995; Siva-Jothy, 1995; Westneat and Birkhead, 1998). This stress-mediated hypothesis for the evolution of condition-dependent traits is based on the immunosuppressive effects of glucocorticoids in both birds and mammals (review in Buchanan, 2000; Sapolsky, 1992b, , 2000), and studies suggesting that condition-dependent sexual signals are influenced by physiological stress (Buchanan, 2000; Evans et al., 2000; Husak and Moore, 2008), and predicts that high levels of glucocorticoids should be linked to increased pathogen loads, and decreased expression of secondary sexual traits.

While the relationship between testosterone and sexually selected traits is well established (Andersson, 1994), fewer studies have investigated the relationships among stress hormones, sex steroids, and ornament expression (Evans et al., 2000; Husak and Moore,

2008; Owen-Ashley et al., 2004; Parker et al., 2002). However, evidence from birds and amphibians shows a negative influence of stress on secondary sexual traits: elevated glucocorticoid levels during development have a negative influence on the quality of bird song (Buchanan et al., 2004; Spencer et al., 2003; Wada et al., 2008), the quality and attractiveness of toad vocalisations is negatively related to glucocorticoids, but not testosterone (Leary et al., 2006a; Leary et al., 2006b), and experimentally elevated increases in glucocorticoids are linked to decreased expression of melanin-based ornaments in barn owls (*Tyto alba*), although natural variation in glucocorticoids is not linked to ornament expression (Roulin et al., 2008). Notably, there are as yet no studies that integrate the role of glucocorticoids into a study of testosterone and secondary sexual ornaments in mammals.

We provide the first test of this hypothesis in mammals (Hypothesis 5), predicting that if red coloration in male mandrills acts as an honest signal of glucocorticoid levels (Buchanan, 2000; Evans et al., 2000; Poiani et al., 2000), i.e. the ability to and still be able to maintain fully developed ornaments, then we predict negative relationships between glucocorticoids and each of parasite levels (as an indicator of health) (Prediction 5A), red colour (Prediction 5B), and testosterone levels (Prediction 5C).

<Insert Table 1 about here>

## **METHODS**

## Study population and subjects

The mandrill colony at the Centre International de Recherches Médicales, Franceville (CIRMF), Gabon was established in 1983/4, when 15 animals (7 males, 8 females) were released into a 6.5 ha forest enclosure (E1). Between 1984 and 2004 no subsequent additions were made to the colony, other than by breeding, although animals have been removed occasionally. A second semi-free-ranging group was established in 1994 in a smaller enclosure (E2, 3.5 ha) by transferring 17 mandrills (including 6 adult females and 4 adult males) from the first enclosure. Two adult males (12A4 aged 13.5 yr and 2F aged 15.5 yr) were added to this group in May 2004. These two males were born in E1, removed at age 3 yr (12A4) and 14 yr (2F) and housed in outdoor social cages at the Primate Centre before being introduced into E2. The mandrills foraged freely and received daily supplements of monkey chow, fruit and vegetables. Water was always available from a stream, which runs through all three enclosures. Provisioning should ameliorate any confounding effects of seasonal ecological stress and food availability on cortisol levels (Muller and Wrangham, 2003).

The size and age-sex composition of the study groups during the study period is summarized in **Table 2** and corresponded to the smaller end of group sizes observed in the wild (Rogers et al., 1996). Subjects included all adult males (aged 9+ yr, Setchell et al., 2006a) living in E1 (n=8), and E2 (n=7) between February 2004 and March 2005. One adult male spontaneously transferred from E1 to E2 in December 2004, and thus contributed to both groups, giving a total of 16 males (rather than 17). During the study, 19

infants were born into E1 and 4 were born into E2. Two adult males, two adult females and four juveniles were added to E2 in June 2004. One infant and one adult male died in E1. Two adolescent males died or escaped from E2 (one in June 2004, one in December 2004). Of 18 females of reproductive age in E2, 14 were implanted with a contraceptive implant (melengestrol acetate, MGA, provided by Contraception Advisory Group of the American Zoological Association). These females showed no sexual swellings during the study period. The presence of these implanted females served to increase competition for receptive females when they were present in E2, as few such females were available, but reduced the number of months for which they were available. So... (hypotheses?). This has the potential to influence our test of Hypothesis 3, but

- |   |   |   |
|---|---|---|
| 3 | Receptive females influence glucocorticoid levels | <p><b>3A:</b> higher when receptive females are present than when they are not</p> <p><b>3B:</b> interaction between influence of dominance rank and presence of receptive females, such that dominant males show higher <i>fiC</i> but only when receptive females are present</p> |
|---|---|---|

<Insert Table 2 about here>

### Dominance rank

JMS made observations of male behavior *ad libitum* during twice daily observation periods (approx. 10h00-11h30 and 15h30-17h30). We calculated *dominance rank* using dyadic interaction matrices for each month of the study, based on all interactions where one male avoided or fled when another male approached. Males ranked 1 (alpha) out-ranked all

other males, males ranked 2 out-ranked all but the alpha male, etc. Changes in alpha male were abrupt and unambiguous. Two changes in alpha male occurred in E1, and one in E2, during the study. In E1, the dominance hierarchy was stable from five months before the beginning of the study until 31 July 2004, when the alpha male died of injuries likely sustained when falling out of a tree. Rank instability occurred between August and October 2004, with the second-ranking male taking-over as alpha male for 40 days, before being challenged, injured and deposed by a third male on September 20<sup>th</sup> 2004. This unstable period occurred during the mating season. The hierarchy then remained stable until the end of the study (March 05). At the beginning of the study, the alpha male in E2 had been dominant for 8 years. He was deposed in December 2004 by the male who jumped into E2 from E1, who then remained alpha male until the end of the study period. This take-over occurred outside the mating season. We defined months as 'stable', when no changes in alpha male occurred, and 'unstable' where a change in alpha male occurred, or had occurred in the previous month.

### **Presence of receptive females**

We noted the reproductive state of females daily as one of the following:

- **Cycling:** females in any stage of the menstrual cycle, during which females show conspicuous perineal swellings (Dixson, 1998). Females show conspicuous swellings for  $11 \pm 1$  (mean  $\pm$  SEM, n=45 cycles) days per cycle (Setchell and Wickings, 2004a).

- Pregnant: assigned post hoc from the birth of an infant, beginning with the final detumescence of the perineal skin, mean $\pm$ SEM 175 $\pm$ 1 days (Setchell et al., 2002).
- Lactating: the period following the birth of an infant to the resumption of cycling. Mean $\pm$ SEM post-partum amenorrhoea following birth of a live infant is 242 $\pm$ 92 days (Setchell and Wickings, 2004b).
- ‘Contracepted’: females with a contraceptive implant and not showing cyclical sexual swellings.

We split the days of the study into ‘mating’ and ‘non-mating’, depending on the presence (‘mating’) or absence (‘non-mating’) of females with maximal sexual swellings in the group (termed ‘receptive’ females). Receptive females were present during seven of the 13 months of the study in E1 (June-December 2004), and three months in E2 (June, September and November 2004). Animals housed in E1/E2 were in visual contact with one another, meaning that receptive females in one enclosure might have influenced males in the neighboring enclosure. However, we assumed that visual contact would be less influential than contact with females in a male’s own enclosure, and any interactions between enclosures would serve only to reduce the likelihood of finding a significant relationship between the presence of receptive females and other variables.

### **Male group association**

We scored the group affiliation of each male each day as ‘group associated’: traveling, feeding, and interacting as part of the social group; ‘peripheral’: traveling and feeding on



the edge of the group but often more than 100 m from all other group members; or 'solitary': traveling and feeding alone (Setchell, 2003; Wickings and Dixson, 1992). Peripheral males appeared to track group movements in the enclosures, while solitary males appeared to actively avoid contact with the group. We summarized scores of group association into monthly percentages of days scored in each category for each male.

### **Male coloration**

We used the color of the red skin on the nose as a measure of secondary sexual development (*red coloration*). This measure is closely related to the degree of expression of other secondary sexual traits in male mandrills (Setchell and Dixson, 2001a, 2001c). We quantified facial color monthly for non-anesthetized males using digital images captured using a Nikon Coolpix 5700 digital camera and saved as fine quality jpegs. Images required calibration, to account for exposure and light drift (Gerald et al., 2001). Colony conditions meant that it was impossible either to obtain images of animals in the same frame as a photographic white and black standard, or to place a standard in the same position as the animal and capture a second image immediately following that of the subject. We, therefore, used only images taken when males were in either an open grassy area or in an open feeding pen, and where color ranged the full spectrum from white to black, and used the 'Autolevels' command in Adobe Photoshop 6.0 (Image Mode set to RGB) to define the lightest and darkest pixels in each color channel as white and black. This correction was intended to reduce the influence of variation in ambient lighting conditions on color values as far as possible under colony conditions. Once calibrated, the midnasal strip was outlined

in a standard fashion using the polygonal lasso tool in Adobe Photoshop 6.0. We measured the mean luminosity and the mean red intensity value of the highlighted area using the 'Image > histogram' command. Red intensity divided by luminosity of the image best described the red color of an image (as ranked by independent observers), and was highly and significantly positively correlated with previous measures made using quantified color charts (Setchell and Dixson, 2001c). Although we are aware that this method introduces scatter or 'noise' to the dataset, there is no reason to believe that it introduces systematic bias, and we have shown previously that it is possible to find significant results in predicted directions using these methods (Setchell et al., 2008b; Setchell et al., 2006a, 2006b). We obtained a mean of 1.4 (SEM 0.04, range 1-5) images per male per month and used the mean value where we had more than one image.

### **Fecal sampling**

We collected a total of 317 fecal samples from study subjects between January 2004 and March 2005. We attempted to collect three samples per month for each male, achieving a mean of  $1.6 \pm 1.3$  samples per male per month, and a total of  $20 \pm 3$  samples per male. We collected samples immediately after defecation during either morning (10h00 – 11h30) or afternoon (15h30 – 17h30) observation periods. We noted the identity of the individual, date, time (am or pm) and consistency of the sample, homogenized the feces, and stored one portion (mean  $\pm$  SEM =  $7.12 \pm 1.44$  g) at -20C in 40 ml of 90 % ethanol for hormone analyses, and a second portion (mean  $\pm$  SEM:  $7.6 \pm 0.1$  g) in 20 ml of 10 % formalin solution for parasite analysis.

## **Steroid extraction**

We homogenized fecal samples in their storage ethanol, vortexed them for 1 minute, and shook them for three hours in a rotating shaker. We then centrifuged them for 15 minutes at 2000 rpm, dried a 2 ml aliquot of the supernatant under N<sub>2</sub> at 40 °C, and resuspended this in 1 ml EIA phosphate buffered saline. We dried the fecal pellet to constant mass at 60 °C to determine the dry mass of the sample (mean dry fecal mass was 1.23 ± 1.13 g). We determined extraction efficiency by measuring recovery of <sup>3</sup>H-cortisol (3010 counts per minute, Amersham, Buckinghamshire, UK) added to ten samples prior to extraction, using a 1216 RackBeta liquid scintillation counter. Mean extraction efficiency was 84.4 ± 1.4 %. We corrected hormone concentrations for this extraction loss and expressed them as ng of hormone per mg of dry feces.

## **Assay methods**

We measured immunoreactive cortisol in fecal extracts (*fiC*) by microtitreplate enzyme immunoassay (EIA), using an antiserum (R4866) raised against a steroid bovine albumin in rabbit (Munro and Stabenfeldt, 1985), and horseradish peroxide as a label. This antibody has cross reactivities of 96% with prednisolone, 66% with prednisone, 60% with cortisone, 2.5% with corticosterone, and < 1% with various other steroids (Ziegler et al., 1995). We validated the assay immunologically by demonstrating parallelism, accuracy, and sensitivity. Serial dilutions of pooled fecal extracts gave displacement curves parallel to

that obtained for the cortisol standard for 30-90 % binding, indicating that the amount of cortisol measured varied directly with the volume of extract for this portion of the curve. We determined accuracy by spiking a low and high concentration quality control pool, comprising samples from all adult and several sub-adult males, with standard preparations in the range 500 – 31.25 pg. The recovery of the standard preparations was  $100.9 \pm 6.8$  % ( $r^2 = 0.993$ ,  $n=3$  plates). Assay sensitivity was 1.95 pg/well.

For practical and ethical reasons it was not possible to conduct an ACTH challenge (e.g. Wasser et al., 2000) to establish whether *fiC* accurately reflects acute adrenal activation, and thus to physiologically validate the cortisol antibody for the CIRMF mandrills.

However, we were able to validate the assay biologically by demonstrating that *fiC* levels increased following stressful experiences in both males and females, (see Setchell et al., 2008a for details). These results suggest that the antibody is able to track fluctuations in metabolites that provide biologically relevant information regarding adrenal status, and that elevations in *fiC* may reasonably be regarded as indicative of the physiological stress response in these mandrills.

We diluted fecal extracts 1:6 in assay buffer (0.1 M phosphate buffered saline, pH 7.0, with 0.1% bovine serum albumin) and assayed 50  $\mu$ l aliquots along with 50  $\mu$ l aliquots of reference standard in doubling dilutions (range 1.95-1000 pg/well). We re-assayed samples if duplicates had coefficients of variation greater than 5%, and re-diluted and re-assayed samples binding >90% or <30%. The intra-assay coefficient of variation for a subset of 30 samples was  $3.4 \pm 1.9$  %. We ran high (HQC) and low (LQC) quality controls,

consisting of a pool of all samples, in duplicate on each plate. Inter-assay variation for these controls was 15.1 % (HQC) and 14.4 % (LQC). We also ensured that there was no confound between the assay plate number and any of our predictor variables. We have shown previously that neither storage time nor time of collection (am vs. pm) influence *fiC* levels (Setchell et al., 2008a).

We measured immunoreactive testosterone (*fiT*) using an antiserum and labeled testosterone conjugate (horseradish peroxidase: HRP) provided by Coralie Munro (University of California, Davis). We validated the assay immunologically by demonstrating parallelism, accuracy, and sensitivity, and biologically by comparing *fiT* with free testosterone measured in serum samples, and by comparing immature and prime males (details in Setchell et al., 2008b). Details of assay procedures for quantifying levels of *fiT* are provided in Setchell *et al.* (2008b).

## **Health**

We had insufficient detailed data to examine the relationship between glucocorticoids and body condition or immune parameters. However, we were able to investigate the relationship between *fiC* and gastro-intestinal parasite infection. An independent parasitologist estimated parasite infection in the fecal samples using direct smears and centrifugation/flotation using a Sheather's solution at a specific gravity of 1.18, recording parasitic eggs, larvae, trophs and cysts by species according to characteristic morphology (Setchell et al., 2007). We have described general patterns of parasite prevalence,

abundance and diversity in the mandrill colony elsewhere (Setchell et al., 2007). Briefly, we found three taxa of amoebic protozoa (*Entamoeba coli*, *Endolimax nana*, and *Entamoeba histolytica/dispar* complex), one ciliate protozoa (*Balantidium coli*), and various nematodes in fecal samples collected from study animals during the study period. Because our measures of abundance are limited, and such measures do not necessarily reflect actual infection levels (Hansen and Perry, 1994), we quantified general levels of parasitism as mean species richness (the mean number of taxa found in the feces of an individual male during the study period). Richness is related to sampling effort and season (Setchell et al., 2007), so we used only data for males for which we had three samples for at least six months of the annual cycle (n=13), and ensured that sampling was approximately even across the males and across time.

### **Statistical analyses**

We normalized *fiC* and *fiT* levels via log transformation and used all available data for each male, including male identity as a random factor where appropriate in analyses to account for the fact that we sampled the same individuals repeatedly. Variables describing dominance rank, rank stability, male group association and red coloration describe the month in which a sample was collected. Parasite richness simply described the male. The variable ‘presence of receptive females’ described the day that a sample was collected, allowing for a 24 hour time lag to peak steroid excretion (Bahr et al., 2000). We thus compared the sample with the presence of receptive females on the previous day. Feces represent an integrated source of hormones, and are not a good point source, meaning that

this comparison may be inaccurate. In practice, however, the presence of receptive females on any one day was closely linked to their presence on adjacent days, as the receptive period is longer than one day.

We used a general linear mixed model (GLMM, in SPSS 15) to test predictions relating *fiC* levels to *dominance rank* (Predictions 1A-B), *rank stability* (categorical variable: stable vs. unstable, Prediction 2A) and the *presence of receptive females* (categorical variable: mating vs. non-mating, Prediction 3A). We included the *enclosure* that a male lived in (categorical variable: E1, E2, E3) as an explanatory variable to detect any differences in *fiC* that were due to group membership. We also included *male identity* as a random factor, and tested for main effects and the two-way interaction terms *dominance rank \*rank stability* (Prediction 2B) and *dominance rank \*presence of receptive females* (Prediction 3B). We explored the relationship between *fiC* and *dominance rank* further using non-parametric Spearman correlations based on mean values for each male, and examined significant interaction terms by splitting the dataset into receptive females present/not present and stable/unstable periods.

We used separate GLMM analyses to test Prediction 4, investigating the influence of *group association* (% days group associated per month) and *solitary* (% days spent solitary) on *fiC* because *group association* and *solitary* were correlated with one another (although not equivalent, due to measurement of 'peripheral', see above), and with dominance rank).

To test the predictions of the stress-mediated hypothesis for the evolution of condition-dependent traits, we first examined the relationship between mean *fiC* for each male and his mean *parasite species richness* across the year (Prediction 5A). Next, we used a GLMM to test the relationship between *fiC* and *red coloration* (Prediction 5B), with *red coloration* as the dependent variable, *fiC* as a covariate, and *individual identity* as a random factor. Then we used a similar GLMM with *fiT* as the dependent variable to examine the relationship between *fiC* and *fiT* (Prediction 5C). We also explored relationships between *fiC* and *red coloration* and *fiT* further using correlation analyses based on mean values for each male.

Comparison of *fiC* and *fiT* for the same faecal sample may lead to the problem of correlated errors, where an over- or under-estimate of fecal mass will create a bias in the same direction for both hormone levels, falsely supporting the hypothesis of correlation. To avoid this, we also compared the *fiC* value for a given sample with the *fiT* level for the next sample collected for that male.

## RESULTS

### Social influences on glucocorticoid levels

Using all available data, and including *male ID* as a random factor, we found that *presence of receptive females*, *rank stability*, and the interaction terms *dominance rank \* presence of receptive females* and *dominance rank \* rank stability* were all significant predictors of *fiC* levels, but that *enclosure* and *dominance rank* were not (Table 3). Exploring mean values



for individual males across the study period, we found that mean *fiC* was also unrelated to mean *dominance rank* ( $n = 14$ ,  $r_s = 0.009$ ,  $p = 0.976$ ), but increased in the presence of receptive females (**Fig 1A**) and in unstable situations (**Fig 1B**). These results support Predictions 2A and 3A, but not 1A or 1B (Table 1).

When we split the dataset by *rank stability*, the relationship between *dominance rank* and *fiC* was significant and positive when ranks were stable (GLMM, *fiC* as the dependent variable, *male ID* as a random factor, *dominance rank* as a covariate:  $F_{1,102} = 6.41$ ,  $p = 0.013$ , estimate  $\pm$  SEM =  $0.031 \pm 0.12$ ), indicating that *fiC* was higher in subordinate males under stable conditions. However, the relationship was significant but negative when ranks were unstable ( $F_{1,96} = 10.75$ ,  $p = 0.001$ , estimate  $\pm$  SEM =  $-0.039 \pm 0.012$ ), indicating that under conditions of instability, higher ranking males had higher *fiC*, supporting Prediction 2B.

The significant interaction between *dominance rank* and *presence of receptive females*, suggests that the influence of *dominance rank* on *fiC* differed when receptive females were present from when they were not. When we split the dataset by *presence of receptive females*, the relationship between *dominance rank* and *fiC* was significant and negative when receptive females were present (GLMM, *fiC* as the dependent variable, *male ID* as a random factor, *dominance rank* as a covariate:  $F_{1,46} = 8.63$ ,  $p = 0.005$ , estimate  $\pm$  SEM =  $-0.056 \pm 0.019$ ), meaning that *fiC* was higher in more dominant males. However, this relationship was non-significant when receptive females were not present ( $F_{1,151} = 0.94$ ,  $p = 0.333$ ). These results support Prediction 3B.

*fiC* was not significantly associated with either *group association* ( $F_{1,94} = 0.72, p = 0.398$ ) nor time spent *solitary* ( $F_{1,94} = 0.05, p = 0.816$ ), giving no support to Prediction 4.

< Insert Table 3 about here >

< Insert Fig 1 about here >

### **Stress hormones and secondary sexual traits**

We found a significant, positive relationship between mean *fiC* over the study and mean *parasite species richness* ( $n=13, r = 0.576, p = 0.039$ ), indicating that males with higher *fiC* levels had higher levels of parasite infestation, supporting Prediction 5A. However, we found no support for Prediction 5B: *fiC* was not a significant predictor of *red coloration* (GLMM using all available data, *red coloration* as the dependent variable, *fiC* as a covariate, *male ID* as a random factor:  $F_{1,190} = 0.73, p = 0.393$ ). Comparing mean values for individual males, we also found no significant relationship between *fiC* and *red coloration* ( $n=16, r=0.425, p=0.100$ ).

*fiC* was a significant predictor of *fiT* (GLMM using all available data, *fiT* as the dependent variable, *male ID* as a random factor, *fiC* as a covariate:  $F_{1,205} = 43.65, p < 0.001$ ). However, this relationship was in the opposite direction to that predicted (Prediction 5C). As *fiT* increased, *fiC* also increased (estimate  $\pm$  SEM =  $0.593 \pm 0.090$ ). Comparing mean values

for individual males across the study period, we also found a significant positive relationship between *fiC* and *fiT* ( $n=14$ ,  $r=0.691$ ,  $p=0.006$ , Fig 2). Comparing consecutive samples, to avoid the problem of correlated errors, confirmed the significant, positive relationship between *fiC* and *fiT* (GLMM using all available data, *fiT* as the dependent variable, *fiC* as a covariate, *male ID* as a random factor:  $F_{1,193} = 7.76$ ,  $p=0.006$ ). We interpret this association with caution, because feces contain metabolites of the hormones (rather than the hormones themselves), and is it possible that the cortisol assay cross-reacts with metabolites of testosterone (or vice versa), creating a positive relationship where none exists. It was beyond the scope of this study to use High Performance Liquid Chromatography to investigate this question. However, when we compared *fiC* and *fiT* values for individual samples, the correlation was significant and positive, but only medium in strength ( $r = 0.419$ ,  $p < 0.001$ ,  $N = 317$ ), arguing against an explanation due only to cross-reactivity.

<Insert Fig 2 about here>

## DISCUSSION

### Social influences on glucocorticoid levels

We investigated four main hypotheses concerning social influences on glucocorticoid levels in male mandrills, concerning the possible influence of dominance rank, rank stability, the presence of receptive females, and sociality (summarised in Table 1). We found no

relationship between dominance rank and glucocorticoid levels, either overall, or when we investigated mean levels for individual males. This suggests that the relative allostatic load of dominance does not exceed that of subordinate status (the null hypothesis for Hypothesis 1). However, male glucocorticoid levels increased when the dominance hierarchy was unstable, and the relationship between dominance rank and glucocorticoid levels changed direction according to the stability of the dominance hierarchy, supporting Hypothesis 2: glucocorticoid levels were higher in subordinate males under stable conditions (supporting the 'subordinate stress paradigm', Creel, 2001), but under conditions of instability it was higher ranking males that had higher glucocorticoid levels (supporting the 'costs of dominance' hypothesis', Creel et al., 1996). These findings suggest that the relationship between glucocorticoids and dominance rank is dependent on the social environment, and resemble those reported for baboons (Bergman et al., 2005; Sapolsky, 1992a, , 1993; Sapolsky et al., 1997). They are likely to be due to differences in male ability to predict and control the social environment during stable and unstable periods, leading to differences in the relative allostatic load (Goymann and Wingfield, 2004) of social status in the two conditions. We did not measure rates of aggression, but increased glucocorticoids in higher ranking males during unstable periods may also reflect the metabolic demands of increased rates of aggression among these males, who are most likely to be actively competing for positions at the top of the hierarchy.

Glucocorticoids were higher when receptive females were present, supporting Hypothesis 3. This result may have been influenced by the small number of non contracepted females in E2, which may have acted to increase male-male competition

for those available. However, we found no difference in male glucocorticoid levels between the two enclosures, and our results reflect similar findings in a variety of other primate species (Barrett et al., 2002; Glick, 1984; Lynch et al., 2002; Ostner et al., 2008a; Ostner et al., 2008b; Strier et al., 1999), and likely due to the costs of competition for access to receptive females. Further, the effects of dominance rank and the presence of receptive females on glucocorticoid levels interacted, with dominant males experienced higher glucocorticoid levels than subordinate males during mating periods but not during non-mating periods. This fits with observations that only high-ranking males mate-guard females (Setchell et al., 2005). Mate-guarding is physiologically costly: mate-guarding males fend off challenges from other males, and decrease in body mass across the mating season (Setchell and Dixson, 2001b), probably because mate-guarding constrains their foraging activity, as in baboons (Alberts et al., 1996; Bercovitch, 1983). Mate-guarding males also show increased self-directed behaviour, such as scratching (JMS, unpublished observations), supporting the notion that they are stressed, because such behaviour can be used as a proxy for stress in non-human primates (Castles et al., 1999). Studies of chacma baboons also suggest that mate-guarding is stressful: males entering a sexual consortship (analogous to mate-guarding in mandrills) experience a temporary increase in glucocorticoids (Bergman et al., 2005). The strong link between mate-guarding and paternity, and the great reproductive benefits associated with high rank during the mating season (Charpentier et al., 2005; Setchell et al., 2005), are likely to make these physiological costs worthwhile for high-ranking male mandrills.

We found no support for Hypothesis 4, that peripheral and solitary males ameliorate social stress by avoiding conflict, and thus experience lower glucocorticoid levels than group-associated males. Nevertheless, this leaves open the possibility that these males succeed in at least ameliorating the even higher stress of associating with higher-ranking males, by decreasing their contact with the group. Their cortisol levels may reflect a balance of reduced stress from avoiding conflict, but increased stress due to social isolation, which is also known to be linked with increased cortisol levels (Abbott et al., 2003).

### **Stress hormones and secondary sexual traits**

A major aim of this study was to investigate the role of glucocorticoids in ornament expression, and to test the hypothesis that male ornaments reliably indicate male quality in terms of the ability to cope with stressors without experiencing chronically elevated stress levels, by examining the relationships between glucocorticoids, sexually-selected male coloration, testosterone and health (Hypothesis 5). We found mixed support for the predictions of this hypothesis: males with higher glucocorticoids suffered a higher diversity of parasite infection during the study period, supporting our prediction; however we found no significant relationship between glucocorticoids and ornamentation, and a positive relationship between glucocorticoid and testosterone levels, rather than the negative relationship predicted by the stress-mediated immunocompetence handicap hypothesis, under which only males that are able to withstand stress, and thus have low glucocorticoid levels, should be able to afford high levels of testosterone. The finding that males with higher glucocorticoids suffered a higher diversity of parasite infection during the study

period is in line with evidence that glucocorticoids suppress the immune system in other species (Sapolsky, 1992b, , 2000). However, it is equally likely that parasite infection stimulates the stress response, as part of the immune defence (Lochmiller and Deerenberg, 2000). Either way, this relationship underpins the hypothesis that only males that are able to withstand the physiological stress of infection should be able to develop maximal expression of sexually selected traits (Buchanan, 2000; Evans et al., 2000; Poiani et al., 2000). However, the lack of a significant relationship between red coloration and glucocorticoid levels suggests that glucocorticoids do not play a simple role in translating social conditions or physical health into ornament expression. While our findings for mandrills suffer from the usual problems of observational studies that rely on correlations, they do not provide strong support for the stress-mediated hypothesis for the evolution of sexual signals (Buchanan, 2000; Evans et al., 2000; Poiani et al., 2000). Nevertheless, the lack of a relationship between red colour and glucocorticoids may suggest that high-ranking males are able cope with stress levels and sustain high testosterone (and therefore high colour), while low-ranking males are able to maintain only low levels of testosterone under similar stress levels. Only experiments will allow us to investigate the relationship between glucocorticoids and red colour in detail. Finally, as we note in the Results, we are unable to rule out possible cross-reactivity between the glucocorticoid and testosterone endocrine assays. However, the medium strength of the relationship between the two suggests that cross-reactivity is unlikely to be the only explanation. Moreover, results for other species suggest that the relationship between androgen and glucocorticoid levels varies among species and with context, with reports of positive, negative and no association between the two in both male primates (Bercovitch and Clarke, 1995), and

birds (Astheimer et al., 2000; Silverin, 1998). Given that the relationship between dominance rank and endocrine status is complex and situation-dependent (Goymann and Wingfield, 2004; Wingfield et al., 1990), it comes as no surprise that the relationship between the two types of steroid hormone may also be more nuanced.

We see two possible explanations for the lack of solid support for the stress-mediated hypothesis for the evolution of secondary sexual traits in mandrills. First, a recent review of the role of stress hormones in mate choice noted that the relative influence of different aspects of the glucocorticoid stress response (developmental, baseline and peak levels of glucocorticoids) on sexually selected traits is not yet clear (Husak and Moore, 2008). The fecal glucocorticoid levels that we studied represent an integrated measure of glucocorticoid excretion over a period of days (Whitten et al., 1998), and are likely to lie somewhere between baseline and maximum stress-induced levels of glucocorticoids. They do not, however, tell us anything about developmental stress, and it is interesting to note that studies that support the stress-mediated immunocompetence handicap hypothesis in birds concentrate on the role of developmental stress, rather than adult baseline or peak levels and examine relatively stable sexually-selected traits (brain structures involved in song production and plumage) (Buchanan et al., 2004; Roulin et al., 2008; Spencer et al., 2003; Wada et al., 2008). It may be, therefore, that developmental stress is more important in the development of secondary sexual traits in male mandrills than the measures that we were able to obtain and that the influence of stress is more detectable in more permanent traits (such as overall body size) than in red coloration, which is a dynamic signal of current testosterone and rank status (Setchell et al., 2008b). Second, much of the research



linking stress to signals concerns condition-dependent signals. Although red coloration in mandrills is status dependent, being more developed in dominant males than in subordinates, and changing with changes in dominance rank (Setchell and Dixson, 2001a, 2001c; Setchell et al., 2008b), there is less evidence that it is condition-dependent. In our population, red colour is not linked to measures of faecal parasitism or hematological parameters (as a measure of immunocompetence) (Setchell et al., in press). These studies are not conclusive, due to the healthy, provisioned nature of the colony, but if red colour is not linked, or only weakly linked, to condition, then it may not reflect stress.

In conclusion, our findings for male mandrills support previous work on baboons, showing that the relationship between dominance rank and glucocorticoid levels is complex and contingent on rank stability and the extent of direct competition over receptive females. Although glucocorticoids correlate positively with health, we found no direct evidence for a link between physiological stress and the expression of sexually selected traits in this species of mammal.

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**Table 1: Summary of hypotheses, predictions, and support provided by this study of mandrills**

<b>Hypothesis</b>	<b>Details</b>	<b>Prediction for <i>fiC</i></b>	<b>Supported?</b>	
1	Dominance rank influences glucocorticoid levels	Subordinate males have greater allostatic load than dominants (the subordinate stress paradigm)	<b>1A:</b> higher in subordinate males than dominant males	No
		Dominant males have greater allostatic load than than subordinates ('costs of dominance' paradigm)	<b>1B:</b> higher in dominant males than subordinate males	No
2	Rank instability influences glucocorticoid levels	Allostatic load increases in all males when ranks are unstable	<b>2A:</b> higher during unstable periods than during stable periods	Yes
		Rank stability mediates the relationship between dominance rank and <i>fiC</i>	<b>2B:</b> interaction between the influence of dominance rank and rank stability, such that dominant males show higher <i>fiC</i> when the hierarchy is unstable, while subordinates show higher <i>fiC</i> when hierarchy is stable	Yes
3	Receptive females influence glucocorticoid levels	Competition over access to females results in increased greater allostatic load in all males	<b>3A:</b> higher when receptive females are present than when they are not	Yes
		Increase due to receptive females should be stronger in dominant males (who compete) than in subordinates (who don't)	<b>3B:</b> interaction between influence of dominance rank and presence of receptive females, such that dominant males show higher <i>fiC</i> but only when receptive females are present	Yes
4	Male sociality influences glucocorticoid levels	Subordinate males ameliorate social stress by avoiding other males	<b>4:</b> lower in peripheral and solitary males than in social males	No
5	Stress-mediated hypothesis for the evolution of condition-dependent traits	Red coloration acts as an honest signal of the ability to withstand physiological stress	Negative relationships with	
			<b>5A:</b> parasite levels	Yes
			<b>5B:</b> red colour	No
		<b>5C:</b> Testosterone levels	No	

**Table 2: Size and age-sex composition of the study groups during the study period**

<b>Enclosure</b>	<b>Group composition in Feb 04</b>	<b>Number of females available to cycle*</b>	<b>Number of males aged &gt;9 yr</b>
E1	75 animals (45 females, 30 males)	30	8
E2	72 animals (34 females, 25 males)	4 (all other females aged >3 yr had contraceptive implants)	7

\*Females of reproductive age, without a contraceptive implant (melengestrol acetate, MGA, provided by Contraception Advisory Group of the American Zoological Association). Females with implants showed no sexual swellings during the study period.

**Table 3: Results of the GLMM examining the influence of social factors on  $\log_{10}C$ , including individual identity as a random variable**

<b>Source</b>	<b>Denominator df</b>	<b>F</b>	<b>p</b>
Intercept	20.63	1296.97	<0.001
Enclosure	19.11	0.39	0.540
<i>dominance rank</i>	34.03	0.97	0.332
<i>rank stability</i>	154.25	11.44	0.001
<i>dominance rank * rank stability</i>	126.81	16.56	<0.001
<i>presence of receptive females</i>	185.74	6.97	0.009
<i>dominance rank * presence of receptive females</i>	190.91	4.72	0.031

Numerator df = 1

Fig. 1: Mean  $\pm$  SEM log *fiC* during (A) non-mating and mating periods and (B) stable and unstable periods

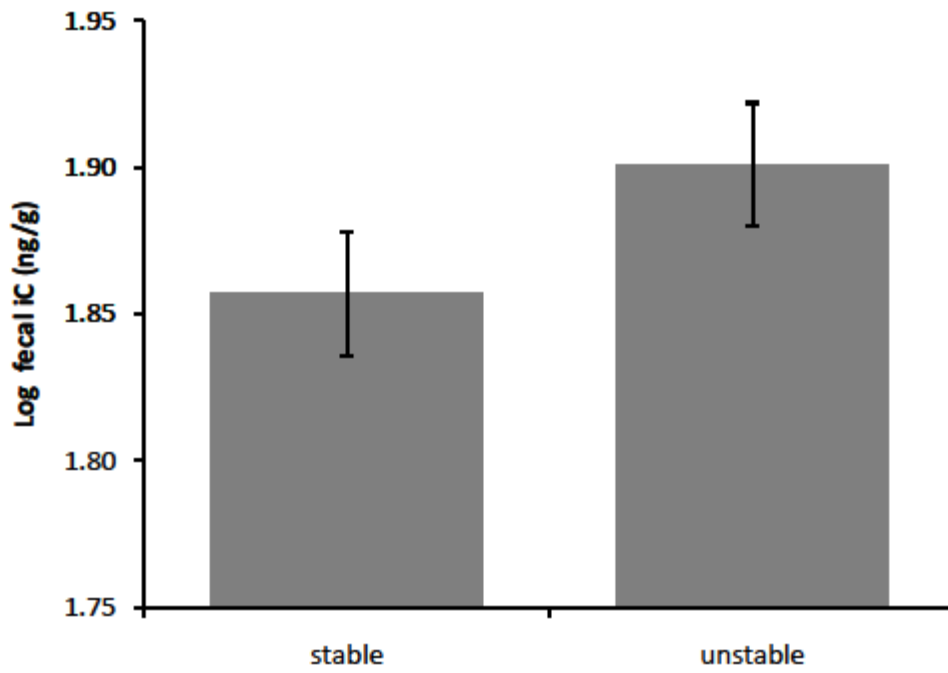
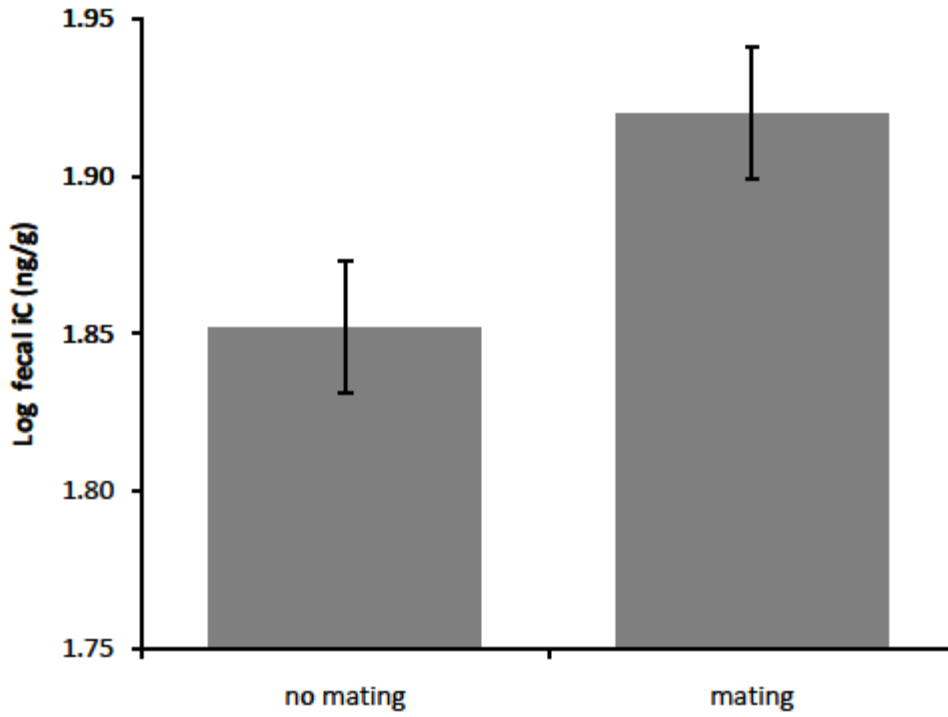


Fig. 2: Mean log *fiT* vs mean log *fiC* for individual males across the study period

