

# Age and socially related changes in fecal androgen metabolite concentrations in free-ranging male giraffes

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## Highlights:

- Season has an impact on fecal androgen metabolite (fAM) levels in free-ranging giraffes
- In the presence of females, old adult bulls have higher fAM levels than younger adult bulls
- In all male groups, young adult bulls have higher fAM levels than old adult bulls
- Sexual activity increases fAM levels, but only in old adult bulls.

**Abstract:**

In many mammal species, androgen levels in males are elevated during periods of mating activity, often to facilitate aggressive behavior between males over access to fertile females. However, this pattern might be less obvious in species with a rather low male-male aggression rate, or in those that are not strictly seasonal breeders. A complex social structure, as well as additional social and environmental factors, might add more to the complexity. Here, we applied a non-invasive method to monitor fecal androgen metabolite (fAM) levels in free-ranging giraffe bulls over a period of months to examine longitudinal patterns of androgen metabolite concentrations in relation to observed male sexual behavior in different age classes. Giraffes are non-seasonal breeders, living in a fission-fusion social system and males show a roaming strategy to search for fertile females. Our results show that season has an impact on fAM levels in free-ranging giraffes, with respective steroid concentrations being higher in summer. In the presence of females, fAM levels of bulls are significantly higher compared to when found in all-male groups, with old adult bulls showing the highest fAM levels. In contrast, young adult bulls have overall slightly higher fAM levels compared to old adult bulls when residing in all male groups. Sexual behavior increases fAM levels only in old adult bulls.

**Keywords:**

non-invasive hormone monitoring, feces, androgens, giraffe bulls, sexual activity, South Africa

Abbreviations: fecal androgen metabolites (fAM)

## 1. Introduction:

In many mammal species males undergo morphological changes during their life-history stages and when reaching puberty, the development of secondary sexual characteristics and spermatogenesis start, both of which are predominantly mediated by alterations in androgen concentrations (Saltzman et al., 2011). Further, the display of male sexual behavior is also influenced by the endocrine milieu and in most mammals spermatogenesis and male sexual activity are synchronized (Miles et al., 2007; Park and Rissman, 2011). In some species reproduction is temporarily restricted to a certain time period, often once or twice a year, to ensure that birth takes place at the most favorable time of the year, mainly when resources are abundant to support lactation and post-weaning growth of the offspring (Revel et al., 2007).

The 'challenge hypothesis' predicts that especially seasonal breeding males need to balance their androgen levels between times of increased male-male competition for mating opportunities with associated elevated concentrations of androgens, and times of reduced or non-breeding occurrence, with comparatively low androgen concentrations (Wingfield et al., 1990). Elevated androgen levels are thus limited to a short period of time, as prolonged elevations in sex steroid levels can have compromising effects, including an increased mortality risk due to an overall increase in male-male aggression, which leads to a higher risk of injury (Wingfield et al., 1990). Furthermore, high androgen levels may also lead to suppressed immune function, resulting for example in a potentially higher parasite load, which in turn can have an impact on overall well-being and lifetime reproductive success ('immunocompetence handicap hypothesis' Folstad and Karter, 1992; Schuurs and Verheul, 1990).

Such alterations in androgen levels have even been described in non-seasonally breeding species such as chimpanzee (*Pan troglodytes schweinfurthii*), with males showing elevated androgen levels when parous females are present and therefore male-male aggression increases (Muehlenbein et al., 2004; Muller and Wrangham, 2004). Furthermore, a temporal increase in aggression related to male

reproductive activity appears to be androgen mediated in African (*Loxodonta africana*) and Asian elephants (*Elephas maximus*) (Ganswindt et al., 2005; Ghosal et al., 2013; Rasmussen et al., 2008).

However, elevations in androgen levels are of course not always primarily linked to male aggression in a reproductive context (Rose et al., 1975). In chacma baboons (*Papio hamadryas ursinus*), males show an increase in androgen levels during important life stages, for example when competing to acquire a higher dominance rank, rather than in a mating situation (Beehner et al., 2006).

However, in species with a rather low male-male aggression rate, diverse results in conjunction with the 'challenge hypothesis' have been described, and in a variety of species androgen levels appear to be elevated due to sexual behavior *per se* rather than associated male-male competition. In tufted capuchins (*Cebus apella nigrinus*) e.g. elevated androgen levels have been linked to female consortship rather than to intra-sexual aggressive behavior (Lynch et al., 2002). Despite a breeding season, characterized by synchronized female sexual activity, tufted capuchin monkeys seem to maintain comparable levels of androgens all year round, with the exception of a defined peak during the conception period. Thus, the expression of male sexual behavior and an increase in androgen levels seems socially mediated by higher female sexual activity and not due to increased male-male aggression (Lynch et al., 2002). A similar relationship between androgen levels and sexual activity has been found in the spotted hyena (*Crocuta crocuta*), where males show a low rate of reproductive aggression towards each other and changes in male androgen levels appears to be closely related with the presence of cycling females (Dloniak et al., 2006).

The investigation of the relationship between androgens and male reproductive behavior is a growing field, but the potential role of contributing social and environmental factors are still less understood. This is especially true in species that are not strictly seasonal breeders and live in a complex social structure, respective patterns are difficult to extract, with the influence of extrinsic factors adding to the complexity. One reason why our current knowledge is still limited in this regard might be the result of logistical challenges of longitudinal monitoring of androgen levels in free-ranging populations. In many studies concerning wild animals, it is not possible to collect blood

samples on a regular basis, which has resulted in the development of non-invasive methods to collect samples without disturbing the animals under investigation. In this regard, fecal sampling as a non-invasive alternative for hormone monitoring has proven to be most suitable for determining the endocrine milieu in a variety of species (Schwarzenberger, 2007). Feces can be collected easily, animals are usually not disturbed during sample collection, thus sampling is safe and feedback-free due to the absence of capture and handling (Möstl and Palme, 2002). Furthermore blood hormone levels are usually affected by circadian or pulsatile hormone release, whereas fecal steroid concentrations give an overview over the hormone secretion during a longer timeframe and therefore reduces the influence of short-term fluctuations (Möstl and Palme, 2002).

In our study, we frequently collected fecal samples to examine longitudinal patterns of androgen metabolite concentrations for free-ranging male giraffes in relation to observed male sexual behavior for different age classes. Giraffes occur in a highly flexible fission-fusion system which is characterized by the splitting and reunion of subgroups within a population on a daily basis (Bercovitch and Berry, 2013; Dagg, 2014; Kummer, 1971). Male giraffes assume a more solitary lifestyle once they reach adulthood at an age of about 5 years, and adopt a roaming strategy to search for fertile females (e.g. Dagg, 1971; Pratt and Anderson, 1982). When males encounter cows they start to stimulate the anogenital region of the female to stimulate urination. After the female has urinated, the male tests the urine by taking it into his mouth and displays so called “flehmen” behaviour (Bercovitch et al., 2006).

Giraffes do not have a strict mating season, but birth peaks within the rainy season have been reported from different locations (e.g. Bercovitch and Berry, 2010; Dagg, 1971; Pratt and Anderson, 1982). It has also been shown that androgen levels in male giraffes are not only age dependent, but also show a seasonal pattern and appear to be linked to sexual activity (Seeber et al., 2013). This seasonal pattern in androgen levels seem to also be influenced by differences in group structure occurrence found between winter and summer months (Wolf et al., resubmitted).

## **2. Material and methods:**

### *2.1 Study site and animals*

Data collection took place in an approximately 70km<sup>2</sup> fenced Game Reserve in Kwazulu-Natal, South Africa. The reserve mainly consists of acacia & marula woodland and mixed acacia woodland, with an average annual rainfall of 400-600 mm, occurring mostly in summer (between September and March) (Shannon et al., 2006). A giraffe population of 80 individuals (27 males, 41 females, and 12 infants of unknown sex) was observed over a period of 12 months (November 2014 – October 2015). Giraffes were individually identified by their unique pelage pattern, and assigned to respective age classes following Langman (1977). Infants are defined as individuals younger than 3 months and still accompanied by their mothers, juveniles younger than 1.5 years, sub-adults up to about 4 years of age, and adult individuals over the age of 4 years. From observations in captive populations male giraffes are assumed to become sexually mature at an age of about three years (Hall-Martin et al., 1978; Reuther, 1961).

Initially, we classified adult bulls into three age classes A, B, and C (Pratt and Anderson, 1982). Class A bulls were the oldest and tallest, with massive muscular on the base of the neck, big ossicones, and well developed bone structure on the forehead as described by Pratt and Anderson (1982). Class B bulls were younger, had less stout neck muscles, and developed bone structure on the forehead, whereas class C show only slight muscular on the neck and no or little additional bone mass on the skull. However, while in our study it was not possible to clearly distinguish between B and C bulls, we subsequently assigned only two age classes, old bulls (Ad1, n = 5) – formerly Class A) and younger bulls (Ad2, n = 6– formerly class B and C). Subadult and juvenile individuals were categorized as young (n = 8).

### *2.2 Social and Behavioral data*

Giraffe groups were categorized either as 'all-male groups' (comprising only bulls) or 'mixed sex groups' (comprising all groups in which at least one female was present). All-male groups were

encountered 75x in winter and 30x in summer, whereas mixed sex groups were observed 147x in winter and 203x in summer (Wolf et al., resubmitted).

A male was considered to be sexually active if it emitted any of the following behaviors towards a female: courtship behavior, investigation of the females anogenital region, urine testing, mating and mate guarding (Seeber et al., 2013). Conversely, a bull was considered to be sexually inactive if he did not show any of these behaviors.

Intra-sexual competition was determined for bulls only and assessed by monitoring dominance and aggressive behavior. Dominance behavior was considered to be present when the following behaviors were observed: yielding from another bull, displacement, dominance gestures and sparring behavior (Seeber et al., 2012). Aggressive behavior was considered for being present if fighting with physical contact took place between two individual bulls. Dominance as well as aggressive behavior were considered to be present when observed at least 3 times per day. Subsequently, intra-sexual competition was defined as present on a day if dominance or aggressive behavior was noted present.

### *2.3 Fecal sample collection, extraction, and assay*

A total of 585 fecal samples (n = 260 in summer (Ad1 = 90, Ad2 = 100, young = 70), n = 325 in winter (Ad1 = 71, Ad2 = 150, young = 104)) were collected and analyzed for fecal androgen metabolite (fAM) concentrations. Numbers of collected samples for age-classes in respective social settings are given in Wolf et al. (resubmitted). Fecal material was collected within a maximum of 20 minutes post-defecation, to minimize bacterial degradation of the fAM in the sample (Heistermann, 2010; Möstl and Palme, 2002). Upon collection, samples were taken from different places of the dropping, placed in a polypropylene tube, stored on ice immediately, and frozen within six hours. In the lab, feces were freeze-dried, pulverized, and sieved through a thin metal strainer to remove fibrous material (Fieß et al., 1999). Between 0.10 - 0.11 g of fecal powder was extracted by vortexing for 15 min. with 80% ethanol in water (3 ml). After centrifugation for 10 min. at 1500 g, supernatants were aliquoted and stored at -20°C until assay procedure (Fieß et al., 1999; Seeber et al., 2013).

Fecal androgen metabolite (fAM) concentrations were determined using an enzyme immunoassay first described by Palme and Möstl (1993). This assay has previously been used to reliably monitor fAM alterations in giraffe (Seeber et al. 2013) and other mammals (Ganswindt et al., 2002). The assay procedure followed established protocols (Ganswindt et al., 2002). Sensitivity of the assay at 90% binding was 12 ng/g feces (dry weight). Inter-assay coefficients of variation, determined by repeated measurement of high- and low-value quality controls, ranged between 14.8% and 16.2%.

#### *2.4 Statistical analyses*

To investigate the effects of age-classes and the effect of absence and presence of females on fecal androgen metabolite (fAM) levels in male giraffes, we ran a general linear mixed model (GLMM, Baayen et al., 2008) with fAM levels as response variable using the function lmer of the R-package lme4 (Bates et al., 2013). To meet the assumption of normal distribution, fAM values were log-transformed.

We included age class (Ad1, Ad2 and Young, i.e., juvenile and sub-adult combined), sexual activity, intra-sexual competition and absence and presence of females as test predictors with fixed effects (Mundry, 2014). To test for differences in sexual activity across different age classes we included the interaction between age class and sexual activity. To test for differences of intra-sexual competition in the presence of females across the different age classes, we included a three-way interaction between the respective main effects. To control for potential seasonal effects, we included season (as fixed effect) by including Julian date as a circular variable. Therefore, day of sampling was turned into radians by dividing it by the average of a year (365.25 days) and then multiplied by  $2*\pi$  (Stolwijk et al., 1999). The resulting sine and cosine were included in the model under the assumption of one seasonal peak per annum. Individual ID was included as random effect. We included random slopes of all fixed effects, except age class, and for the interaction between intra-sexual competition and age class, to keep type I error rate at the nominal level of 5% (Barr et al., 2013; Schielzeth and Forstmeier, 2009).



Model stability was established by excluding random effect levels one at a time and by comparing the estimates of the fixed effects with the ones obtained based on the complete data set. Variance Inflation Factors (VIF; Field, 2009) were determined to check for collinearity, which revealed collinearity to not be an issue (maximum VIF: 1.107). We used a likelihood ratio test to determine the significance of the full model as compared to the null model (Dobson, 2002; Forstmeier and Schielzeth, 2011) and to determine the significance of individual effects (Barr et al., 2013). For easier interpretation of the main effects, interactions that were not significant were excluded from the final model (Schielzeth, 2010). To further investigate the effect of season on the fAM levels, we compared the final model including season with the final model lacking season using an ANOVA. Post hoc tests to further investigate differences between absence and presence of females, and age class on fAM levels were conducted using the function `ghlt` of the package `multcomp`. To compare differences in individual median fAM levels of sexually active and inactive bulls, a paired t-test was used. All statistical analyses were done using R, version 3.0.2 (R Core Team, 2013).

### **3. Results**

The overall median fAM levels for Ad1 and Ad2 bulls were 7.62  $\mu\text{g/g}$  dry weight (DW) and 7.59  $\mu\text{g/g}$  DW, respectively. The median fAM levels found in the young age class was 4.01  $\mu\text{g/g}$  DW. In the model we examined the effect of age classes and the effect of absence and presence of females in a group on fAM levels in males. Here the full – null model comparison was highly significant ( $\chi^2 = 77.78827$ ,  $df = 14$ ,  $P < 0.001$ ). However, neither the three-way interaction, nor the two-way interaction revealed significance. Therefore, all non-significant two-way interactions were removed from the model, and a reduced model was performed, to enable better interpretation of the main effects. In the final model, we found a tendency towards bulls that are sexually active to have higher fAM levels compared to males which were not sexually active ( $\chi^2 = 3.522$ ,  $df = 1$ ,  $P = 0.061$ ; table 1). Further, the interaction between age class and group composition (i.e., all-male vs. mixed sex groups)

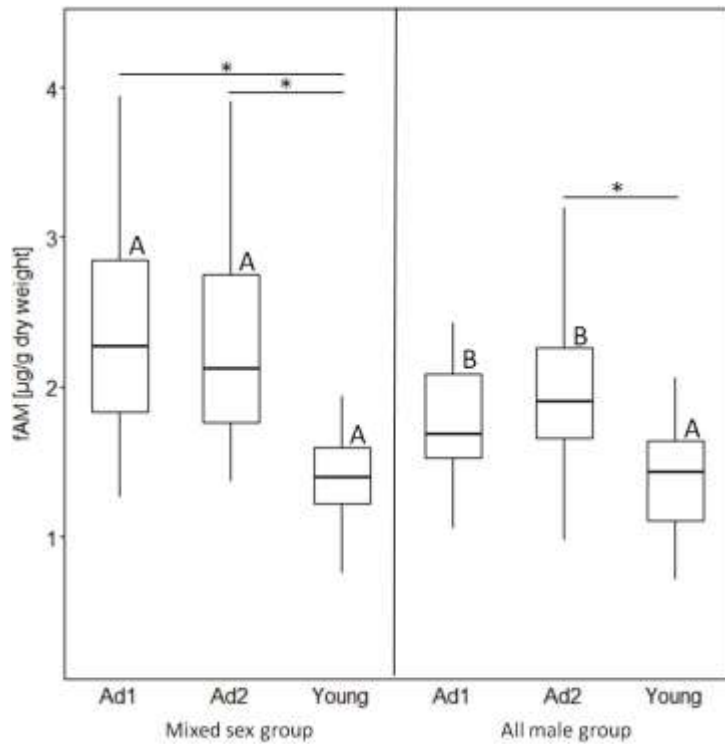
was significant ( $\chi^2= 11.707$ ,  $df= 2$ ,  $P=0.003$ ; table 1), displaying that bulls of the three age classes showed different changes in fAM levels depending on the absence or presence of females.

**Table 1:** Statistical outcome of the final model of the impact of behavioral and social factors on fecal androgen metabolites. Significant effects are indicated in bold.

Term	Estimate	SE	DF	$\chi^2$	P-value
Intercept	2.357	0.066			
<i>Test predictors</i>					
<b>Sexual activity</b>	<b>0.206</b>	<b>0.102</b>	<b>1</b>	<b>3.522</b>	<b>0.061</b>
Intra-sexual competition	-0.052	0.051	1	1.040	0.308
<b>Age class * group composition</b>	<b>0.603</b>	<b>0.158</b>	<b>2</b>	<b>11.707</b>	<b>0.003</b>
<i>Control predictors</i>					
Season sinus <sup>(1)</sup>	-0.065	0.043	2	9.957	0.007
Season cosines <sup>(1)</sup>	0.108	0.037			

\* indicating interaction terms; <sup>(1)</sup> DF and p values indicate the comparison of the full model with the reduced model without season

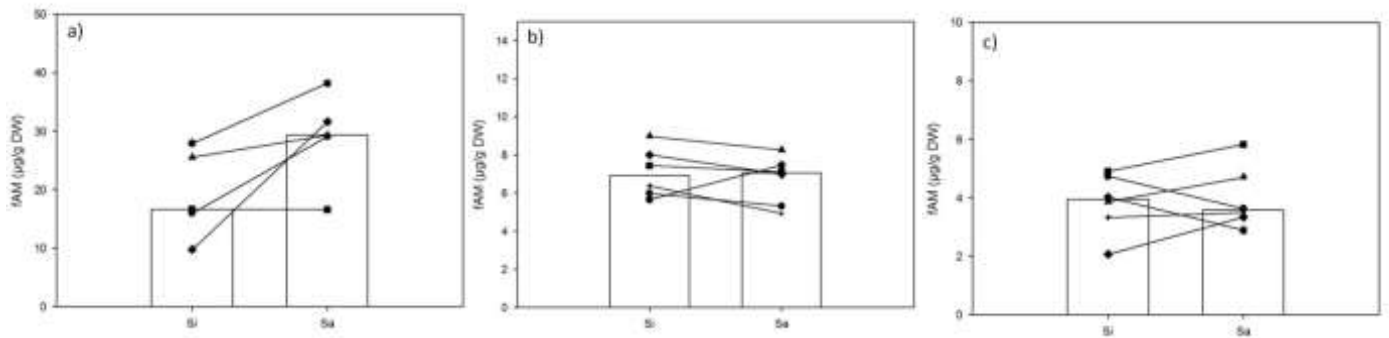
Post hoc analyses revealed that adult bulls (Ad1 and Ad2) had higher fAM levels when females were present (fig.1). The average increase for Ad1 males from being in an all-male compared to mixed sex group was 34%, and only 11% for Ad2 bulls. In young bulls fAM levels did not change in the presence of females (fig.1). Within mixed sex groups, adult bulls (Ad1 and Ad2) had significantly higher fAM levels compared to younger bulls ( $p<0.001$ ), with overall fAM levels being 72% (Ad1) and 67% (Ad2) higher compared to the youngsters (fig 1). Within all-male groups, this difference was only significant when comparing fAM levels of young bulls with those of Ad2 bulls ( $p<0.001$ ; fig.1). In all-male groups, overall fAM levels were 28% higher in Ad1 bulls and 42% higher in Ad2 bulls compared to steroid levels of the young bull group.



**Figure 1:**

metabolite (fAM) levels for the three age classes of bulls in mixed sex, or all male groups. Statistically significant differences between the age classes are indicated by asterisks. Different letters indicate statistically significant differences within the same age class when in different group settings.

The model showed a trend for sexual activity having an impact on fAM levels (table 1). We further investigated this by comparing individual median fAM levels of bulls being either sexually active or inactive. Our results showed a trend for sexually active Ad1 bulls to have higher fAM levels ( $t = -2.576$ ,  $df = 4$ ,  $p=0.06$ ) with an overall increase of 76% (see fig. 2). In contrast, no significant differences in individual fAM levels were found in bulls from the other two age classes when comparing different sexual stages (Ad2:  $t = 0.844$ ,  $df = 5$ ,  $p=0.43$ ; young bulls:  $t = -0.375$ ,  $df = 5$ ,  $p=0.72$ ).



**Figure 2:** Fecal androgen metabolite (fAM) levels of Ad1 (a), Ad2 (b), and young (c) bulls in mixed sex groups, when either sexually inactive (Si) or sexually active (Sa)

Investigating only the effect of season on fAM levels we found a significance ( $\chi^2 = 9.96$ ,  $df = 2$ ,  $P < 0.007$ ), indicating that season has an impact on fAM levels in giraffes, with higher concentrations during the summer month. In summer, overall individual median fAM levels were  $9,67 \mu\text{g/g DW}$  for Ad1,  $9,87 \mu\text{g/g DW}$  for Ad2 and  $3,59 \mu\text{g/g DW}$  for young bulls. Whereas, overall individual median fAM levels were  $6,24 \mu\text{g/g DW}$  for Ad1,  $7,15 \mu\text{g/g DW}$  for Ad2 and  $3,87 \mu\text{g/g DW}$  for young bulls in winter.

Older bulls were observed more often being engaged in intra-sexual competition (Ad1 54% and Ad2 45%, respectively) compared to subadult/juvenile (37%) males. However, the overall number of aggressive interactions between bulls was rather low. Within the 12 month period only three fights were observed, but none ended in any form of visible injury to either of the bulls. Thus, intra-sexual competition in the study population was mainly determined by dominance gestures, displacement by an older bull, and sparring bouts between younger individuals.

#### 4. Discussion

Our results show that alterations of fAM levels in giraffe bulls are seasonally dependent, with overall lower levels arising during the winter months. Such a pattern is in line with previous behavioral

findings, showing that group structure in giraffes changes with season, the bulls tending to associate more in all-male groups during the winter months (Wolf et al. submitted). A similar seasonal pattern for androgen alterations has been found in a different southern African giraffe population (Seeber, 2012). Although these two studies took place during different years and at different sites, a comparable seasonal change in fAM levels points towards a general pattern, and three possible circumstances may explain the decline in fAM levels during the winter months. Firstly, birth synchrony has been reported for giraffes although they are non-seasonal breeders (e.g. Dagg, 1971; Pratt and Anderson, 1982). For example Hall-Martin and colleagues (1975), showed that the majority of births in a giraffe population in the eastern Transvaal, South Africa occurred between December and March. Similarly, in our study population the majority of births took place between October and January (South African summer). Therefore, male giraffe fAM levels appear to be positively correlated with a higher number of fertile females being present during the summer rainy season. Such a seasonal pattern would be supported by the 'challenge hypothesis', which predicts that males show temporary elevations in androgen levels during the mating period to facilitate intersexual aggression, and consequently exhibit lower androgen levels during non-mating periods (Wingfield et al., 1990). Secondly, fAM concentrations in bulls might also be influenced by changes in food consumption. It has been shown for several species that changes in diet may affect steroid hormone metabolism and thus can influence the amount of excreted fecal material, thereby altering the estimation of hormone metabolite concentrations (reviewed in Goymann, 2012). In our study, the observed giraffes were facing a severe drought during winter, and although a similar pattern in fAM levels have been described for a different population of giraffes a few years ago, we cannot exclude the possibility of dietary influences, as no quantitative or qualitative data on food consumption were collected. Finally, all-male groups have been encountered 1.5 times more often in winter. The reduced fAM levels during winter may be a result of preferred association in all-males groups during this time of the year, when mating opportunities and subsequently intra-sexual competition for mates are reduced.

This explanation is also supported by the fact that in our study fAM levels in adult bulls were elevated when males were associated with females; which is in line with previous findings for other species. For example, higher androgen metabolite concentrations have also been reported for chimpanzees, when swollen parous females were present (Muehlenbein et al., 2004; Muller and Wrangham, 2004). However, this increase in androgen levels in chimpanzees appears to be mainly related to increased male-male aggression over dominance and not to sexual behavior *per se* (Muehlenbein et al., 2004; Muller and Wrangham, 2004). In our study, however, the similar increase in fAM levels in the presence of females, is unrelated to observed aggressive or dominance behavior. Consequently, as bulls in our study showed an overall lower rate of aggression, the increase in fAM levels may possibly be due to social stimuli rather than intra-sexual aggression. Contact with a sexually active female may trigger an increase in androgen concentrations as has been shown e.g. in some primate species, such as squirrel monkeys (Schiml et al., 1996) or rhesus macaques (Bernstein et al., 1974). In a study on sheep it has also been shown, that the presence of estrous ewes leads to an increase in androgen levels in rams even outside the breeding season (Gonzalez et al., 1988; Illius et al., 1976).

As our study animals are a fenced population, a certain degree of genetic relatedness between all individuals is assumed. This could be a contributing factor to the overall lower rate of aggression observed in study population, as well as impacting on the androgen mediated aggression determined.

That sexual behavior is in some way related to an increase in fAM concentrations in adult bulls is supported by our data showing that in mixed sex groups, the older adult giraffe bulls (Ad1) had a further increase in fAM levels when sexually active. A similar finding was presented in a recent study on giraffes, where the oldest bulls also showed significantly higher fAM levels, when sexually active (Seeber et al., 2013). For a number of species, times of sexual activity are often correlated with elevated androgen levels, as for example shown in bison (Mooring et al., 2004) or African and Asian elephant (Ganswindt et al., 2005; Ghosal et al., 2013), with already minor changes in fAM levels being related to male sexual activity in elephants (Rasmussen et al., 2008). In contrast, elephant bulls in

must show much higher androgen levels than necessary to stimulate sexual behavior and therefore appear more likely to be linked to intra-sexual competition and mate guarding (Rasmussen et al., 2008). While in the present study, adult males (Ad1 and Ad2), but not younger bulls had higher fAM levels year around, it is possible that older giraffe bulls may also maintain increased fAM levels to be prepared for potential fights over mating possibilities or for mate guarding as seen in elephants (Rasmussen et al., 2008).

Young bulls (aged between one and four years) differ from the seasonal and social group specific alteration in fAM levels seen in adult (Ad1 and Ad2) bulls. Independently of the social group structure, young bulls showed the lowest fAM levels amongst males in our study population. One explanation for this may be that the young bull group includes individuals that have not reached sexual maturity yet, or are not fully physically mature. Giraffe bulls are assumed to become sexually mature at an age of approximately three years (Hall-Martin et al., 1978), and although the majority of the individuals assigned to our young age category are presumably slightly older, age estimates for maturation in giraffe bulls are mainly based on observations in captive populations (Hall-Martin et al., 1978; Reuther, 1961), and thus it might be questionable if such young males can be actually seen as mature in a free-ranging setting (Hall-Martin et al., 1978). Interestingly, even the youngest bulls in our study population showed interest in females by investigative behavior and urine testing, but that behavior was not correlated with an increase in fAM levels. Therefore, it is possible that sexual behaviors seen in the young bulls are more likely to be a copy of the observed behaviors of adult bulls. Thus our study provides evidence from wild male giraffes that younger bulls of the age between one and four years are perhaps not fully mature and therefore, their fAM levels show differences in the presence or absence of females in comparison to mature males. Future studies with individuals of known ages with long-term data collected could be combined with behavioral observations to assess maturation onset in wild male giraffes.

In our study, adult bulls had lower fAM levels in all-male groups when compared to those joining mixed sex groups. When comparing age classes in this setting, Ad2 males had significantly higher fAM

levels compared to young bulls. Old bulls (Ad1) are thought to be the most dominant ones (Pratt and Anderson, 1985), and in some species higher rank is correlated with higher androgen levels (Muehlenbein et al., 2004). As this applies also to our study subjects when females are present, Ad1 bulls may not need to engage in further establishment of their rank when only with males as they are already settled within the hierarchy. This assumption is supported by their behaviors, as Ad1 bulls rarely engaged in sparring bouts, whereas younger adult bulls (Ad2) might still need to establish their rank. The overall lower fAM levels during the absence of females in all adult males, and especially in older males (Ad1), is possibly in line with the 'immunocompetence handicap hypothesis' (ICHH). The ICHH proposes that there is a trade-off between mating effort and immune function, based on the prediction that on one hand, higher androgen concentrations increases mating success, but on the other, it has immune suppressive effects (Folstad and Karter, 1992; Roberts et al., 2004). Therefore, Ad1 males might have increased fAM levels during times of female presence to support mating success, but fAM levels are decreased in the absence of females to maintain immune function. It could be argued that already mature but not old aged males (Ad2) have perhaps better immune function than Ad1 bulls, because the immune system shows a decline with increasing age (Miller, 1996).

In summary, our study shows that changes in fAM levels in giraffe bulls are related to changes in the structure of the social groups. In mixed sex groups fAM levels of bulls are significantly elevated than when compared to all-male groups. Furthermore, we were able to show that only fully mature males exhibit increases in fAM levels when sexually active, which is more pronounced in older aged (Ad1) bulls compared to mature, but younger males (Ad2), and that these changes seem not to be stimulated by aggressive behavior between males as it is posited by the 'challenge hypothesis'. Giraffes live in a complex social system and androgen levels in bulls are not only related to age, but also influenced by the presence and absence of females.



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### **References:**

- Baayen, R.H., Davidson, D.J., Bates, D.M., 2008. Mixed-effects modeling with crossed random effects for subjects and items. *J. Mem. Lang.* 59, 390–412.
- Barr, D.J., Levy, R., Scheepers, C., Tily, H.J., 2013. Random effects structure for confirmatory hypothesis testing: Keep it maximal. *J. Mem. Lang.* 68, 255–278.
- Bates, D., Maechler, M., Bolker, B., Walker, S., 2013. lme4: Linear mixed-effects models using Eigen and S4. R package version 1.0-5.
- Beehner, J.C., Bergman, T.J., Cheney, D.L., Seyfarth, R.M., Whitten, P.L., 2006. Testosterone predicts future dominance rank and mating activity among male chacma baboons. *Behav. Ecol. Sociobiol.* 59, 469–479.
- Bercovitch, F.B., Bashaw, M.J., del Castillo, S.M., 2006. Sociosexual behavior, male mating tactics, and the reproductive cycle of giraffe *Giraffa camelopardalis*. *Horm. Behav.* 50, 314–321.
- Bercovitch, F.B., Berry, P.S.M., 2013. Herd composition, kinship and fission–fusion social dynamics among wild giraffe. *Afr. J. Ecol.* 51, 206–216.
- Bercovitch, F.B., Berry, P.S.M., 2010. Ecological determinants of herd size in the Thornicroft’s giraffe of Zambia. *Afr. J. Ecol.* 48, 962–971.

- Bernstein, I.S., Rose, R.M., Gordon, T.P., 1974. Behavioral and environmental events influencing primate testosterone levels. *J. Hum. Evol.* 3, 517–525.
- Dagg, A.I., 2014. Giraffe: biology, behaviour, and conservation. Cambridge University Press, New York.
- Dagg, A.I., 1971. *Giraffa camelopardalis*. *Mamm. Species* 1–8.
- Dloniak, S.M., French, J.A., Holekamp, K.E., 2006. Faecal androgen concentrations in adult male spotted hyaenas, *Crocuta crocuta*, reflect interactions with socially dominant females. *Anim. Behav.* 71, 27–37.
- Dobson, A.J., 2002. An Introduction to generalized linear models. Chapman & Hall / CRC Press, Boca Raton.
- Field, A.P., 2009. Discovering statistics using SPSS:(and sex and drugs and rock'n'roll). Sage Publications, Los Angeles.
- Fieß, M., Heistermann, M., Hodges, J.K., 1999. Patterns of Urinary and Fecal Steroid Excretion during the Ovarian Cycle and Pregnancy in the African Elephant (*Loxodonta africana*). *Gen. Comp. Endocrinol.* 115, 76–89.
- Folstad, I., Karter, A.J., 1992. Parasites, bright males, and the immunocompetence handicap. *Am. Nat.* 139, 603–622.
- Forstmeier, W., Schielzeth, H., 2011. Cryptic multiple hypotheses testing in linear models: overestimated effect sizes and the winner's curse. *Behav. Ecol. Sociobiol.* 65, 47–55.
- Ganswindt, A., Heistermann, M., Borrigan, S., Hodges, J.K., 2002. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. *Zoo Biol.* 21, 27–36.
- Ganswindt, A., Rasmussen, H.B., Heistermann, M., Hodges, J.K., 2005. The sexually active states of free-ranging male African elephants (*Loxodonta africana*): defining musth and non-musth using endocrinology, physical signals, and behavior. *Horm. Behav.* 47, 83–91.

- Ghosal, R., Ganswindt, A., Seshagiri, P.B., Sukumar, R., 2013. Endocrine Correlates of Musth in Free-Ranging Asian Elephants (*Elephas maximus*) Determined by Non-Invasive Faecal Steroid Hormone Metabolite Measurements. PLoS ONE 8, e84787.
- Gonzalez, R., Orgeur, P., Signoret, J.P., 1988. Luteinizing Hormone , Testosterone and Cortisol responses in rams upon presentation of estrous females in the nonbreeding season. Theriogenology 30, 1075–1086.
- Goymann, W., 2012. On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual. Methods Ecol. Evol. 3, 757–765.
- Hall-Martin, A.J., Skinner, J.D., Dyk, J.M.V., 1975. Reproduction in the giraffe in relation to some environmental factors. Afr. J. Ecol. 13, 237–248.
- Hall-Martin, A.J., Skinner, J.D., Hopkins, B.J., 1978. The development of the reproductive organs of the male giraffe, *Giraffa camelopardalis*. J. Reprod. Fertil. 52, 1–7.
- Heistermann, M., 2010. Non-invasive monitoring of endocrine status in laboratory primates: methods, guidelines and applications. Adv. Sci. Res. 5, 1–9. doi:10.5194/asr-5-1-2010
- Illius, A.W., Haynes, N.B., Lamming, G.E., 1976. Effects of ewe proximity on peripheral plasma testosterone levels and behaviour in the ram. J. Reprod. Fertil. 48, 25–32.
- Kummer, H., 1971. Primate societies: group techniques of ecological adaptation. Harlan Davidson, Wheeling (Illinois).
- Langman, V.A., 1977. Cow-calf Relationships in Giraffe (*Giraffa camelopardalis giraffa*). Z. Für Tierpsychol. 43, 264–286.
- Lynch, J.W., Ziegler, T.E., Strier, K.B., 2002. Individual and Seasonal Variation in Fecal Testosterone and Cortisol Levels of Wild Male Tufted Capuchin Monkeys, *Cebus apella nigrinus*. Horm. Behav. 41, 275–287.

- Miles, D.B., Sinervo, B., Hazard, L.C., Svensson, E.I., Costa, D., 2007. Relating endocrinology, physiology and behaviour using species with alternative mating strategies. *Funct. Ecol.* 21, 653–665.
- Miller, R.A., 1996. The aging Immune System: Primer and Prospectus. *Science* 273, 70–74.
- Mooring, M.S., Patton, M.L., Lance, V.A., Hall, B.M., Schaad, E.W., Fortin, S.S., Jella, J.E., McPeak, K.M., 2004. Fecal androgens of bison bulls during the rut. *Horm. Behav.* 46, 392–398.
- Möstl, E., Palme, R., 2002. Hormones as indicators of stress. *Domest. Anim. Endocrinol.* 23, 67–74.
- Muehlenbein, M.P., Watts, D.P., Whitten, P.L., 2004. Dominance rank and fecal testosterone levels in adult male Chimpanzees (*Pan troglodytes schweinfurthii*) at Ngogo, Kibale National Park, Uganda. *Am. J. Primatol.* 64, 71–82.
- Muller, M.N., Wrangham, R.W., 2004. Dominance, aggression and testosterone in wild chimpanzees: a test of the “challenge hypothesis.” *Anim. Behav.* 67, 113–123.
- Mundry, R., 2014. Statistical Issues and Assumptions of Phylogenetic Generalized Least Squares, in: Garamszegi, L.Z. (Ed), *Modern Phylogenetic Comparative Methods and Their Application in Evolutionary Biology*. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 131–156.
- Palme, R., Möstl, E., 1993. Biotin-Streptavidin Enzyme Immunoassay for the Determination of Oestrogens and Androgens in Boar Faeces, in: *Proceedings of the 5th Symposium on the Analysis of Steroids*. Szombathely, Hungary, pp. 111–117.
- Park, J.H., Rissman, E.F., 2011. Behavioral Neuroendocrinology of Reproduction in Mammals, in: Norris, D.O., Lopez, K.H. (eds), *Hormones and Reproduction of Vertebrates*. Academic Press, Saint Louis, pp. 139–173.
- Pratt, D.M., Anderson, V.H., 1985. Giraffe social behaviour. *J. Nat. Hist.* 19, 771–781.
- Pratt, D.M., Anderson, V.H., 1982. Population, distribution, and behaviour of giraffe in the Arusha National Park, Tanzania. *J. Nat. Hist.* 16, 481–489.
- R Core Team, 2013. *R: A language and environment for statistical computing*. Vienna, Austria.

- Rasmussen, H., Ganswindt, A., Douglas-Hamilton, I., Vollrath, F., 2008. Endocrine and behavioral changes in male African elephants: Linking hormone changes to sexual state and reproductive tactics. *Horm. Behav.* 54, 539–548.
- Reuther, R.T., 1961. Breeding Notes on Mammals in Captivity. *J. Mammal.* 42, 427.
- Revel, F.G., Ansel, L., Klosen, P., Saboureau, M., Pévet, P., Mikkelsen, J.D., Simonneaux, V., 2007. Kisspeptin: A key link to seasonal breeding. *Rev. Endocr. Metab. Disord.* 8, 57–65.
- Roberts, M.L., Buchanan, K.L., Evans, M.R., 2004. Testing the immunocompetence handicap hypothesis: a review of the evidence. *Anim. Behav.* 68, 227–239.
- Rose, R.M., Berstein, I.S., Gordon, T.P., 1975. Consequences of social conflict on plasma testosterone levels in rhesus monkeys. *Psychosom. Med.* 37, 50–61.
- Saltzman, W., Tardif, S.D., Rutherford, J.N., 2011. Hormones and Reproductive Cycles in Primates, in: Norris, D.O., Lopez, K.H. (eds), *Hormones and Reproduction of Vertebrates*. Academic Press, Saint Louis, pp. 291–327.
- Schielzeth, H., 2010. Simple means to improve the interpretability of regression coefficients: Interpretation of regression coefficients. *Methods Ecol. Evol.* 1, 103–113.
- Schielzeth, H., Forstmeier, W., 2009. Conclusions beyond support: overconfident estimates in mixed models. *Behav. Ecol.* 20, 416–420.
- Schimpl, P.A., Mendoza, S.P., Saltzman, W., Lyons, D.M., Mason, W.A., 1996. Seasonality in squirrel monkeys (*Saimiri sciureus*): Social facilitation by females. *Physiol. Behav.* 60, 1105–1113.
- Schuurs, A., Verheul, H.A.M., 1990. Effects of gender and sex steroids on the immune response. *J. Steroid Biochem.* 35, 157–172.
- Schwarzenberger, F., 2007. The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species. *Int. Zoo Yearb.* 41, 52–74.
- Seeber, P.A., 2012. Association between reproductive behaviour and androgen metabolite excretion in free-ranging male giraffes (*Giraffa camelopardalis*) (Master of Science). University of Pretoria, Pretoria.

- Seeber, P.A., Ciofolo, I., Ganswindt, A., 2012. Behavioural inventory of the giraffe (*Giraffa camelopardalis*). BMC Res. Notes 5, 650.
- Seeber, P.A., Duncan, P., Fritz, H., Ganswindt, A., 2013. Androgen changes and flexible rutting behaviour in male giraffes. Biol. Lett. 9, 20130396.
- Shannon, G., Page, B., Slotow, R., Duffy, K., 2006. African elephant home range and habitat selection in Pongola Game Reserve, South Africa. Afr. Zool. 41, 37–44.
- Stolwijk, A.M., Straatman, H., Zielhuis, G.A., 1999. Studying seasonality by using sine and cosine functions in regression analysis. J. Epidemiol. Community Health 53, 235–238.
- Wingfield, J.C., Hegner, R.E., Dufty, A.M., Ball, G.F., 1990. The “Challenge Hypothesis”: Theoretical Implications for Patterns of Testosterone Secretion, Mating Systems, and Breeding Strategies. Am. Nat. 136, 829–846.
- Wolf, T.E, Bennett, N.C., Burroughs, R., Ganswindt, A. resubmitted. The impact of age-class and social context on fecal glucocorticoid metabolite levels in free-ranging male giraffes.
- Wolf, T.E., Ngonga Ngomo, A.C., Bennett, N.C., Burroughs, R., Ganswindt, A. resubmitted. Seasonal changes in social networks of giraffe