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### Article

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

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

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20 **Abstract**

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

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

41

## 42 **Introduction**

43 Reproductive events are important parts of an animal's annual's life history and of critical  
44 importance in determining an individual's fitness and hence the viability of a population (Olive  
45 et al. 2000). Reproductive hormones, which are secreted by the hypothalamic-pituitary-  
46 gonadal (HPG) axis, are responsible for regulating behavioural, physical and physiological  
47 parameters during reproductive events (Johnson 1986; Nieschlag et al. 2012). In seasonal  
48 breeders, periods of reproductive activity, though often short in duration, are characterised by  
49 group instability (in social species), heightened intra-sexual competition and high energy  
50 demands, all of which can activate the stress-response (Creel 2005; DeVries et al. 2003). This  
51 is an important mechanism allowing an organism to restore homeostasis through the activation  
52 of the hypothalamic-pituitary-adrenal axis (HPA axis) and the subsequent secretion of  
53 glucocorticoids (O'connor et al. 2000). Consequently, glucocorticoid concentrations are often  
54 used as an index of perceived stress in an organism (Sapolsky et al. 2000). Secreted  
55 glucocorticoids stimulate cardiovascular activity and energy mobilisation, while triggering  
56 important behavioural changes in order to cope with perceived stressors (Reeder and Kramer  
57 2005). However, a functional cross-talk has been found to exist between the HPA and HPG  
58 axes, with substantial increases in glucocorticoid concentrations inhibit the secretion of  
59 reproductive hormones, directly influencing the reproductive capabilities of an individual  
60 (Dobson and Smith 2000).

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

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

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

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

## 94 **Methods and Materials**

### 95 *Study site*

96 We conducted the study at Ithumela Primate Sanctuary (IPS, Buffelsdrift Conservancy, South  
97 Africa, 25°35'55.79"S, 28°19'30.82"E) between March and November 2013. We collected  
98 temperature and rainfall data for the area from the South African Weather Service. The study  
99 site has a hot, wet season from October to March, whereas a cold, dry season occurs from  
100 April to September. During the study the maximum temperatures varied between 13.8 °C and  
101 35.8 °C (mean  $\pm$  SD: 25  $\pm$  3.06 °C), whereas minimum temperatures varied between -1 °C  
102 and 17.6 °C (mean  $\pm$  SD: 8.29  $\pm$  4.61 °C). A total of 209 mm of rainfall occurred during the  
103 study period, with the majority occurring in March (34.8 mm), April (81.6 mm), and September  
104 (78 mm).

### 105 *Study animals*

106 The African lesser bushbaby is a small nocturnal prosimian distributed throughout sub-  
107 Saharan Africa (Bearder 1987). Male and female *G. moholi* individuals have separate, but  
108 overlapping, home ranges, with frequent interaction occurring among individuals (Bearder &  
109 Martin 1979). *G. moholi* has been described as polygynandrous, with two mating periods per  
110 year (May and September; Pullen, Bearder & Dixon 2000; Scheun et al., 2016b). Our study  
111 animals comprised seven male and female individuals held in captivity at IPS, as well as 14  
112 males and 12 females from the surrounding wild population of Buffelsdrift Conservancy. All  
113 individuals were marked with subcutaneously injected passive identification transponders  
114 (ID100 Trovan, EURO I.D., Weilerswist, Germany). The seven adult male and female *G.*  
115 *moholi* were housed in mating pairs in separate cages at IPS. Although this pairing of *G.*  
116 *moholi* individuals is unnatural, compared to the natural social structure of the species  
117 (Bearder and Martin 1979), this was done to observe mating instances as well as track  
118 reproductive hormone patterns in the species during mating and pregnancies (Scheun et al.  
119 2016b). The average age of the seven captive females was 3.4  $\pm$  1.3 years of age (range: 2-

120 5 years), while males were  $2.9 \pm 0.7$  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 ( $3 \times 1.5 \times 2.8$  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



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

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

### 181 *Hormone extraction and analysis*

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

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

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

### 203 *Data analysis*

#### 204 *A priori* model-building and selection

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

217

$$218 \quad y_i \sim N(\mu_i, \sigma^2) \quad (1)$$

$$219 \quad g(\mu_i) = n_i \quad (2)$$

$$220 \quad n_i = \sum_{j=1}^s \beta_j x_{ij} + \sum_{k=1}^r Z_{ik} \mu_{ik} \quad (3)$$

$$221 \quad u_{ik} \sim N(0, \sigma_k^2) \quad (4)$$

222

223 We standardized variables using Package 'arm': numeric variables to  $\bar{x} = 0$ ;  $\sigma = 0.5$  and  
224 binary variables to  $\bar{x} = 0$  with a difference of 1 between categories (Gelman 2008). We used  
225 variance inflation factors (VIFs, Anderson et al. 2001) to assess multicollinearity, using an *a*  
226 *priori* cut-off of VIF = 5 for rejecting collinear variables. We evaluated candidate models with  
227 Akaike's Information Criterion (Akaike 1974) with small sample size correction (AICs,  
228 Anderson 2008). We performed multi-model inference and model averaging (Burnham and  
229 Anderson 2002) using Akaike weights ( $w_i$ ) of all candidate models. We assessed goodness of  
230 fit of parameter estimates using 85% confidence intervals (Anderson 2008; Arnold 2010) and  
231 assessed variation explained by the global model using  $\Omega_0^2$  (Xu 2003).

#### 232 *Post hoc* graphical comparisons

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

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

249 parturition). Number of faecal samples per free-ranging animal was low (range: 1-6, median 2  
250 for males; range: 1 to 5, median 2 for non-reproductive females; range: 1-4, median 2.5 for  
251 reproductive females) compared to captive animals (range: 67-76, median 73 for non-  
252 reproductive males; range: 16-22, median 19 for reproductive males; range: 29-78, median  
253 35 for non-reproductive females; range: 36-40, median 36.5 for reproductive females; 8 to 10,  
254 median 8 for late reproductive females).

## 255 **Results**

### 256 *A priori models of faecal glucocorticoid metabolite variability*

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

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

### 272 *Post hoc longitudinal profiles of faecal glucocorticoid metabolites*

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

#### 287 *Post hoc graphical comparison by population and reproductive status*

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

294 For male bushbabies, captivity status seemed to have little effect on fAM  
295 concentrations during the non-reproductive period (Fig. 3 b). Within captive males, fAM  
296 concentrations increased from the non-reproductive to the reproductive period (Fig. 3 b).  
297 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

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

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

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

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

358         Although a general season-related pattern of glucocorticoid concentrations has been  
359 described for a number of species in the wild (Romero 2002), this pattern can be substantially  
360 altered in captivity by various extrinsic factors. Such circumstances, including restriction of  
361 movement, absence of predation and refugia, forced proximity to humans, and unnatural  
362 grouping of often gregarious and non-gregarious species, can result in a prolonged elevation  
363 of glucocorticoid concentrations (Morgan and Tromborg 2007). Thus, some captive individuals  
364 exhibit chronically elevated glucocorticoid concentrations (a new and higher basal  
365 concentration level, Dickens et al. 2009). Animals in our captive population were housed as  
366 mating pairs, while being confined to a small area. Both of these factors are atypical for free-  
367 ranging *G. moholi*, in terms of social behaviour and movement dynamics (Bearder 1987). Such  
368 chronic adrenocortical activity, in response to a suboptimal captive setup, has been found in  
369 primate species such as the gray mouse lemur (*Microcebus murinus*, Perret and Predine  
370 1984) and spider monkeys (*Ateles geoffroyi rufiventris*, Davis et al. 2005), but also in captive-  
371 held, non-primate mammal species such as the giant panda (*Ailuropoda melanoleuca*, Liu et  
372 al. 2006) and the tigrina (*Leopardus tigrinus*, Moreira et al. 2007). The high variability of fGCM  
373 concentrations as a result of the captive setup may mask putative patterns in adrenocortical  
374 activity during key life stages in bushbabies and potentially in other species. This masking  
375 effect may have resulted in the relatively weak association of predictor variables assessed in  
376 our linear mixed models. Although the fGCM patterns were highly variable and presumably  
377 chronically elevated as a result of the captive setup of our study, no disruption of reproductive



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

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

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

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

404 reproductive and adrenocortical activity in free-ranging bushbabies, and designed to assess  
405 the putative effect of captivity on adrenocortical and gonadal hormone production in captive  
406 versus free-ranging bushbabies. As more than 26 billion animals, from over 10, 000 species,  
407 are kept in captive setups such as zoos, farms and conservation centres (Mason and Veasey  
408 2010) it is important to clarify what effects captivity itself may have on the adrenocortical  
409 activity of a species. Studies on captive and domesticated species have shown that the  
410 perception of confinement-specific stressors is species-specific, making a generalised  
411 assessment difficult (Romero 2002). It is thus important to not only clarify the role of  
412 reproductive season on adrenocortical activity, but also the possible effect of captivity on the  
413 stress concentrations of caged individuals. The potential effect of captivity, the need for  
414 representative faecal sampling throughout the life history stage under study and the potential  
415 use of reproductive hormone concentrations as proxies for reproductive activity (specifically  
416 the progression of pregnancy), are factors that could be applied to non-invasive studies of the  
417 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').

	<b>Model: log(FGCM)~</b>	<b>* logL</b>	<b>†K</b>	<b>‡AICc</b>	<b>§Δ</b>	<b>  w<sub>i</sub></b>
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 (logL), † number of parameters (K), ‡ Akaike's Information Criterion with small sample correction (AICc), § AICc distance from the best model (Δ) and || Akaike weight (w<sub>i</sub>)

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).

<b>Model: log(FGCM)~</b>	<b>* logL</b>	<b>†K</b>	<b>‡AICc</b>	<b>§Δ</b>	<b>  w<sub>i</sub></b>
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<b>1</b>	FAM + (1 animal)	-456.1	4	920.4	0.0	0.26
<b>2</b>	FAM + repr + (1 animal)	-455.2	5	920.6	0.2	0.23
<b>3</b>	FAM + lact + (1 animal)	-456.0	5	922.0	1.6	0.11
<b>4</b>	FAM + preg + (1 animal)	-456.0	5	922.1	1.8	0.11
<b>5</b>	FAM + preg + repr + (1 animal)	-455.1	6	922.3	1.9	0.10
<b>6</b>	FAM + lact + repr + (1 animal)	-455.1	6	922.4	2.1	0.09
<b>7</b>	FAM + lact + preg + (1 animal)	-455.7	6	923.5	3.2	0.05
<b>8</b>	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 ( $\Delta$ ) 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 ( $\sum w_i$ ) 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 adrenocorticotrophic 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

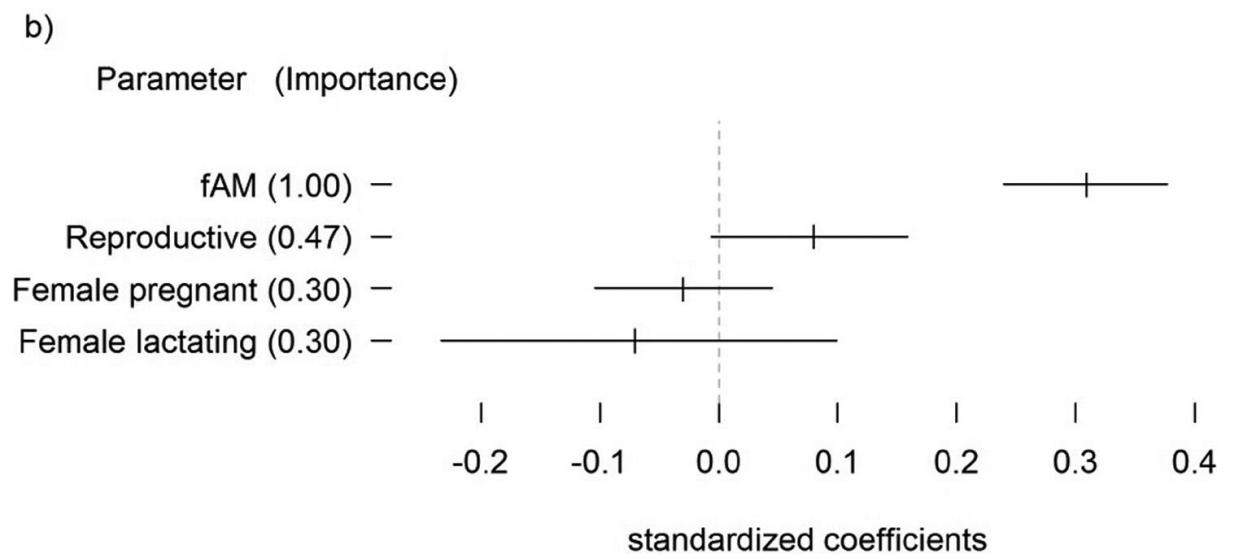
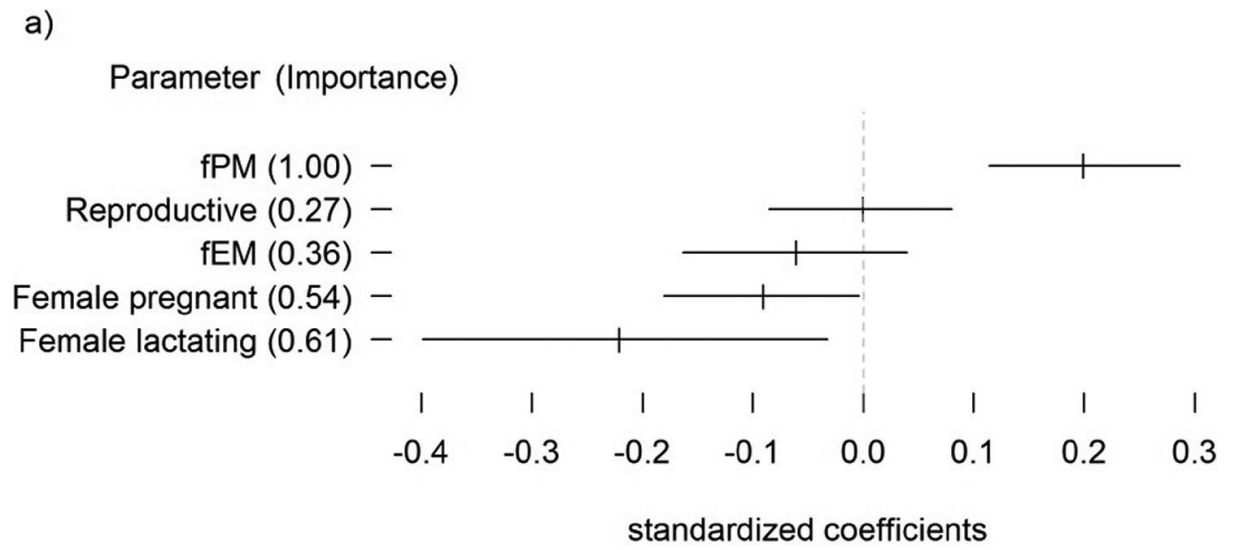


Figure 2

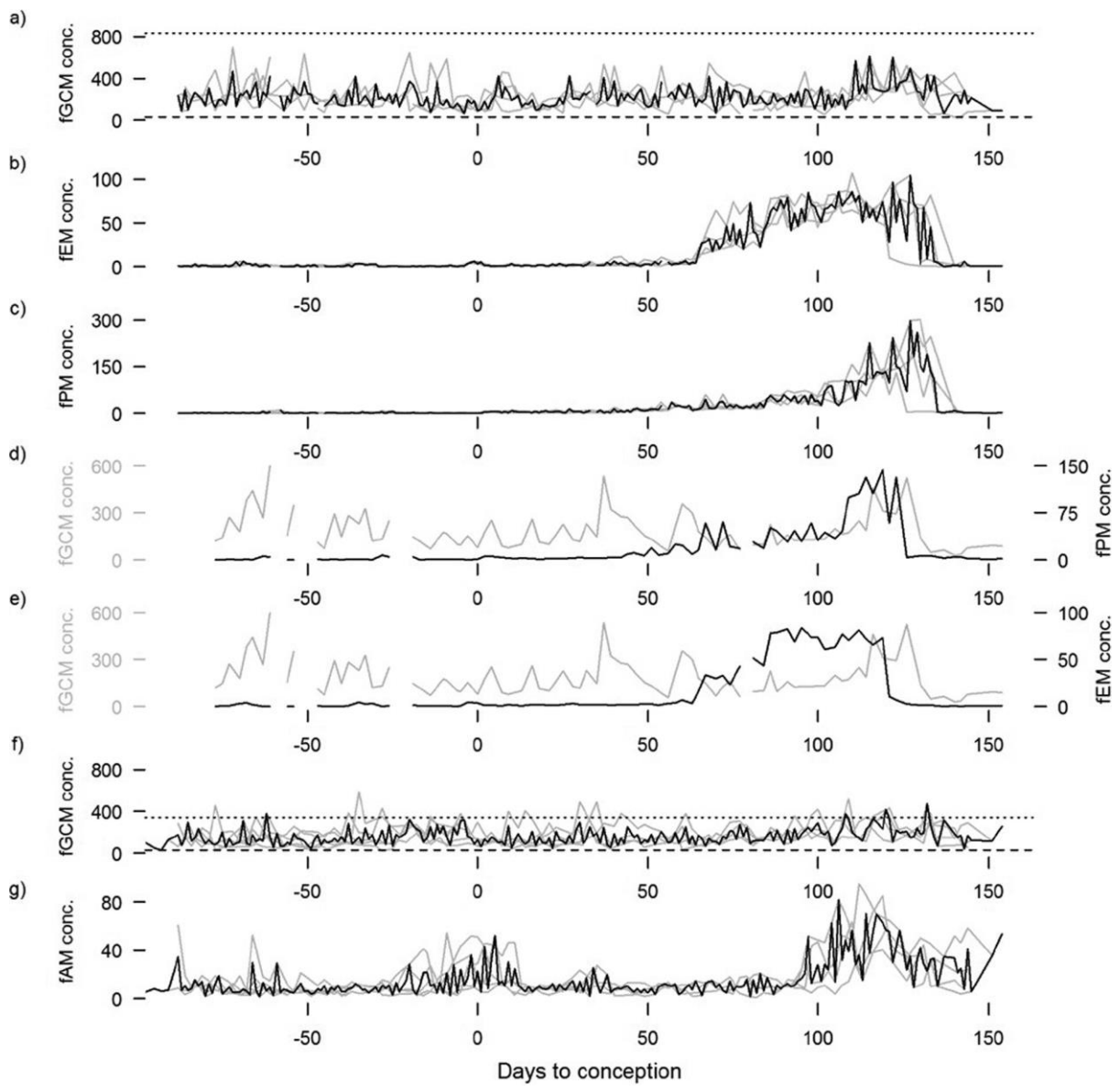


Figure 3

