Putative drivers of adrenocortical activity in captive African lesser bushbaby, *Galago moholi*

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Abstract

In seasonal breeders periods of reproductive activity, often coincide with high levels of glucocorticoids. We studied seven male and female African lesser bushbabies, *Galago moholi*, over two mating periods via non-invasive faecal hormone metabolite monitoring to investigate the relationship between reproductive and adrenocortical hormone activity. We used linear mixed-effect models to investigate the effect of physiological (endocrine) variables on faecal glucocorticoid metabolite concentrations. Our results indicate faecal androgen (males) and progestagen metabolite concentrations (females) as the variables best able to explain variability in faecal glucocorticoid metabolite concentrations. However, the models explained only a fraction (26 and 12%, respectively) of the observed variability and graphical analysis suggests a biologically relevant difference in faecal glucocorticoid metabolite concentrations between captive and free-ranging animals during non-reproductive periods. Thus, captivity may have affected glucocorticoid output in our focal animals, potentially weakening the expected relationship between reproductive activity and faecal glucocorticoid metabolite variability. Due to the ease of faecal and observational sample collection, a large number of studies monitoring adrenocortical activity in wildlife are conducted using only captive settings, with inferences unquestioned when applied to free-ranging scenarios. Our study cautions against this practice, as particular housing or management conditions may influence the pattern of adrenocortical activity.

Keywords: *Galago moholi,* African lesser bushbaby, reproduction, stress; glucocorticoids; non-invasive hormone monitoring, captivity

Introduction

Reproductive events are important parts of an animal's annual's life history and of critical importance in determining an individual's fitness and hence the viability of a population (Olive et al. 2000). Reproductive hormones, which are secreted by the hypothalamic-pituitary-gonadal (HPG) axis, are responsible for regulating behavioural, physical and physiological parameters during reproductive events (Johnson 1986; Nieschlag et al. 2012). In seasonal breeders, periods of reproductive activity, though often short in duration, are characterised by group instability (in social species), heightened intra-sexual competition and high energy demands, all of which can activate the stress-response (Creel 2005; DeVries et al. 2003). This is an important mechanism allowing an organism to restore homeostasis through the activation of the hypothalamic-pituitary-adrenal axis (HPA axis) and the subsequent secretion of glucocorticoids (O'connor et al. 2000). Consequently, glucocorticoid concentrations are often used as an index of perceived stress in an organism (Sapolsky et al. 2000). Secreted glucorticoids stimulate cardiovascular activity and energy mobilisation, while triggering important behavioural changes in order to cope with perceived stressors (Reeder and Kramer 2005). However, a functional cross-talk has been found to exist between the HPA and HPG axes, with substantial increases in glucocorticoid concentrations inhibit the secretion of reproductive hormones, directly influencing the reproductive capabilities of an individual (Dobson and Smith 2000).

The regulation of parts of the HPG axis, such as gonadotrophin-releasing hormone (GnRH), by parts of the HPA axis such as corticotrophin-releasing factors (CRF) occurs through both direct and indirect mechanisms. The indirect regulation is thought to occur by modulation of various components of the HPA-axis such as the activation of the sympathetic nervous and limbic systems, as well as glucocorticoid production and excretion (Chand and Lovejoy 2011; Sapolsky 1985). Evidence for such regulation can be seen through the administration of corticotrophic-releasing hormones which results in a sudden decrease of GnRH and luteinizing hormones (Feng et al. 1991). The more direct regulation of the HPG-

axis occurs through the suppression of GnRH-expression neurons, by CRF, at signal transduction and transcription regulation levels (Kinsey-Jones et al. 2006; Tellam et al. 1998). Although the regulatory effect of CRF on the HPG-axis has been observed numerous times, instances exist where an increase in cortisol concentrations in non-human primates does not translate into suppression of reproductive function (Cameron 1997; Vugt et al. 1997).

Similarly, increased production of reproductive hormones can exert both a positive and negative feedback pattern on adrenocortical activity (Stavisky et al. 2003; Viau 2002). One mechanism responsible for the modulation of adrenal activity is the binding of testosterone and oestrogen cognate receptors within the central nervous system, influencing the stress response (Handa et al. 1994). Such actions suggest that reproductive hormones directly regulate HPA activity in order to avoid the numerous deleterious effects of elevated glucocorticoid secretion on reproductive function. As with the regulatory effect of CRF on the HPG-axis, elevated reproductive hormones do not necessarily result in the suppression of adrenocortical activity in mammal species (Ziegler et al. 1995). As the interplay between the HPG and HPA axes can be species specific, a general link should not be assumed for all mammal species.

Aside from the possible HPG-HPA cross-talk affecting adrenocortical activity in mammals, other extrinsic factors such as predator-prey interactions (Monclús et al. 2009) and social interactions (Girard-Buttoz et al. 2014) have been shown to alter adrenocortical activity in mammals. In an attempt to better understand the association between reproductive function and adrenocortical activity, we monitored reproductive hormones and mating activity as well as glucocorticoid concentrations in several captive pairs and a surrounding free-ranging population of African lesser bushbabies (*Galago moholi*). We hypothesized that both reproductive hormones and mating activity will be major drivers for alterations in adrenocortical activity in both male and female individuals.

Methods and Materials

Study site

We conducted the study at Ithumela Primate Sanctuary (IPS, Buffelsdrift Conservancy, South Africa, 25°35'55.79"S, 28°19'30.82"E) between March and November 2013. We collected temperature and rainfall data for the area from the South African Weather Service. The study site has a hot, wet season from October to March, whereas a cold, dry season occurs from April to September. During the study the maximum temperatures varied between 13.8 °C and 35.8 °C (mean ± SD: 25 ± 3.06 °C), whereas minimum temperatures varied between -1 °C and 17.6 °C (mean ± SD: 8.29 ± 4.61 °C). A total of 209 mm of rainfall occurred during the study period, with the majority occurring in March (34.8 mm), April (81.6 mm), and September (78 mm).

Study animals

The African lesser bushbaby is a small nocturnal prosimian distributed throughout sub-Saharan Africa (Bearder 1987). Male and female *G. moholi* individuals have separate, but overlapping, home ranges, with frequent interaction occurring among individuals (Bearder & Martin 1979). *G. moholi* has been described as polygynandrous, with two mating periods per year (May and September; Pullen, Bearder & Dixson 2000; Scheun et al., 2016b). Our study animals comprised seven male and female individuals held in captivity at IPS, as well as 14 males and 12 females from the surrounding wild population of Buffelsdrift Conservancy. All individuals were marked with subcutaneously injected passive identification transponders (ID100 Trovan, EURO I.D., Weilerswist, Germany). The seven adult male and female *G. moholi* were housed in mating pairs in separate cages at IPS. Although this pairing of *G. moholi* individuals is unnatural, compared to the natural social structure of the species (Bearder and Martin 1979), this was done to observe mating instances as well as track reproductive hormone patterns in the species during mating and pregnancies (Scheun et al. 2016b). The average age of the seven captive females was 3.4 ± 1.3 years of age (range: 2-

120 5 years), while males were 2.9 ± 0.7 years of age (range: 2-4) years old. Thus all captive
121 individuals were older than 8.5 months, the minimum reproductive age of *G. moholi* (Nekaris
122 and Bearder 2007). Throughout the study period trained personnel from IPS, as well as local
123 veterinarians, conducted frequent health care assessments of all captive individuals. All
124 individuals were found to be healthy throughout the study period. For the captive setup we
125 designed enclosures (3 × 1.5 × 2.8 m) which allowed for easy separation (< 30 min) of paired
126 animals during periods of sample collection. Each enclosure consisted of three compartments,
127 the middle of which functioned as the sleeping area. Upon their exit each individual would
128 move to one of the side compartments, through a small opening which contained a trap door
129 mechanism, allowing for the successful separation of individuals and eliminating the chances
130 of cross contamination of samples. A small amount of hair was removed from the tail of all
131 captive males. This allowed for individual identification and sample assignment. We fed
132 captive individuals a combination of yogurt, fresh fruit and dry cat food (Whiskas, South Africa)
133 at 18:00 each night (which lasted their entire active phase), with fresh water being available
134 *ad libitum.* Close proximity and contact of captive individuals by the researchers were kept at
135 a minimum throughout the study. For the free-ranging setup we trapped individuals from the
136 surrounding area using walk-in live (40 x 15 x 15 cm) and Sherman traps (7 x 7 x 30.5 cm, H.
137 B. Sherman Traps, Tallahassee, Florida, USA) baited with banana, honey and peanut butter.
138 As a result of wild individuals roaming freely, data could only be collected during time of
139 capture. We collected faecal samples from free-roaming individuals to evaluate whether the
140 hormone data from the captive setup were representative of a free-ranging *G. moholi*
141 population. We performed the study with the approval of the University of Pretoria Animal Use
142 and Care Committee (Reference EC056-12).

143 *Faecal sample and data collection*

144 During the study, we collected fresh faecal samples three times a week from all captive
145 animals. Our cages allowed for a separation of both sexes until samples of each individual
146 were obtained and individuals were reunited. In addition, we set traps tri-weekly and collected

147 all fresh faecal material from trapped free-ranging individuals. As an increase in glucocorticoid
148 concentration is only observed in faecal matter approximately 12 hours following a stressful
149 event (Scheun et al. 2015), we were confident that capture stress would not reflect in the
150 collected samples. For our captive population, we collected a total of 631 faecal samples from
151 the males (range: 87-94 per animal) and 626 faecal samples from the females (range: 84-93
152 per animal) during the study period. For free-ranging animals we collected 39 faecal samples
153 from males (from 14 animals) and 38 faecal samples from females (from 12 animals). As a
154 result of the low number of samples collected from each free-ranging animal (range: 1-5), we
155 were unable to conduct any statistical analysis on the free-ranging sample set.

156 We noted the reproductive status of males and females in captive and free-ranging
157 groups. To do so we conducted nightly observations (*ad libitum* sampling, 20:00 h - 04:00 h,
158 Dr Juan Scheun, Altmann 1974) throughout the study, using red-filtered light, on all animals
159 to assess the incidence of reproductive behaviour (i.e the period of reproductive vs non-
160 reproductive activity). As such we did not set out to quantify the occurrence of behaviours, as
161 this has been done previously for the species both in captivity as well as the natural
162 environment (Bearder and Martin 1979; Lipschitz et al. 2001; Pullen et al. 2000), but simply to
163 determine whether individuals were sexually activate in either population (as seen by mating
164 activity). As female vaginal opening only occurs during periods of mating, this was used to
165 determine mating periods in captive and free-ranging individuals. We categorised reproductive
166 status in females as an animal being ‘pregnant’, ‘non-reproductive’ or ‘lactating’. We assessed
167 pregnancy status in females by increased mass of an animal between weighing events, the
168 presence of a foetus through the careful palpation of the lower stomach or backdating from
169 the parturition. To confirm lactation we applied pressure to the mammary glands of female
170 post-partum to attain whether milk production was present. We defined males as
171 reproductively active when increased male-female follows, excessive male-female grooming,
172 regular vaginal sniffing and licking, attempted mounts and intromission were observed
173 (Lipschitz et al. 2001). An increase in androgen concentrations and testis volume was further

used as evidence of reproductive activity in males, as observed in other seasonal breeders (Goeritz et al. 2003; McLachlan et al. 1996).

As a result of the low recapture rate, we were unable to determine the reproductive status of free-ranging males (18 individuals), but managed to reliably determine reproductive status for the captive males (7 individuals). For free-ranging females, pregnancy could be reliably determined 50 days post conception (6 individuals), while pregnancy status for captive females could be determined accurately from the date of conception until parturition.

Hormone extraction and analysis

We froze fresh faecal material directly after collection and stored all samples at -20 °C until hormone extraction. We lyophilised, pulverised, and sieved faecal samples through a thin mesh to remove fibrous material (Fieß et al. 1999). We then extracted 0.050-0.055 g of faecal powder by vortexing for 15 min with 1.5 ml of 80 % ethanol. Subsequently, we centrifuged steroid extracts for 10 min at 1500 *g*, after which, supernatants were transferred into new microcentrifuge tubes and stored at -20 °C until hormone analysis.

Faecal glucocorticoid metabolite (fGCM) concentrations as well as reproductive steroid concentrations (for males: faecal androgen metabolites [fAM], for females: faecal oestrogen metabolites [fEM] and faecal progestagen metabolites [fPM]) were determined via enzyme-immunoassay (EIA) techniques. Details for the respective EIAs, including cross-reactivities, are given in Palme and Mostl (1997) for measuring fGCMs, in Palme and Möstl (1993) for fAMs and fEMs and in Schwarzenberger et al. (1996) for fPMs. Sensitivities of the respective assays were 3 ng/g dry weight (DW) for fGCMs and fEMs, 7.5 ng/g DW for fAMs and 1.5 ng/g DW for fPM. Serial dilutions of extracted *G. moholi* faecal samples gave displacement curves that were parallel to the respective standard curve. Intra- and inter-assay coefficients of variation, determined by repeated measurements of high- and low- value quality controls, ranged between 6.9 % and 13.1 %. Reliability of the EIA for monitoring adrenocortical activity has been shown in (Scheun et al. 2015). EIA parameters, as well as biological validations, for

the fAM, fEM and fPM are given in Scheun et al. (2016a) and (Scheun et al. 2016b). We conducted all assays at the Endocrine Research Laboratory at the Faculty of Veterinary Science, University of Pretoria.

Data analysis

A priori model-building and selection

We explored *a priori* population-level covariates of captive male and female bushbaby fGCMs using fGCM concentrations in 638 faeces from seven males and in 630 faeces from seven females. We modelled natural-log-transformed fGCM concentrations as the response variable (*yi*'s, Eqn 1) in linear mixed models, fitted with the 'identity' link function (Eqn 2), using *lmer* in Package 'lme4' (Bates et al. 2012) in R, v 3.2.0 (R Team, 2014). We used all global model subsets (all were plausible) and omitted interaction terms, allowing for balanced-design model averaging. The global model for the male bushbabies included a total of four fixed effects (*βj*'s, Eqn 3): fAM; reproductive status (reproductive, non-reproductive); female pregnant (yes, no); female lactating (yes, no). The global model for the female bushbabies included a total of five fixed effects: fEM; fPM; reproductive status (reproductively active, reproductively non-active); pregnant (yes, no); lactating (yes, no). We modelled repeated measures on each animal as random effects (u_{ik}'s, Eqn 3,4): animal (1|animal).

$$y_i \sim N\,(\mu_i, \sigma^2) \quad (1)$$

$$g(\mu_i) = n_i \quad (2)$$

$$n_i = \sum_{j=1}^{s} \beta_j x_{ij} + \sum_{k=1}^{r} Z_{ik}\mu_{ik} \quad (3)$$

$$u_{ik} \sim N\,(0, \sigma_k^2) \quad (4)$$

We standardized variables using Package '*arm'*: numeric variables to $\bar{x}$ = 0; σ = 0:5 and binary variables to $\bar{x}$ = 0 with a difference of 1 between categories (Gelman 2008). We used variance inflation factors (VIFs, Anderson et al. 2001) to assess multicolinearity, using an *a priori* cut-off of VIF = 5 for rejecting collinear variables. We evaluated candidate models with Akaike's Information Criterion (Akaike 1974) with small sample size correction (AICs, Anderson 2008). We performed multi-model inference and model averaging (Burnham and Anderson 2002) using Akaike weights (w_i) of all candidate models. We assessed goodness of fit of parameter estimates using 85% confidence intervals (Anderson 2008; Arnold 2010) and assessed variation explained by the global model using Ω_0^2 (Xu 2003).

Post hoc graphical comparisons

After the *a priori* linear mixed model analyses, we performed *post hoc* graphical analyses of the faecal metabolite (glucocorticoid, androgen, progestagen, and oestrogen) data (formal analysis was inappropriate for *post hoc* comparisons). We plotted longitudinal faecal hormone metabolite data for the four captive study pairs that conceived (range: 87- 93 samples for each individual). For the fGCM data, we provided the baseline concentration for free-ranging animals for comparison, which we derived using the median of baselines of free-ranging animals (18 males of unknown reproductive status and 11 non-reproductive females; thick dashed line, Fig. 2a and 2f). FGCM concentrations of free-ranging individuals were used as baseline fGCM concentrations as this represents the stress levels present in the natural environment. We also included the peak fGCM response to an adrenocorticotropic hormone challenge (the median of peak responses for three captive male and females; thin dashed line, Fig. 2a and 2f; Scheun et al. 2015). We compared the median faecal hormone metabolite concentrations for free-ranging and captive bushbabies in non-reproductive and reproductive periods.

When we compared fPM and fEM concentrations between captive and free-ranging animals, we used only data from the same reproductive period (50 days post conception to

parturition). Number of faecal samples per free-ranging animal was low (range: 1-6, median 2 for males; range: 1 to 5, median 2 for non-reproductive females; range: 1-4, median 2.5 for reproductive females) compared to captive animals (range: 67-76, median 73 for non-reproductive males; range: 16-22, median 19 for reproductive males; range: 29-78, median 35 for non-reproductive females; range: 36-40, median 36.5 for reproductive females; 8 to 10, median 8 for late reproductive females).

Results

A priori models of faecal glucocorticoid metabolite variability

For the female bushbabies in our study, the covariate that best explained variation in fGCMs was fPM concentration (Table 1, Fig. 1). We selected this variable in all of our best candidate models (ΔAICc < 2, Table 1). Female pregnancy and lactation status also explained some of the variation in fGCMs, but both of these variables had high variability in parameter estimates, had a small effect size, and were not selected in all the top models (Fig. 1). Our global model, which included all four variables, explained 12% of variation in fGCMs, with $\Omega_0^2 = 0.12$. Variance inflation factors for all covariates were below 2.1, suggesting that multicolinearity was not problematic in our models.

For male bushbabies, the covariate that best explained variation in fGCMs was fAM concentration (Table 2, Fig. 1). We selected this variable in all of our best candidate models (ΔAICc < 2, Table 2). Reproductive status of the male and the lactation and reproductive status of that animal's female all had high variability in parameter estimates or had a small effect size (Fig. 1). Our global model (containing all variables) explained 26% of variation in fGCMs, with $\Omega_0^2 = 0.26$. Variance inflation factors for all covariates were below 2, suggesting that multicolinearity was not problematic in our models.

Post hoc longitudinal profiles of faecal glucocorticoid metabolites

Longitudinally, fGCM concentrations displayed high variability during non-reproductive and reproductive periods for both males and females (Fig. 2 a, f). In females, both the fEM and fPM concentrations increased approximately 60 days after conception, declining to their pre-conception baseline values approximately 135 days after conception (Fig. 2 b, c). The highest fGCM concentrations appeared to coincide with the final 21 days of gestation and the peak fPM concentrations (Fig. 2 a, c, d). In males, fAM concentrations increased around the period of first conception, and again around the period of parturition and second conception (Fig. 2 g). At the study population level, the putative association (suggested by our models) in captive animals between fGCM concentration and fPM concentration (for females) or fAM concentration (for males) appears weak (Fig. 2 a, c, f, g). In both captive females and males, the fluctuations in fGCM concentrations appear to be higher than the median non-reproductive baseline values for free-ranging animals (dashed lines, Fig. 2 a, f), and in males appear to approach the median peak fGCM response for captive animals challenged with adrenocorticotropic hormone (dotted lines, Fig. 2 a, f).

Post hoc graphical comparison by population and reproductive status

Captive bushbabies of both sexes appeared to have higher fGCM concentrations than those of free-ranging animals (Fig. 3 a). Within a captive animal, fGCM concentrations appear to increase from the non-reproductive to the reproductive period for males and from the non-reproductive to the late reproductive period for females (Fig. 3 a). While free-ranging males and females had similar concentrations of fGCM, captive females in the late reproductive period may have higher fGCM concentrations than captive males (Fig. 3 a).

For male bushbabies, captivity status seemed to have little effect on fAM concentrations during the non-reproductive period (Fig. 3 b). Within captive males, fAM concentrations increased from the non-reproductive to the reproductive period (Fig. 3 b). Captive female bushbabies had higher fPM and fEM levels than free-ranging individuals during

298 reproductive and non-reproductive periods (Fig. 3a), although it is more likely that the low
299 sample size within a free-ranging animal resulted in missing the peak hormone concentrations.

300 In the captive setup two periods of mating activity were observed during the study,
301 namely at the end of May and mid-September. During the May mating event all seven male
302 and females were involved in mating activity, with four females conceiving. However, as a
303 result of ongoing pregnancies during the September mating event, only three of the seven
304 females were observed mating with their paired males (see Scheun et al. 2016b for more
305 information on mating activity). Mating activity in the free-ranging population was observed
306 during the same period, though for only a brief period of time compared to the captive setup
307 (2 days in total).

308 **Discussion**

309 This study is the first to describe a putative link between reproduction and adrenocortical
310 activity of a nocturnal prosimian by monitoring reproductive and stress hormone metabolite
311 concentrations in faeces. Although results from our models indicate that fAM and fPM
312 concentrations, for male and females respectively, best explain adrenocortical activity,
313 additional unknown factors seem to be driving fGCM patterns in the captive population. The
314 association between reproductive factors and adrenocortical activity is consistent with
315 previous studies on a range of primates, including muriquis (*Brachyteles arachnoids*
316 *hypoxanthus*, Strier et al. 1999), long-tailed macaques (*Macaca fascicularis*, Stavisky et al.
317 2003) and common marmosets (*Callithrix jacchus*, Saltzman et al. 1994). Our study highlights
318 the influence of certain reproductive parameters such as mating activity and pregnancy status,
319 specifically as modelled by gonadal hormone production, on mammalian adrenocortical
320 activity. An important outcome from this study is the relatively weak relationship between male
321 mating status and adrenocortical activity. Although a putative correlation has been found
322 between reproductive status (mating: yes/no) and androgen concentrations in seasonal
323 breeders (Wingfield et al. 1990), our observation only highlights the importance of fAM

concentrations, but not reproductive status in explaining fGCM variability in bushbabies. This suggests that the use of simple yes/no dichotomies may not always be sufficient for modelling complex physiological associations. Similarly, although female pregnancy status was marginally important in our model, the pregnancy progression, which was modelled via fPM concentrations, performed considerably better in explaining fGCM variability.

These results suggest that studies investigating drivers of fGCMs should include robust *a priori* considerations of causality, defining the potential relationship between all hormone metabolite concentrations and the physiological processes that produce the respective hormones, during the model-building phase. The low level of variability explained by our models indicated that additional factors are likely responsible for a considerable proportion of the fGCM variability observed during our study. We thus incorporated *post hoc* analyses to suggest potential factors driving bushbaby adrenocortical activity for future investigation.

The *post hoc* results suggested higher baseline (non-reproductive) adrenocortical activity in captive versus free-ranging males, and in captive versus free-ranging females. While the baseline fGCM concentrations for free-ranging males and females were well below median fGCM concentrations of captive individuals prior to the adrenocorticotropic hormone challenges conducted on the species (Scheun et al. 2015), baseline fGCM concentrations for captive females approached the concentrations elicited during that challenge, and captive males exceeded the adrenocorticotropic hormone challenge concentrations in multiple samples. As an adrenocorticotropic hormone challenge can elicit a near-maximum physiological stress response (Palme 2005) depending on the dose administered, our results suggest that factors associated with our applied captive setup represent biologically significant physiological challenges for *G. moholi*. These inferences were drawn from *post hoc* analysis and should be interpreted accordingly. The results do, however, suggest avenues for future research focused on possible drivers of adrenal activity in the captive setup. Further, the apparent correlation found in the *post-hoc* graphical analysis between fGCM and fPM,

particularly 21 days prior to parturition, may be driven by the pregnancy-related physiological adaptations. As foetal development progresses throughout pregnancy, a steady increase in progestagen and glucocorticoid concentrations is required to support this, reaching maximum levels shortly before parturition (Fieß et al. 1999; Lindsay and Nieman 2005). Additionally, the increase in androgen concentration in male individuals prior to and during periods of conception is required to activate both reproductive activity and sperm production (Nieschlag et al. 2012; Scheun et al. 2016a).

Although a general season-related pattern of glucocorticoid concentrations has been described for a number of species in the wild (Romero 2002), this pattern can be substantially altered in captivity by various extrinsic factors. Such circumstances, including restriction of movement, absence of predation and refugia, forced proximity to humans, and unnatural grouping of often gregarious and non-gregarious species, can result in a prolonged elevation of glucocorticoid concentrations (Morgan and Tromborg 2007). Thus, some captive individuals exhibit chronically elevated glucocorticoid concentrations (a new and higher basal concentration level, Dickens et al. 2009). Animals in our captive population were housed as mating pairs, while being confined to a small area. Both of these factors are atypical for free-ranging *G. moholi*, in terms of social behaviour and movement dynamics (Bearder 1987). Such chronic adrenocortical activity, in response to a suboptimal captive setup, has been found in primate species such as the gray mouse lemur (*Microcebus murinus*, Perret and Predine 1984) and spider monkeys (*Ateles geoffroyi rufiventris*, Davis et al. 2005), but also in captive-held, non-primate mammal species such as the giant panda (*Ailuropoda melanoleuca*, Liu et al. 2006) and the tigrina (*Leopardus tigrinus*, Moreira et al. 2007). The high variability of fGCM concentrations as a result of the captive setup may mask putative patterns in adrenocortical activity during key life stages in bushbabies and potentially in other species. This masking effect may have resulted in the relatively weak association of predictor variables assessed in our linear mixed models. Although the fGCM patterns were highly variable and presumably chronically elevated as a result of the captive setup of our study, no disruption of reproductive

function was found in five of the seven captive females. As the stress response and the effect of chronically elevated stress is individual specific (Romero 2002), the suppression of reproductive function could have occurred for two of the seven females, both showing irregularity in terms ovarian hormone cycles (Scheun et al. 2016b).

Many studies describing adrenocortical activity are conducted on captive populations only, due to ease of sample collection and animal observation. Our results suggest that long-term captivity can cause extensive and unpredictable changes in adrenocortical activity, disrupting the natural hormone cascade that might be observed in free-ranging animals (Künzl and Sachser 1999; Romero and Wingfield 1999). Because of this disruption, the inference from captive studies should be applied to free-ranging populations with extreme caution.

Finally, the differences observed graphically in reproductive hormone metabolite concentrations between the captive and free-ranging females in our study may be an artefact of the reduced sampling in free-ranging compared to captive females. In captive females, the fPM and fEM concentrations followed non-uniform longitudinal profiles with peaks toward the end of pregnancy, which may have been missed in free-ranging females. Studies attempting to describe the putative role of reproduction in adrenocortical activity, or attempting to compare hormone concentrations between populations, should ensure that identical sampling protocols (representative of the entire life history stage in question) are followed for all study populations.

Although our applied models explained relatively little of the variability in fGCM, we were successful in positing factors potentially responsible for adrenocortical activity in captive African lesser bushbabies. FGCM variability in males is best described by fAM concentrations, which may be a proxy for male mating activity, while female fGCM variability is explained by fPM concentrations, which are potentially a proxy for the progression of pregnancy. As a result of the possible influence of captivity on adrenocortical activity, future studies should apply caution when using captive studies to infer patterns of adrenocortical and gonadal activity in free-ranging animals. Follow up studies are needed, specifically designed to assess

reproductive and adrenocortical activity in free-ranging bushbabies, and designed to assess the putative effect of captivity on adrenocortical and gonadal hormone production in captive versus free-ranging bushbabies. As more than 26 billion animals, from over 10, 000 species, are kept in captive setups such as zoos, farms and conservation centres (Mason and Veasey 2010) it is important to clarify what effects captivity itself may have on the adrenocortical activity of a species. Studies on captive and domesticated species have shown that the perception of confinement-specific stressors is species-specific, making a generalised assessment difficult (Romero 2002). It is thus important to not only clarify the role of reproductive season on adrenocortical activity, but also the possible effect of captivity on the stress concentrations of caged individuals. The potential effect of captivity, the need for representative faecal sampling throughout the life history stage under study and the potential use of reproductive hormone concentrations as proxies for reproductive activity (specifically the progression of pregnancy), are factors that could be applied to non-invasive studies of the stress physiology of any species.

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Tables

Table 1. The results from the mixed effects candidate models, modelling natural-log-transformed fGCM levels in females of the African lesser bushbaby (*G. moholi*; 630 samples from seven females').

	Model: log(FGCM)~	* log*L*	†*K*	‡AICc	§Δ	‖*w*$_i$
1	FPM + lact + preg + (1|animal)	-542.5	6	1097.0	0.0	0.19
2	FPM +(1|animal)	-545.1	4	1098.2	1.2	0.10
3	FPM + lact + (1 |animal)	-544.1	5	1098.3	1.3	0.10
4	FPM + fEM + lact + preg + (1|animal)	-542.2	7	1098.5	1.5	0.09
5	FPM + preg + (1| animal)	-544.3	5	1098.7	1.6	0.08
6	FPM + fEM + lact + (1|animal)	-543.4	6	1098.9	1.8	0.07
7	FPM + lact + preg + repr + (1|animal)	-542.4	7	1099.1	2.0	0.07
8	FPM + fEM + (1|animal)	-544.6	5	1099.2	2.2	0.06
9	FPM + repr + (1|animal)	-545.1	5	1100.3	3.2	0.04
10	FPM + fEM + preg + (1|animal)	-544.1	6	1100.3	3.3	0.04
11	FPM + lact + repr + (1|animal)	-544.1	6	1100.4	3.3	0.04
12	FPM + fEM + lact + preg + repr + (1|animal)	-542.2	8	1100.6	3.5	0.03
13	FPM + preg + repr + (1|animal)	-544.3	6	1100.7	3.7	0.03
14	FPM + fEM + lact + repr + (1|animal)	-543.4	7	1100.9	3.9	0.03
15	FPM + fEM + repr + (1|animal)	-544.6	6	1101.3	4.2	0.02
16	FPM + fEM + preg + repr + (1|animal)	-544.1	7	1102.3	5.3	0.01

* Log likelihood (log*L*), † number of parameters (*K*), ‡ Akaike's Information Criterion with small sample correction (AICc), § AICc distance from the best model (Δ) and || Akaike weight (w_i)

Table 2.

The results from the mixed effects candidate model, modelling natural-log-transformed fGCM levels, in males of the African lesser bushbaby (*G. moholi*; 638 samples from seven males).

	Model: log(FGCM)~	* log*L*	†*K*	‡AICc	§Δ	‖*w*$_i$

1	FAM + (1|animal)	-456.1	4	920.4	0.0	0.26
2	FAM + repr + (1|animal)	-455.2	5	920.6	0.2	0.23
3	FAM + lact + (1 |animal)	-456.0	5	922.0	1.6	0.11
4	FAM + preg + (1|animal)	-456.0	5	922.1	1.8	0.11
5	FAM + preg + repr + (1|animal)	-455.1	6	922.3	1.9	0.10
6	FAM + lact + repr + (1|animal)	-455.1	6	922.4	2.1	0.09
7	FAM + lact + preg + (1|animal)	-455.7	6	923.5	3.2	0.05
8	FAM + lact + preg + repr + (1|animal)	-454.9	7	924.0	3.6	0.04

* Log likelihood (log*L*), † number of parameters (*K*), ‡ Akaike's Information Criterion with small sample correction (AICc), § AICc distance from the best model (Δ) and || Akaike weight (w_i)

Figure legends

Figure 1. Standardized parameter estimates (Gelman, 2008) with 85% confidence intervals (Arnold, 2010), after model averaging of all candidate models, for models of a) female and b) male African lesser bushbabies *(G.* moholi) faecal glucocorticoid metabolites. Parameters were faecal progestagen metabolite (fPM) concentration, faecal estrogen metabolite (fEM) concentration, faecal androgen metabolite concentration (fAM), reproductive status (reproductive), lactation status of the animal or the animal's paired female (female lactating), and pregnancy status of the animal or the animal's paired female (female pregnant). After parameter labels, we report relative importance-sum of Akaike weights (∑wi) over all models that include the parameter.

Figure 2. Longitudinal profiles of a) faecal glucocorticoid metabolite (fGCM) concentrations, b) faecal estrogen metabolite (fEM) concentrations, c) faecal progestagen metabolite (fPM) concentrations for four captive female African lesser bushbabies (*G. moholi*) and f) fGCM concentrations, g) faecal androgen metabolite (fAM) concentrations for four captive male African lesser bushbabies (*G. moholi*). Individual animals are indicated with grey lines and study population medians with black lines. Dotted lines indicate the median peak fGCM response for three animals from each sex challenged with adrenocorticotropic hormone. Dashed lines indicate the median or baseline fGCM concentration for wild bushbabies for each sex. Individual female longitudinal fPM, fEM and fGCM profile is shown (d, e).

Figure 3. Post hoc comparison of faecal hormone metabolite concentrations between male and female bushbabies, between free-ranging (wild [W]) and captive [C] African lesser bushbabies (*G. moholi*), and among bushbabies of varying reproductive status (unknown [unk], non-reproductive [NR], reproductive [R] and late reproductive [LR]) for a) glucocorticoids and b) reproductive hormones (androgens, progestagens, and estrogens). Dots represent median values for a given animal. Horizontal black lines indicate group medians. Grey lines link paired values within an animal.

Figure 1

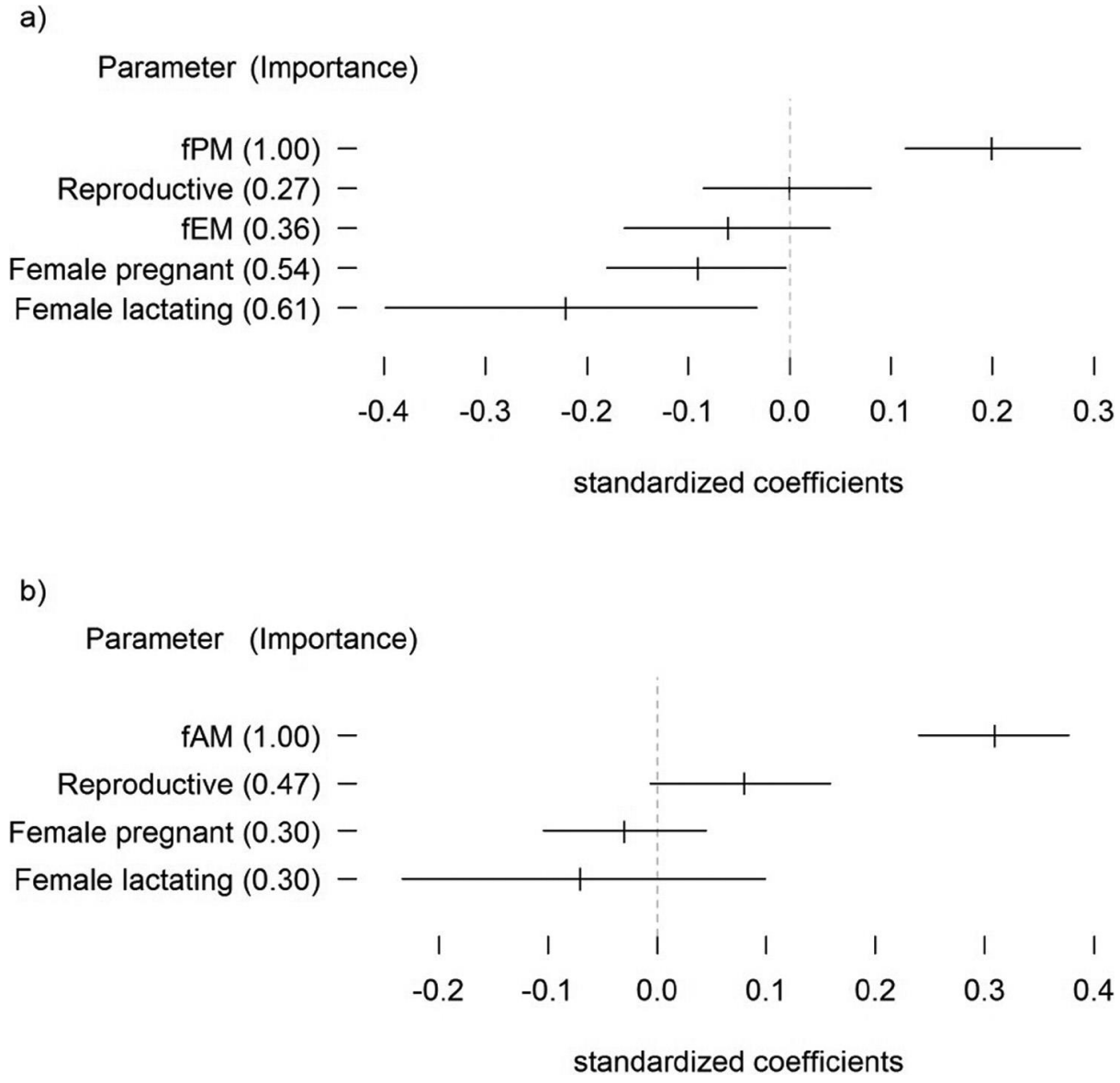
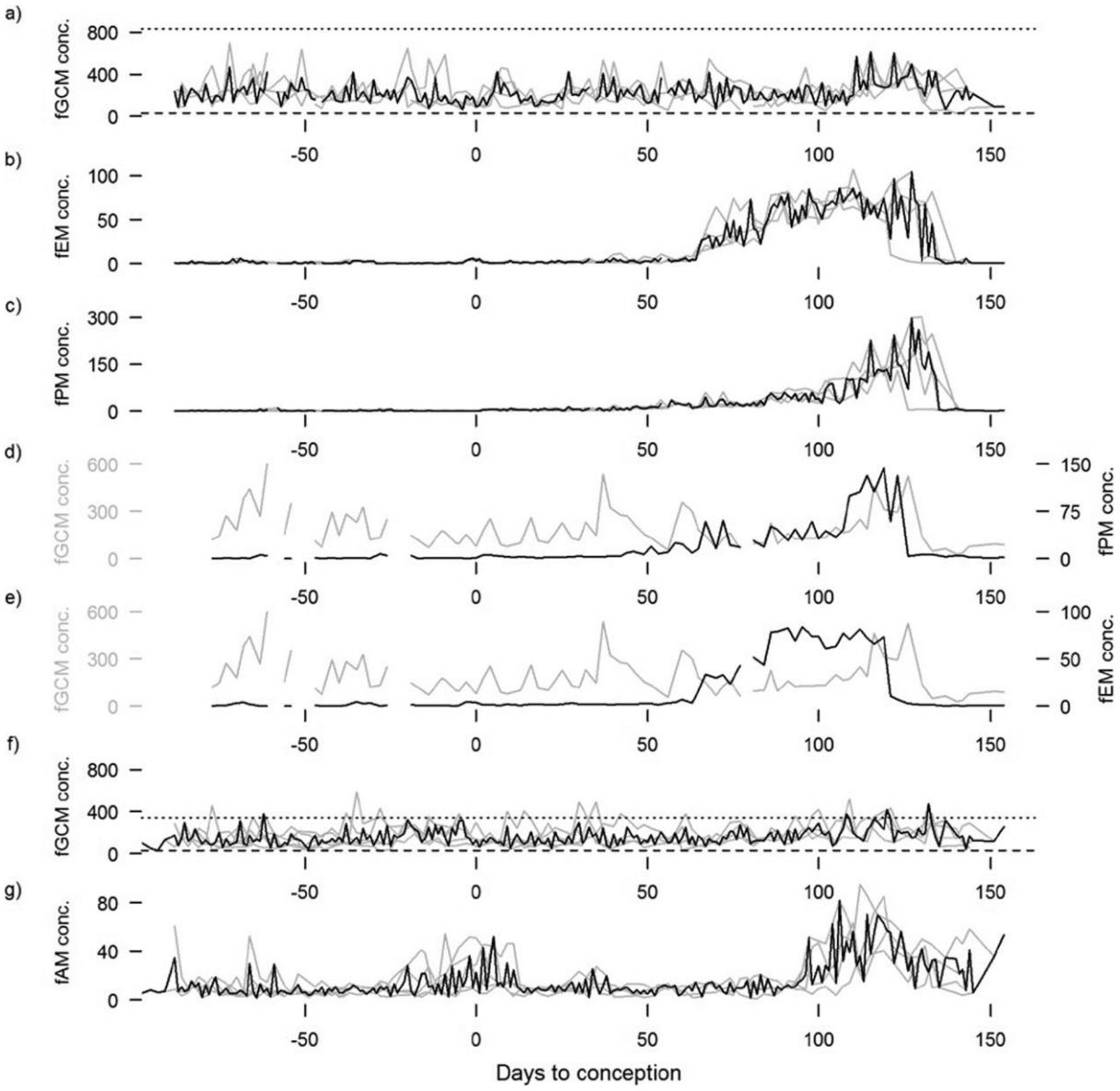


Figure 2


a)
fGCM conc.
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b)
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e)
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f)
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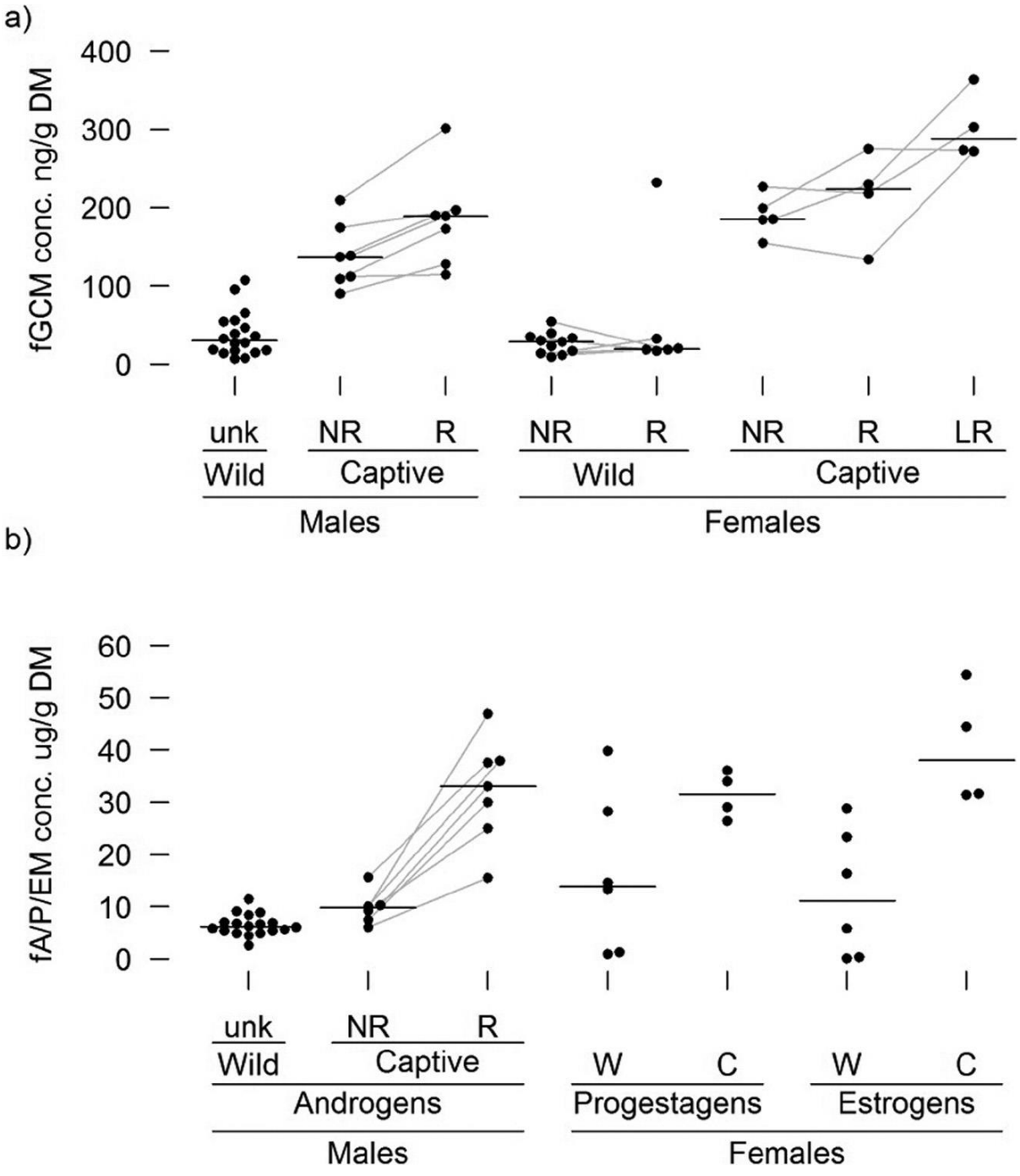
100
150
Days to conception

Figure 3


a)
fGCM conc. ng/g DM
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Wild
Captive
Wild
Captive
Males
Females
b)
fA/P/EM conc. ug/g DM
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Captive
Androgens
Progestagens
Estrogens
Males
Females