Female reproductive activity and its endocrine correlates in the African lesser bushbaby, *Galago moholi*

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Abstract

Steroid hormones play an important role in female reproductive physiology and behaviour and are often used to monitor important female reproductive events. However, such studies are often attempted on captive populations alone, delivering limited data. One such example is the African lesser bushbaby, *Galago moholi*, for which contradicting observational data exist between captive and free-ranging populations, while hormonal analyses have only been obtained from a single captive population. To extend and rectify the limited information, we monitored faecal progestagen and oestrogen metabolite levels across various important life history stages of both captive and free-ranging *G. moholi*. We additionally recorded changes

in vaginal state as well as the occurrence of reproductive and aggressive behaviour throughout the study. Data from our captive population revealed an ovarian cycle length of 33.44 ± 0.59 days (mean ± SD), with follicular and luteal phases of 14.2 ± 1.0 and 19.1 ± 1.5 days, respectively, and an average pregnancy length of 128 ± 3.3 days. The initiation of female reproductive activity was closely linked to an oestrus-related increase in faecal oestrogen metabolite levels. Four of the seven captive females monitored in our study conceived during the May mating period, with one additional female fertilised in September, supporting the idea that the September mating period functions as a back-up for female *G. moholi.* Identified benchmark faecal progestagen metabolite levels (non-pregnant: > 1 µg/g dry weight (DW), pregnant: > 9 µg/g DW) should help researchers to determine pregnancy-status of randomly wild-caught females in even a cross-sectional study set-up.

Keywords: African lesser bushbaby, progestagens, oestrogens, faecal steroid metabolites, oestrus, pregnancy

Introduction

Steroid hormones play a vital role in female reproductive physiology and behaviour, most notably in regulating and maintaining cyclicity, secondary sexual development, behavioural oestrus, pregnancy, foetal development and parturition (Bazer et al. 1998; Gesquiere et al. 2007; Ma et al. 2003; Stouffer and Hearn 1998). In this regard, both oestrogen and progestagen changes have been used to successfully monitor key reproductive events (Carroll et al. 1990; Heistermann et al. 1996; Pepe and Albrecht 1995) and often allow to explain the mechanisms underlying female reproductive strategies (Brockman et al. 1995; Wallen and Zehr 2004). However, studies examining longitudinal female reproductive hormone patterns in free-ranging species are still scarce and were in the past often limited by the effort and disturbance caused by sampling methods, such as taking blood. Although blood hormone analysis is a common approach for monitoring ovarian endocrine activity, especially

when working with captive animals in a controlled setup, general difficulties in animal constraint and frequent sample collection have resulted in the development of non-invasive alternatives such as the use of faeces as hormone matrix (Heistermann 2010; Hodges et al. 2010; Schwarzenberger 2007). However, even for hormone analyses via faecal samples, continuous access to the study individuals is required to determine longitudinal hormone metabolite patterns. This challenge in acquisition has resulted in the majority of studies monitoring endocrine activity on captive or semi-captive populations, rarely comparing their findings to free-ranging populations (Buesching et al. 1998; Perret 1986; Williams et al. 1991).

G. moholi has a polygamous mating system with two mating periods a year (Bearder 1987; Doyle et al. 1971; Pullen et al. 2000). Despite the categorisation of a primary (May) and subsidiary, post-partum mating period (September), available data on the importance of the two mating periods seem to differ. Whereas Doyle et al. (1971), studying captive individuals, suggested that females utilise both mating equally, Pullen et al. (2000) found free-ranging females rarely utilising the second mating period. Furthermore, various discrepancies have been found between studies attempting to define behavioural oestrus (Bearder and Martin 1979; Doyle et al. 1971; Lipschitz 1996), as well as pregnancy length (Doyle et al. 1971; Izard and Nash 1988; Nekaris and Bearder 2007). As discrepancies such as these are based on behavioural data alone, the use of endocrine data may offer a more robust method for determining the importance or duration of the above mentioned reproductive parameters.

The use of non-invasive steroid hormone monitoring may present a relatively easy way of frequently monitoring the reproductive status of captive and even free-ranging female *G. moholi*. In this regard, however, only a single, short-term study has attempted to describe the natural pattern of reproductive steroid secretion in captive individuals (Lipschitz 1996). This was accomplished through the use of urinary steroid analysis, a sampling matrix that may be suboptimal for such a small mammal (Heistermann 2010). No further work to elucidate the pattern of reproductive steroid hormone secretion across various important life history stages,

such as follicular and luteal phases, mating, pregnancy and parturition events, have since been attempted in *G. moholi*.

To rectify this shortage, we quantified hormone metabolite levels to describe the longitudinal pattern of faecal progestagen metabolite (fPM) and faecal oestrogen metabolite (fEM) secretion across various important life history stages of *G. moholi* females and tried to define the importance of the subsidiary mating period. Finally, we aimed to develop a technique to assist researchers in the field to determine pregnancy status of randomly wild-caught galagos from a single faecal sample.

Material and methods

Study site and animals

We conducted the study at Ithumela Primate Sanctuary (Buffelsdrift, South Africa, 25°35'55.79"S, 28°19'30.82"E) from March to November 2013 on seven adult female *G. moholi,* each paired with an adult male (all individuals > 2 years). Each captive pair was housed in a cage (3 x 1.5 x 2.8 m), with nest boxes, specifically designed to allow for easy separation of pairs while preventing cross-contamination of faecal samples. We fed a combination of fresh fruit, yogurt and cat pellets, with water being available *ad libitum.* In addition, we aimed to gather information on reproductive cycles of free-ranging females and therefore trapped individuals from the surrounding population tri-weekly, using 15 Sherman and 15 walk-in live traps baited with honey, peanut butter and banana. All traps were checked hourly for the presence of individuals. Galagos were handled for no more than five minutes before being released at the capture location. The study was performed with approval of the University of Pretoria Animal Use and Care Committee (Reference EC056-12).

Faecal sample and behavioural data collection

We collected fresh faecal samples from all seven captive females three times a week. In addition, we took body measurements, which included body weight and length as well as vaginal status (open/closed) on a weekly basis during non-reproductive periods and tri-weekly during periods when females were reproductively active. We also collected fresh faecal material and took respective body measurements from all free-ranging females caught during the study period (n = 12, recapture rate: 2.6 – 20.1%). Free-ranging individuals were captured with self-made live walk-in traps (35 x 15 x 15 cm) as well as Sherman traps (7 x 7x 30.5 cm, H. B. Sherman Traps, Tallahassee, FL, USA) baited with honey, peanut butter and banana. We collected a total of 626 faecal samples (range: 84 – 93 per individual) from the seven captive females, and 38 faecal samples (range: 1-6 per individual) from free-ranging females during the eight month study period.

In addition, we conducted observations of the captive females, using *ad libitum* sampling (Altmann 1974), between 20h00 and 04h00 between three to five times a week, to assess the presence of reproductive and aggressive behaviour. Reproductive activity included female presenting (i.e. “sit in front of a male”), male “grabbing”, “mounting” and “thrusting”, and finally “intromission”. Aggressive behaviour was classified as individuals chasing each other, usually with high degrees of vocalisation, and culminating in grappling and biting (see ethogram of both reproductive and aggressive behaviours: Lipschitz 1997 and Lipschitz et al. 2001). Female behavioural oestrus was defined as periods of increased female receptivity, allowing males to successfully mount and accomplish intromission. In addition, we determined reproductive status (pregnant vs non-pregnant) of our captive female individuals through direct observations of mating, distinct oestrous behaviour (Lipschitz et al. 2001), as well as by backdating parturition events. Nest boxes of captive pregnant females were examined each evening and morning during the final trimester (from ~ 100 days of gestation) to determine the exact date of parturition. Similarly, for free-ranging females, pregnancy status was assessed

through the presence of a foetus (via palpation of the lower stomach), excessive weight loss between captures (> 20 g) , lactation during birthing seasons and the analysis of steroid hormone metabolite levels from collected faecal samples.

Steroid extraction and analysis

Fresh faecal material was placed into a 1.5 ml microcentrifuge tube within 5 min of defecation for our captive animals, and collected within an hour of defecation in the free-ranging population. Subsequently, samples were frozen immediately and stored at -20 °C until hormone extraction. Frozen faeces was lyophilized, pulverized and then sieved through a thin mesh to remove any fibrous material present (Fieß et al. 1999). Following this, we extracted 50 – 55 mg of faecal powder by vortexing for 15 minutes with 1.5 ml of 80 % ethanol. Subsequently, we centrifuged each sample for 10 min at 1500 *g*, and supernatants were transferred into new microcentrifuge tubes and stored at -20 °C until hormone analysis.

We measured faecal extracts, via enzyme immunoassays (EIA), for faecal oestrogen (fEM) and progestagen metabolites (fPM), using antibodies against 17β-oestradiol-17-HS and 5β-pregnane-3α-ol-20-one-3HS:BSA, respectively. Details of the two EIA's, including cross-reactivities, are described by Palme and Möstl (1993) for the fEM EIA and by Schwarzenberger et al. (1996) for the fPM EIA. Both assays utilised during this study were biologically validated by demonstrating its ability to distinguish between reproductive (pregnancy) and non-reproductive (non-pregnancy) periods. For this, fEM and fPM levels of four individuals, who were fertilised during our study, were compared prior to and during pregnancy. According to Bayesian analysis, there is a 100% probability of fEM and fPM concentrations being higher during reproductive (pregnancy) than non-reproductive (non-pregnancy) periods for all four females. The effect size ranged from 4 to 29 µg/g dry weight (DW) for fEMs and 13-24 µg/g DW for fPMs. Additionally, faecal hormone metabolite levels during pregnancy increased substantially compared to non-pregnancy periods, with fEM concentrations increasing between 8- and 43- fold, while fPM concentrations increased 11-

and 25- fold. Therefore, both EIAs used can robustly monitor fEM and fPM activity in female *G. moholi*.

For steroid hormone determination, 50-µl aliquots of standards (range: 0.1 – 250 pg for the fEM EIA and 0.1-200 pg for the fPM EIA), quality controls and diluted faecal extracts were pipetted in duplicate into microtiterplate wells. Then 50 µl of bioinylated 17β-oestradiol or 5β-pregnane label and antiserum were added and the plates incubated over night at 4 °C. Following incubation the plates were washed four times and 150 µl (20ng) of streptavidin-peroxidase was added to each well. Following incubation in the dark for 30 min at 4 °C, plates were washed again before 150 µl peroxidase substrate solution was added, and plates further incubated for 30-60 min. The reaction was terminated by adding 50 µL of 4N H_2SO_4 and the absorbance measured at 450 nm. Serial dilutions of extracted faecal samples gave displacement curves that were parallel to the respective standard curves in both assays. The sensitivity of the EIAs were 6.6 µg/g DW for the fEM assay and 2.4 µg/g DW for the fPM assay as calculated at the 90 % binding efficiency point. Intra- and inter-assay coefficients of variation, determined by repeated measurements of high and low value quality controls, were 6.9 and 7.4 % (Intra-assay) and 7.0 and 7.9 % (Inter-assay) for the fEM EIA, while, for the fPM EIA, the values were 5.3 and 8.1 % (Intra-assay) and 6.2 and 8.9 % (Inter-assay), respectively. Laboratory analysis was conducted at the Endocrine Research Laboratory at the Faculty of Veterinary Science, University of Pretoria.

Data analysis

We calculated baseline fEM and fPM levels for each individual using an iterative process according to Brown et al. (1999), whereby all values greater than mean + 2 standard deviation (SD) were removed, the average recalculated, and the process repeated until there were no values exceeding the mean + 2 SD threshold, thus yielding the baseline level. Mathematical justification for using a restricted mean approach when calculating baseline levels has been

discussed e.g. by Miller (1991). We defined periods of elevated fEM and fPM concentrations as the occurrence of two or more consecutive samples that exceeded the calculated individual baseline level.

Cyclicity in terms of regular ovarian endocrine activity was defined by a regular cyclic pattern in fEM and fPM concentrations. We calculated ovarian cycle length as the time period it takes from an initial rise in fEM concentrations above individual baseline till the following rise in fEM concentrations above individual baseline levels. Follicular phase length was determined from the period of initial increase in fEM concentration until peak fEM levels during each ovarian cycle. Similarly, luteal phase length was calculated from the period of initial increase of fPM concentration, which often occurred during peak fEM concentrations, until fPM levels returned to the lowest observed values of a specific ovarian cycle (Baird et al. 1975). Oestrus was determined as the period where increases in fEM levels, during the follicular phase, coincided with observed vaginal opening and mating.

We calculated gestation length from time of presumed conception, classified as the day prior to fPM increase above individual baseline (Harlow et al. 1983), until parturition for each female conceived in May. To determine a potential fPM threshold value for pregnancy status, all fPM values from the seven captive females were indexed according to reproductive status (pregnant (1) and non-pregnant (0)) and subsequently sorted according to concentration and grouped into concentration ranges (0-1 µg/g, 1-2 µg/g, 2-3 µg/g, etc.). Finally, the percentage of pregnant vs non-pregnant samples within each concentration category was determined. All results are given as mean ± standard deviation (SD) unless stated otherwise.

Results

Ovarian endocrine activity

Five of the seven captive females showed regular ovarian endocrine activity throughout the study period (Fig.1 and 2a) with an average ovarian cycle length of 33.4 ± 0.6 days (range: 32- 34 days, Tab. 1). The duration of respective follicular and luteal phases were 14.0 ± 1.0 and 19.1 ± 1.5 days, respectively (Tab. 1). The remaining two females showed high degrees of irregular ovarian endocrine activity, hindering the ability to calculate ovarian cycle, follicular and luteal length for both individuals (Fig. 2b). As a result of the low capture-recapture rate we were unable to determine cyclicity in free-ranging females.

Table 1. Individual reproductive status, baseline fEM and fPM levels, ovarian cycle length, follicular- and luteal phase length as well as pregnancy length for each of the seven captive females. ND = Not determined; NA = Not applicable.

Female (age)	**Reproductive state**	**Hormone baseline levels (µg/g DW)**		**Ovarian cycle (Nr of cycles)**	**Follicular cycle length**	**Luteal cycle length (days)**	**Pregnancy length (days)**
		fEM	**fPM**				
1 (5)	Cyclic	0.77	1.70	32.7 ± 1.4 (6)	15.0 ± 2.7	17.6 ± 4.0	*ND
2 (2)	Cyclic	2.34	0.85	33.5 ± 2.1 (3)	15.5 ± 6.4	17.5 ± 3.3	124
3 (3)	Cyclic	1.20	1.70	33.0 ± 0.0 (3)	13.5 ± 4.9	19.5 ± 4.9	129
4 (4)	Non-cyclic	1.61	2.20	* N/A	*N/A	*N/A	*N/A
5 (5)	Non-cyclic	0.68	3.45	* N/A	*N/A	*N/A	*N/A
6 (3)	Cyclic	3.07	1.40	34.0 ± 1.4 (3)	14.0 ± 0.0	20.0 ± 1.4	131
7 (3)	Cyclic	2.24	3.21	34.0 ± 1.4 (3)	13.0 ± 1.4	21.0 ± 0.0	131
Mean	-	1.70 ± 0.89	2.07 ± 0.95	33.44 ± 0.59	14.2 ± 1.0	19.1 ± 1.5	128.7 ± 3.3

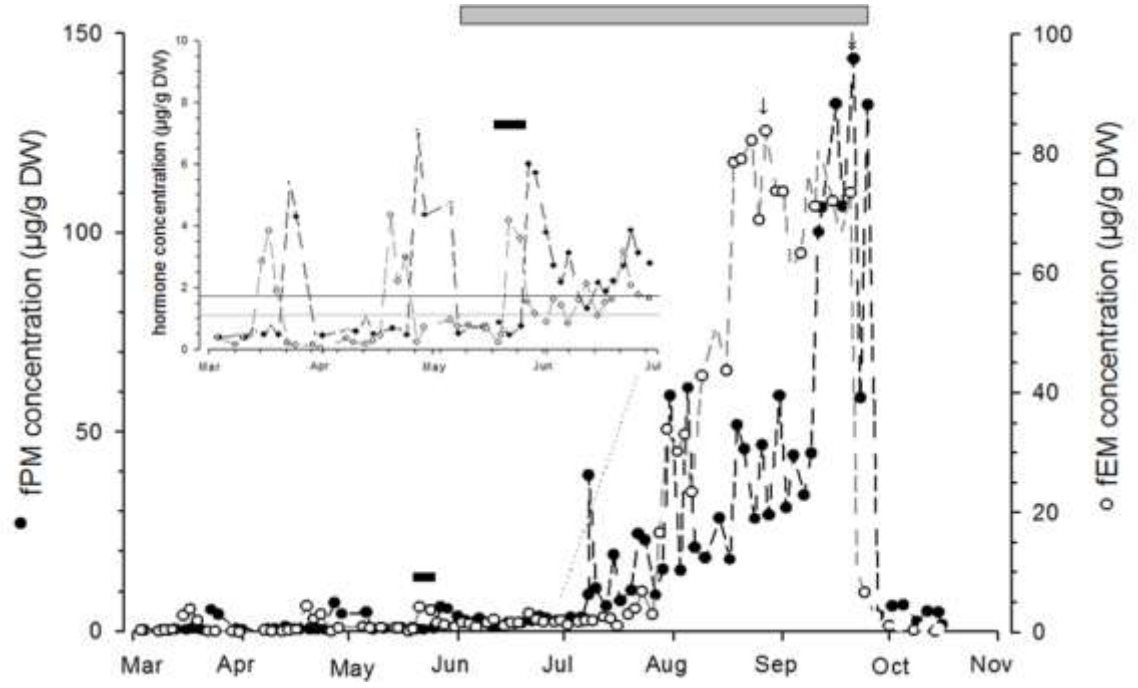

Figure 1. Individual longitudinal fPM and fEM profile of one of the four monitored female African lesser bushbabies (*G. moholi*). FEM and fPM baseline levels are indicated by *grey* and *black lines*, respectively. Black bars indicate periods of mating activity (oestrus) which coincided with an increase in fEM concentration above baseline levels. Peak fEM (late-August) and fPM (late-September) concentrations during pregnancy are indicated by↓ and ↓**,** respectively. Duration of pregnancy is indicated by the *grey bar*. The insert enlarges the period of cyclicity prior conception.

FEM levels during breeding periods

All seven females showed an increase in fEM concentration above individual baseline levels during May (Fig 1, 2a and b). This increase in fEM concentration preceded both vaginal opening and reproductive activity, which included high levels of allo-grooming, sniffing of female genitalia by males, mounting and intromission, by one day. Behavioural oestrus occurred in all seven captive females during the May mating period when fEM concentrations reached elevated levels. This lasted 3.8 ± 0.8 days for the five regular cycling females and 5.0 ± 0.0 days for the two females displaying irregular ovarian endocrine activity, before being terminated as fEM levels returned to baseline values. Male mating effort that occurred after behavioural oestrous and cornification of the vagina, resulted in male-female aggressive interactions. Four of the seven captive females, all of which displayed regular ovarian cycles, were fertilised during the May breeding period.

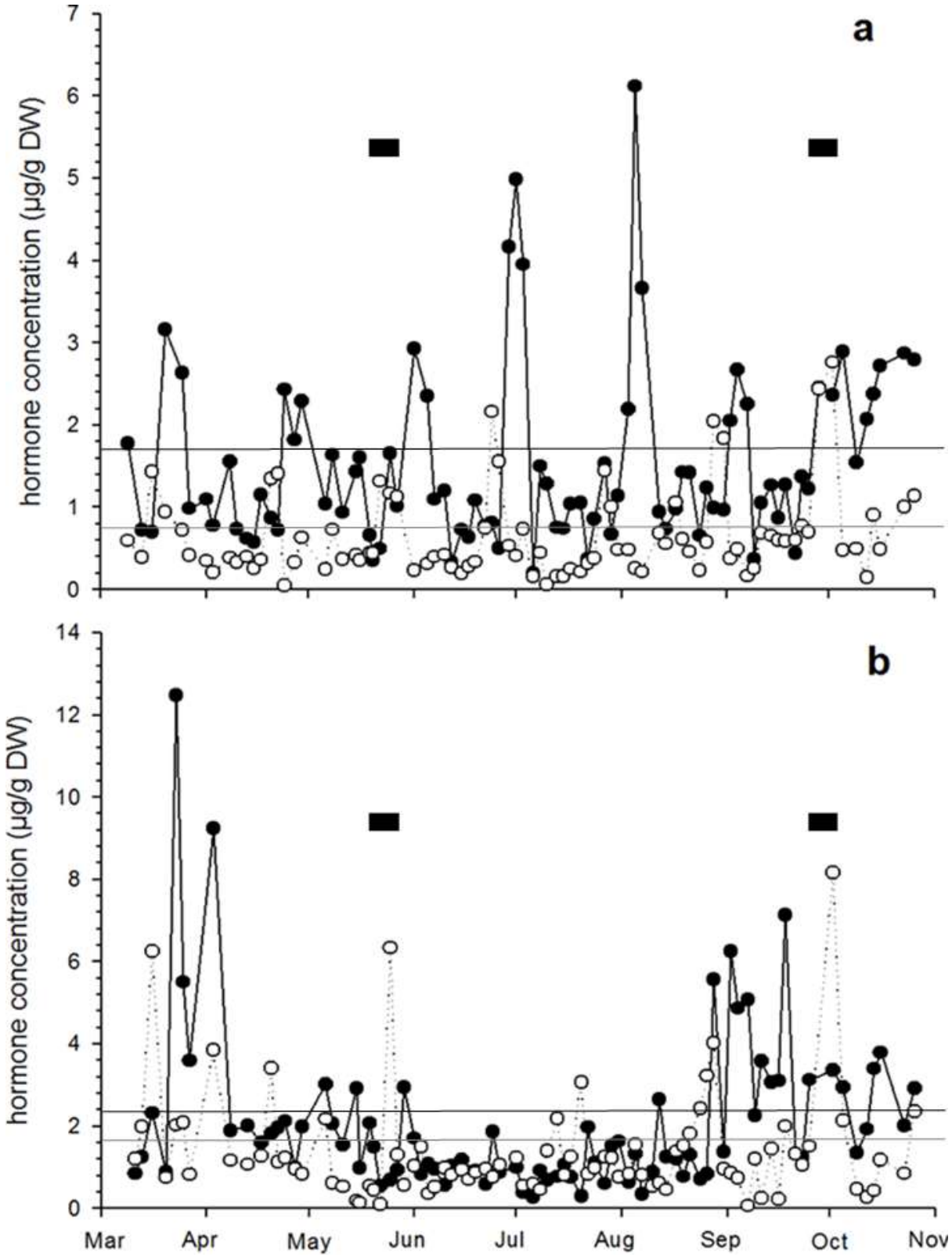

Figure 2 a and b. Longitudinal fPM (●) and fEM (o) profiles of the cyclic *G. moholi* female fertilised during September (a), as well as the profile of one of the two non-cyclic *G. moholi* females (b) monitored. FEM and fPM baseline levels are indicated by *grey* and *black lines* respectively. *Black bars* indicate periods of mating activity (oestrus) which coincided with an increase in fEM concentration to above baseline levels for both individuals.

Following the successful birthing event of all four pregnant females in September and October of the study, no physiological or behavioural signs of post-partum oestrus were observed, although a short period of vaginal opening was seen after parturition (~ 2 days, n = 4, Fig 1). The remaining three females, who were not fertilised during the May period, all displayed clear signs of behavioural oestrous during the September mating event. Behavioural oestrous length during this period was 4.0 ± 0.0 days for the cyclic female (Fig 2a) and 4.5 ± 0.5 days (Fig 2b) for both irregular cyclic females.

FEM and fPM levels during pregnancy and the post-partum period

The five females showing regular cycles through the study period all became pregnant; four during the May (Fig 1) and the fifth during the September mating period (Fig 2a). We were able to monitor all four May pregnancies until parturition.

Following conception in May, both fPM and fEM concentrations initially returned to baseline levels for 7.1 ± 4.9 days and 33.8 ± 7.1 days (n=4), respectively, before increasing and reaching peak values during late August and September (Fig. 1). The decrease in both fEM and fPM concentrations in week 18 -19 of gestation occurred within 48 hours of the onset of labour and the birthing event (Fig. 1), as determined by nest box examination of pregnant females. The average pregnancy length for the four females was 128.0 ± 3.3 days (Tab 1). Our data indicated two fPM thresholds for the female reproductive state, as all samples determined ≤ 1 µg/g DW belonged to animals not being pregnant (captive: n = 139; free-ranging: n = 11; Tab 2), whereas all samples ≥ 9 µg/g DW belonged to pregnant females (approximately mid-July; captive: n = 146; free-ranging: n= 7; Tab 2). All four females gave birth to male-female twin pairs, with an average weight at birth of 10.8 ± 1.3 g for males and 11.25 ± 1.7 g for females, respectively.

Table 2. Total number of samples collected within each progestagen concentration range for both captive and free-ranging populations. Additionally the percentage of samples collected from non-pregnant (NP) and pregnant (P) individuals within each range is shown

Captive female samples				Free-ranging female samples			
Progestagen concentration (µg/g DW)	**Total number of samples**	**Percentage NP samples**	**Percentage P samples**	**Progestagen concentration (µg/g DW)**	**Total number of samples**	**Percentage NP samples**	**Percentage P samples**
0-1	139	100	-	0-1	11	100	-
1-2	144	97	3	1-2	10	50	50
2-3	92	78	22	2-3	3	66	33
3-4	40	63	27	3-4	4	75	25
4-5	17	53	47	4-5	1	100	-
5-6	15	67	33	5-6	-	-	-
6-7	15	40	60	6-7	-	-	-
7-8	9	33	67	7-8	1		100
8-9	9	22	78	8-9	1		100
>9	146	-	100	>9	7	-	100
Total	**626**			**TOTAL**	**38**		

Free-ranging females

As a result of the low recapture rate of free-ranging females (n=4; all four recaptured females were pregnant) between gestation and the birthing periods we were unable to monitor long-term reproductive hormone metabolite patterns and/or lactation length in specific individuals. Therefore we only confirmed the pregnancy status of individuals via the occurrence of a sudden loss in weight between pregnancy and birthing periods (18. 06 ± 0.02 % of total body weight [~35 g] for the four recaptured females), the presence of a foetus (via palpation), as well as the observation of lactation during the birth season. Furthermore, pregnant free-ranging females showed higher fPM and fEM concentrations during pregnancy, than the respective hormone values determined from identified non-pregnant animals during the same period (Fig 3). All pregnant females caught by mid-July had fPM concentration higher than the estimated pregnancy baseline value calculated above (> 9 µg/g DW, Tab 2); while all confirmed non-pregnant individuals caught during this period had fPM levels well below this (2-5 µg/g DW, Tab 2). As a result of the relatively low fEM and fPM concentrations and

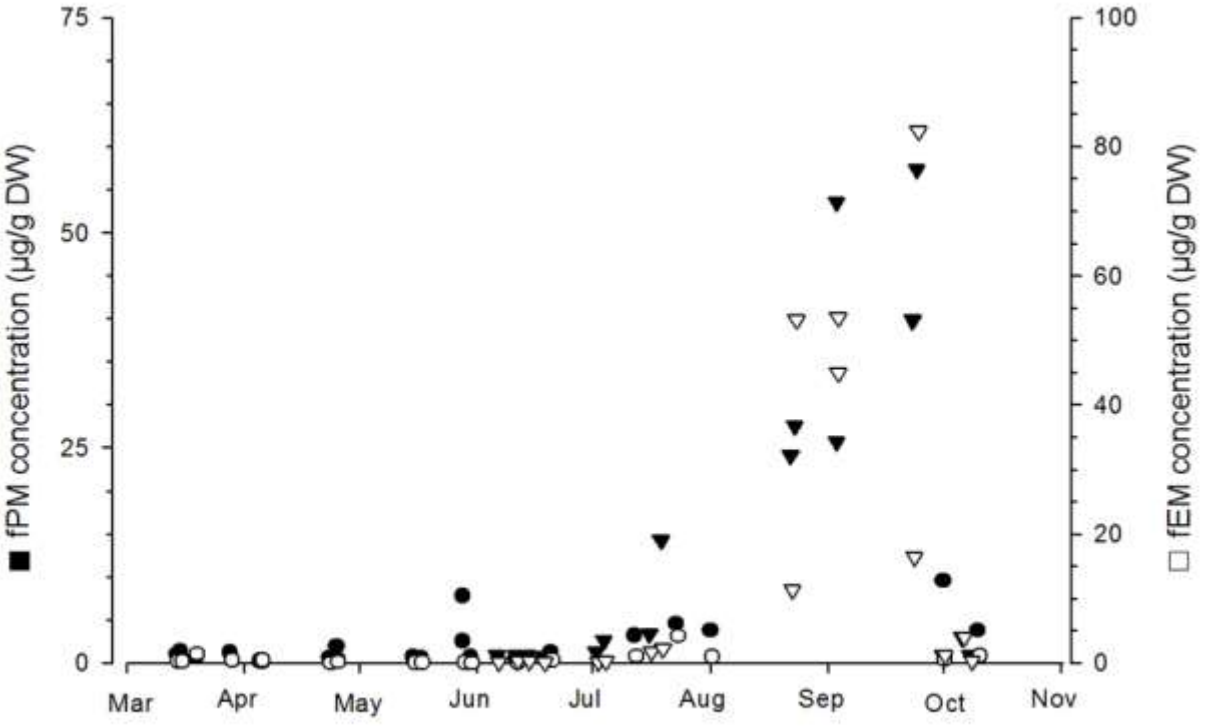

Figure 3. Faecal PM (■) and EM (□) hormone data from free-ranging *G. moholi* individuals caught at Ithumela Primate Sanctuary premises and surroundings, South Africa. ▲/Δ values indicate samples collected from individuals confirmed pregnant via palpation of the lower stomach.

absence of vaginal opening following parturition of pregnant free-ranging females, we were unable to confirm the presence of post-partum oestrus and mating in individuals that were pregnant during the winter. Recaptured females who were not fertilised during the May period displayed vaginal opening during the September breeding period.

Discussion

This study has shown that non-invasive, faecal hormone metabolite monitoring can be a robust tool for describing the pattern of oestrogen and progestagen secretion during key life stages of captive and free-ranging female *G. moholi*.

Five of our seven monitored females displayed regular ovarian cycles, observed as regular fluctuations of fPM and fEM concentrations, presumably representing regular follicular and luteal activity, throughout the study. Interestingly, the calculated ovarian cycle length obtained in the current study (33.4 days) is less than the 38.5 days observed by Lipschitz (1996), but rather similar to that of the closely related and similar sized Senegal galago (31.7-32.9 days, *G. senegalensis*, Darney and Franklin 1982; Manley 1966). The difference observed in ovarian cycle length between *G. moholi* populations may be the result of different non-invasive methodological approaches (urine vs faecal steroid metabolite analysis) and sampling intervals. Furthermore, the determination of ovarian cycle length by Lipschitz (1996) remains elusive and may have differed from what was used during this study, giving a longer average cycle length.

Despite the fact that some of our study animals showed irregular fluctuations in fEM and fPM levels, all seven captive females showed vaginal openings and behavioural oestrus, indicating that regular ovarian endocrine activity seems not to be a prerequisite for female *G. moholi* entering oestrus. Environmental cues, such as food availability, temperature and constant social stimuli have all been attributed to the initiation of oestrus (Perret 1986; Savini et al. 2008) and might play a role for initiating oestrus in *G. moholi* as well. In our study, the fEM increase seen in conjunction with female reproductive activity seems to play an important role in initiating not only physical changes (vaginal opening), but also respective behavioural alterations (mating acceptance) and presumably female receptivity, as seen in various primate species such as common marmosets (*Callithrix jacchus*, Barnett et al. 2006), Japanese macaques (*Mucaca fuscata*, O'Neill et al. 2004), or grey mouse lemurs (*Microcebus murinus*, Perret 2005). A similar pattern of oestrogen elevations and associated physical and behavioural changes have been described for ovariectomised *G. moholi,* subsequently injected with oestradiol-17β, clearly underlining the initiating role of oestrogens for female *G. moholi* reproductive activity (Lipschitz 1997). Interestingly, only females displaying ovarian cyclicity successfully conceived in our study. Thus the ability to successfully regulate ovarian

endocrine activity seems an important factor to enhance reproductive capabilities, as the loss of or even an irregular oestrogen or progestagen production is known to negatively affect reproductive success (Schwartz 2000).

The apparent inability of females to regulate ovarian endocrine activity has previously been attributed to various factors such as food availability (Wade and Schneider 1992), age (Matt et al. 1987; Nelson et al. 1992) and stress (Dobson and Smith 2000; Tilbrook et al. 2000). As regular cyclicity, as well as pregnancy is considered to be relatively energetically expensive, a shortage of high quality foods during this time may cause temporal changes in related hormone secretion, subsequently influencing reproductive success (Strum and Western 1982; Wade and Schneider 1992). As our study animals were continuously fed on high quality protein pellets, fresh fruits and insects, this seems a rather unlikely explanation for our data. Despite the absence of seasonality in terms of food availability, individuals were still exposed to seasonal changes in day length and temperature, both important factors driving seasonal breeding and reproductive hormone secretion (Wingfield 1984). A negative correlation between fertility and age has been observed for a number of primate species including hanuman langurs (*Presbytis entellus*, Borries et al. 1991), rhesus macaques (*Macaca mulatta*, Gilardi et al. 1997; Shideler et al. 2001) and olive baboons (*Papio anubis*, Strum and Western 1982). Although the average age of our captive female group was 3.6 ± 1.3 years (range: 2-5), thus at the chronological age of peak reproductive potential, the biological age of the ovaries could be at an advanced stage, influencing ovarian endocrine activity. Despite the ability of captive galagos to reach 16 years of age (Fischer and Austad 2011), studies of wild populations suggest a much shorter lifespan of approximately five years in wild, free-ranging individuals (Dausmann et al. 2012). The absence of regular cyclicity in two of our seven females, aged four and five respectively, likely point to the end of asymmetric aging process in which the follicular resources needed are presumably exhausted (Hermes et al. 2004). One of the four females fertilised during the study, however, was five years old, which means such ageing might be individual-specific and not to be assumed for all female

G. moholi monitored in such studies. Finally, a prolonged activation of the hypothalamic-pituitary-adrenal axis has been shown to affect the hypothalamic-pituitary-gonadal axis (Ferin 1999; Kirby et al. 2009), inhibiting gonadotrophin release, which consequently alters oestrogen and progestagen secretion (Brann and Mahesh 1991; Tilbrook et al. 2000). The unnatural pairing of animals, movement restriction and regular interactions with humans could all be perceived as stressors, subsequently influencing reproductive hormone activity. Perception of a stressor, however, is individual-specific and might therefore explain why only some of our females showed irregular ovarian endocrine activity.

The observed mating behaviour as well as the determined length of mating activity (3.8-5.0 days) in this study is similar to that described by Doyle et al. (1971) and Lipschitz (1996), although we did not observe signs of female approach or presentation towards males. The fact that captive *G. moholi* display prolonged periods of mating activity compared to that found in free-ranging individuals (~ 1 day, Bearder and Martin 1979), might be a feature of the captive setup as suggested by (Lipschitz 1996).

The pattern of fEM and fPM concentrations following successful conception found in this study is similar to that found in other primate species (Albrecht et al. 2000; Albrecht and Pepe 1990), domestic livestock (Spencer and Bazer 2002) and various other sub-primate species (Bazer et al. 1998). The observed decrease in fEM and fPM concentrations towards end of pregnancy, during week 18-19, should reflect the change in status of the foetal-maternal interface, which consequently initialises parturition (Audus et al. 2002; Mesiano et al. 2002; Mesiano and Welsh 2007). The average pregnancy length of 128 days determined is comparable to the 121 and 133 days described by Doyle et al. (1971) and Lipschitz (1996), using observational data and the presence of spermatozoa in vaginal smears, but far less than the 141 days mentioned in studies using external parameters for determining pregnancy length (*reviewed in* Nekaris and Bearder 2007), indicating that using only behavioural or physical cues might result in inaccurate determination of pregnancy length for *G. moholi*.

The birth of twins in all four cases, as well as the average new-born weight of male and female individuals, is also in line with that found in previous *G. moholi* studies (~ 11 g, Bearder 1987; Izard and Nash 1988). Although vaginal opening was observed in all four lactating females during the post-partum period, the presence of baseline fPM or fEM levels during this period, as well as low levels of female receptivity, means we cannot exclude that vaginal opening at this stage might only be a result of the birthing event rather than post-partum oestrus. While only one of our study females became pregnant during September, the majority of our study animals conceived during the first mating period in May, and not again in September, supporting the idea that the September mating period functions as a back-up for female *G. moholi* who were unable to find a mate or become pregnant during the initial period, as suggested by Pullen et al. (2000).

Although pregnancy can be determined with relative ease in captive females, through palpation or frequent hormone monitoring, the elusive nature of *G. moholi* in the wild makes frequent monitoring of reproductive status logistically challenging. For this reason, a single sample pregnancy test would benefit researcher working with free-ranging populations. The validity of the pregnancy threshold value (> 9 µg/g DW, mid-July) calculated from the captive *G. moholi* setup was tested against our free-ranging female population. From mid-July onwards, all free-ranging females identified as pregnant had fPM levels above 9 µg/g DW, confirming that this threshold value can be used as a relatively robust benchmark for pregnancy determination. However, these threshold values are only applicable if respective fPM concentrations are determined through the exact same procedures, especially steroid extraction and EIA analysis. In addition, factors such as diet as well as differences in gut microbiomes may lead to differences in fPM concentrations between individuals or populations (Goyman et al. 2012), and should be considered by future researchers utilising this benchmark to define pregnancy in this species. With the determined benchmark fPM levels in this study, less than 1 µg/g DW reflecting non-pregnancy and fPM concentrations exceeding 9 µg/g DW

reflecting pregnancy, researchers are now able to determine the pregnancy-status of randomly wild-caught *G. moholi* females at least to some extent.

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