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Non-invasive assessment of glucocorticoid and androgen metabolite levels in cooperatively breeding Damaraland mole-rats (*Fukomys damarensis*)

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Highlights

- Non-invasive monitoring of adrenocortical activity in Damaraland mole-rats.
- Faecal androgen metabolite concentrations in male and female Damaraland mole-rats.
- Glucocorticoids/Androgens vary little among colony members during the dry season.
- Captive males and females have higher androgen concentrations than wild individuals.

Abstract

Dominant females of cooperative breeding species often use aggression to suppress reproduction of subordinate females, resulting in subordinates experiencing stress-related increases in glucocorticoid levels, which may cause reproductive down-regulation. This would suggest a general pattern with higher glucocorticoid levels in subordinate compared to dominant individuals; however, the opposite was found in a number of cooperatively breeding species. Furthermore, breeding females of the cooperatively breeding Damaraland mole-rats (*Fukomys damarensis*) exhibit very high androgen concentrations during the wet season, presumably to support their breeding monopoly. Hormone analysis in Damaraland mole-rats have typically been measured using plasma and urine, but faecal analysis offers additional advantages especially for field studies on this species. The present study examines the suitability of Damaraland mole-rat faecal samples for determining glucocorticoid metabolite (fGCM) and androgen metabolite (fAM) concentrations using enzyme immunoassays. Using these assays, we further evaluated the effects of breeding status on fGCM and fAM concentrations in wild-caught and captive Damaraland mole-rats. Wild-caught breeding and non-breeding males and females exhibited no differences in fAM concentrations. Immunoreactive fGCM concentrations were only high in male breeders and comparatively low in non-breeders and breeding females. Concentrations of fAMs and fGCMs were similar in captive males and females, but fAM concentrations were elevated in captive compared to wild-caught individuals, which may be related to a higher reproductive activity due to removal from the breeding female. The relatively uniform fAM and fGCM concentrations found in wild-caught mole-rats may be explained by a stable colony structure during the dry season during which this study was conducted. Limited dispersal opportunities result in lower aggression and stress levels within a colony and as a result lower fAM and fGCM concentrations.

Keywords: Fecal glucocorticoid metabolites, fecal androgen metabolites, reproduction, ACTH challenge, non-breeder, breeder

1. Introduction

Physical (e.g. malnutrition) and psychosocial factors (e.g. intraspecific aggression) that threaten homeostasis of an organism are some of the causative agents of stress and are typically referred to as stressors (Wielebnowski, 2002). The physiological response of an organism to a stressor usually involves the activation of the hypothalamic-pituitary-adrenal axis (HPA-axis) resulting in the release of glucocorticoids (either cortisol or corticosterone or both; Li and O'Byrne, 2014). Although short-term effects of such perceived stressors are adaptive and important for the survival of an individual, high or prolonged elevations of glucocorticoid levels can negatively affect the immune function, spatial learning and memory, as well as reproductive axis (Conrad, 2010; Dobson and Smith, 2000; Martin et al., 2012). For example, elevated levels of glucocorticoids have been found to decrease androgen concentrations in guinea pigs (Fenske, 1997), olive baboons (*Papio anubis*; Sapolsky 1985), mice (Dong et al., 2004), and red squirrels (*Tamiasciurus hudsonicus*; Boonstra and McColl 2000). Further, they may be responsible for low androgen concentrations found in captive cheetahs (*Acinonyx jubatus*; Terio et al. 2004).

In social species, aggression by dominant individuals may result in decreased or no reproductive activity in subordinates, possibly mediated by high levels of glucocorticoids (Hackländer et al., 2003; Hardy et al., 2002). In some cooperatively breeding species, in which subordinates provide care to the offspring of the dominant individuals (Jennions and Macdonald, 1996), the dominant female may use aggression or even eviction from the group as a measure to suppress reproduction in subordinate females (Faulkes and Abbott, 1997; Young et al., 2006). Evicted subordinate meerkats (*Suricata suricatta)*, for example, experience elevated glucocorticoid concentrations, which cause reproductive down-regulation, reduced conception, and increased abortion rates (Young et al., 2006). In meerkats, high glucocorticoid levels are also related to increased pup-feeding rates and extra-territorial movements of male subordinates (Carlson et al., 2006; Young and Monfort, 2009). However, in cooperatively breeding species, dominant individuals often exhibit the highest glucocorticoid levels (Carlson et al., 2004; Creel, 2005; Creel et al., 1997; Saltzman et al., 1998), which may be the result of higher social stress due to more agonistic encounters dominants engage in (Creel et al., 2013). Higher concentrations of both glucocorticoids and androgens probably facilitate the elevated rates of aggression towards subordinates during times when subordinates may challenge the

breeding monopoly of the dominant individuals (Creel et al., 1997; Hunt et al., 2006; Lutermann et al., 2013; van Kesteren et al., 2012; Young et al., 2006).

Most studies on cooperative breeders have used blood plasma or urine to determine androgen and glucocorticoid concentrations, but these samples are often difficult to obtain from wild animals and are not feedback free (Sheriff et al., 2011). Animals need to be restrained or captured first for sample collection and the related stress response appears in plasma samples within minutes (Reeder and Kramer, 2005; Romero et al., 2008). The measurement of hormone metabolites in faeces alleviates many of these problems, since faeces can often be collected without capturing the animals. Furthermore, circulating hormone levels are integrated over longer periods of time in related faecal matter, reducing the problem of measuring pulsatile hormone secretion and episodic fluctuations (Möstl and Palme, 2002; Palme et al., 2005, 1996; Sheriff et al., 2011). Faecal sampling also allows repeated monitoring of hormones over short intervals, which is especially advantageous in smaller mammals, in which repeated blood or urine sampling is impossible (de Bruin et al., 2014). However, metabolism of hormones by the liver and later by bacteria in the intestines is highly species dependent, resulting in the excretion of faecal metabolites specific for a species (Cook, 2012; Touma and Palme, 2005). This necessitates careful validation of hormone assays for every species to ensure that the measured hormone metabolite concentrations actually reflect adrenal and gonadal activity (Cook, 2012; Touma and Palme, 2005). Further, steroid metabolism can differ between the sexes within a species and stress responses in relation to species-specific activity patterns (Touma et al., 2004, 2003) and thus, knowledge of these variances is important for a correct interpretation of endocrine correlates.

African mole-rats (Bathyergidae) exhibit a wide variety of social systems with some species, such as Damaraland mole-rats (*Fukomys damarensis*), exhibiting cooperative breeding and eusociality (Faulkes et al., 1997). Only one female, often referred to as the queen, and one or two males breed in these mole-rat societies (Bennett and Jarvis, 1988). The remaining members of the colony comprise non-breeding individuals of both sexes, which help with colony and tunnel maintenance, foraging and raising of the young (Jarvis and Bennett, 1993). In Damaraland mole-rats, reproduction in non-breeding females is suppressed by the dual actions of incest avoidance and a physiological suppression through the breeding female (Bennett et al., 1996; Burland et al., 2004). Female subordinates show suppression

of ovulation (Bennett, 1994) as well as reductions in pituitary sensitivity and gonadotrophin releasing hormone (GnRH) secretion (Molteno et al., 2004; Molteno and Bennett, 2000), and decreased oestrogen receptor α and androgen receptor expression (Voigt et al., 2014), indicating suppression of reproduction at different levels of the reproductive and hypothalamo-pituitary-gonadal (HPG) axis. However, reproductive suppression is weakened during the wet seasons when non-breeding females are presented with dispersal opportunities (Molteno and Bennett, 2002; Young et al., 2010). Lutermann et al. (2013) observed that breeding females exhibit higher plasma androgen levels during the wet season possibly indicating higher aggression. Increased aggression may support the breeding monopoly of the breeding female during times when the non-breeding females' physiological suppression is weakened (Lutermann et al., 2013; Molteno and Bennett, 2002; Young et al., 2010). This is further supported by higher urinary glucocorticoid concentrations measured in non-breeding individuals during the wet season (Young et al., 2010). In contrast to females, non-breeding male Damaraland mole-rats show no reduction of the reproductive axis, are capable of spermatogenesis and have similar androgen levels to breeding individuals, suggesting that reproductive suppression is purely behavioural in males (Bennett, 1994). Overall, however, limited data on glucocorticoid or androgen concentration measurements exist for male Damaraland mole-rats (i.e. Bennett, 1994). Thus further studies are needed to determine the effect of dominance status and associated effects on the HPA and reproductive axes of Damaraland mole-rats. In addition, field studies on Damaraland mole-rats require methods for the measurement of hormones, which are largely feedback free.

The objectives of the present study were to 1) examine the suitability of immunoassays for detecting alterations in urinary and faecal glucocorticoid metabolite concentrations in Damaraland mole-rats by performing an adrenocorticotropic hormone stimulation test (ACTH challenge test), 2) examining the suitability of an enzyme-immunoassay (EIA) for detecting alterations in faecal androgen metabolite (fAM) concentrations in Damaraland mole-rats by demonstrating differences in fAM concentrations in relation to different maturation stages and examining potential co-measurement of faecal glucocorticoid metabolites (fGCM), and 3) evaluating the effects of breeding status on fGCM and fAM concentrations in wild-caught and captive Damaraland mole-rats.

We predicted that both fGCM and fAM concentrations would be lower in younger mole-rats than in adults as the reproductive system of young individuals is still developing and as such dominant females are also less likely to harass young individuals to maintain reproductive suppression (see also meerkats: Young et al., 2006). We further expected similar fGCM concentrations in non-breeding and breeding mole-rats as the study was conducted during the dry season when the colony structure is more stable and non-breeding individuals are less likely to disperse and show reproductive activity (Creel et al., 2013; Young et al., 2010). Similarly, we expected higher fAM concentrations in non-breeding and breeding males than females, as androgens are low in non-breeding and breeding females during the dry season (Lutermann et al., 2013). Predictions for fAM and fGCM concentrations in captive as compared to wild-caught Damaraland mole-rats were difficult as results between lab and field experiments are often not comparable (Calisi and Bentley, 2009). Contrary to Young et al. (2010), for example, Clarke et al. (2001) found little evidence for higher urinary cortisol concentrations in non-breeding compared to breeding females in the laboratory. However, we expected that fGCM concentrations may be higher in captive mole-rats due to individual housing, and also that fAM concentrations may be increased in both males and females due to removal of the suppressive effects of breeding individuals (Bennett et al., 1996).

2. Materials and Methods

2.1. Animals and sampling

Damaraland mole-rats used for this study consisted of two groups with different backgrounds. The first group (captive) consisted of five adult females and five adult males, which were kept individually for six months before the start of the experiments. This group was used for conducting an ACTH challenge. The second group (wild-caught) consisted of a total of 63 individuals, which were caught at Tswalu Kalahari Reserve (27°22’S, 22°19'E; 52 individuals) or Winton Guest farm (27°29’S, 22°37’E; 11 individuals) (both Northern Cape, South Africa). The animals were captured in May and June 2015, which corresponds to the dry season in the habitat (Young et al., 2010). All animals were caught using modified Hickman live-traps baited with sweet potato. At Tswalu, entire mole-rat colonies were caught and all individuals were sexed, aged (juvenile, young and adult) and their breeding status (non-breeding, breeding males and queen (the only breeding female in a colony))

was determined. Individuals of <100 g were categorized as young, which were assumed to be <6 months old (Young et al., 2015). The three captured juveniles (body mass <50 g) were excluded from analyses. Queens were easily distinguishable by their perforate vagina and/or swollen teats (Burland et al., 2004). Breeding males were the largest males in a colony and they were characterized by prominent testes and staining around the mouth. A total of eleven colonies with between three and 13 members were collected. Faeces were sampled from all colony members immediately after capture (first sample dropped in trap or holding container) and animals were released again after collection. Animals from Winton (adults only) were also sexed and their breeding status was assessed, but faecal sampling took place within one week after return to the laboratory. There was no difference in fGCM as well as fAM concentrations between the animals from Tswalu and Winton (Mann-Whitney U or Student's t-text: $P \geq 0.19$), thus respective data sets were combined for subsequent analyses.

The necessary permits by the South African nature conservation authorities were obtained. The study was approved by the animal ethics committee of the University of Pretoria (EC098-12).

2.2. ACTH challenge

The ten captive animals were kept individually in collection chambers with a wire-mesh bottom and a collection dish underneath. The wire-mesh bottom prevented any contamination of the samples (especially urine) by food remains or direct contact with the individual. Collection chambers were checked frequently for urine or faeces. Throughout the experiment, animals were fed pieces of sweet potato and apple daily. No additional water was provided as the animals receive all the water from their food (Bennett and Jarvis, 1995). Animals were placed in the collection chambers five days prior to ACTH administration to allow the animals to acclimatize to their new surroundings. Collection of urine and faeces started two days prior to ACTH treatment to obtain baseline values and was continued for five days post treatment. Chambers were checked for samples every hour for the first 24 hours post-ACTH injection and twice a day for the rest of the experimental days before and after treatment. Synthetic ACTH (Synacthen® depot, Novartis, South Africa (Pty) Ltd) was dissolved in a sterile isotonic saline solution and individuals were injected intramuscularly (0.3 ml) around 10:00 a.m. with a dosage of 5-10 IU (50-100 µg)/100

g body weight. The handling time during injection was < 1 min. Sampling commenced one hour after injection. Urine and faeces were collected in Eppendorf tubes using single use plastic pipettes or tweezers, respectively. Tweezers were cleaned thoroughly with ethanol between sampling events. All samples were frozen immediately after collection and stored at -20°C until further preparation. The same set-up was used for faecal collection from wild-caught individuals. Only the first faecal sample dropped by the wild-caught individuals was collected and analysed.

2.3. Sample preparation and analysis

Urine samples (only collected from the 10 captive individuals) were defrosted at room temperature before being analysed with a commercial cortisol radioimmunoassay (Diagnostic Products Corporation, Los Angeles, California, USA). Once liquefied, the samples were thoroughly vortexed and assayed according to manufacturer's guidelines. Intra- and interassay coefficient of variation were 4.2% and 8.7%, respectively. Parallelism of the standard curve with a curve obtained by serial dilutions of Damaraland mole-rat urine was previously assessed by Clarke et al. (2001). Urinary creatinine concentrations were determined using a modified Jaffe reaction (Folin 1914). Respective urinary steroid concentrations are presented as mass per mg creatinine.

Faecal samples were lyophilized, and resulting dried faecal matter pulverised and sieved through a wire-mesh strainer. Between 0.05 and 0.06 g of faecal powder was weighed out per sample and extracted using 1.5 ml of 80% ethanol. The suspension was shaken for 15 min on a multi-vortex and then centrifuged for 10 min at 1500×g. The supernatant was transferred into a clean Eppendorf tube and stored at -20°C until analysis.

A sub-set of faecal steroid extracts (n=66) from five ACTH treated animals were measured for immunoreactive fGCM concentrations using five different EIAs: (i) Cortisol; (ii) 11-oxoaetiocholanolone I (detecting 11,17 dioxoandrostanes); (iii) 11-oxoaetiocholanolone II (detecting fGCMs with a 5β- 3α-ol-11-one structure), (iv) Corticosterone, and (v) 5α-pregnane-3β,11β,21-triol-20-one (detecting fGCMs with a 5α-3β-11β-diol structure). Detailed assay characteristics, including full descriptions of the assay components and cross reactivity's have been provided for the 11-oxoaetiocholanolone I, cortisol and corticosterone EIAs by Palme and Möstl (1997), 11-oxoaetiocholanolone II EIA by Möstl et al. (2002) and for the 5α-pregnane-

3β,11β,21-triol-20-one EIA by Touma et al. (2003). Only three of the five EIAs tested (11-oxoaetiocholanolone I, Corticosterone, and 5α-pregnane-3β,11β,21-triol-20-one) showed an overall individual median increase above 100% post ACTH administration, with the 11-oxoaetiocholanolone I performing best. Subsequently, all steroid extracts were measured for immunoreactive fGCM concentrations using the 11-oxoaetiocholanolone I EIA, and for immunoreactive fAM concentrations using a testosterone EIA (detailed assay characteristics for the testosterone EIA are given by Palme and Möstl, 1993). The sensitivities of the EIAs used were 0.6 ng/g dry weight (DW) for the 11-oxoaetiocholanolone I EIA and 2.4 ng/g DW for the testosterone EIA. Serial dilutions of faecal extracts gave displacement curves that were parallel to the respective standard curve for both assays, with relative variation (%) of the slope of respective trendlines of <5% for the 11-oxoaetiocholanolone I EIA and <4% for the testosterone EIA. Intra-assay coefficients of variation of high- and low-value quality controls were 1.9% and 6.6% for the 11-oxoaetiocholanolone I EIA, and 5.0% and 5.1% for the testosterone EIA. Inter-assay coefficients of variation of high- and low-value quality controls were 12.0% and 13.2% for the 11-oxoaetiocholanolone I EIA, and 8.8% and 11.8% for the testosterone EIA. Faecal steroid concentrations are given as ng/g or µg/g faecal dry weight (DW). EIAs were performed at the Endocrine Research Laboratory, University of Pretoria, as described previously (Ganswindt et al., 2002).

2.4. Data analysis

Mean individual baseline values for urinary cortisol, fGCM and fAM were calculated from the respective steroid concentrations from samples collected during the 48h prior ACTH treatment. Baseline values were used to calculate percentage change of hormone concentrations during the ACTH experiment and were used to compare captive to wild-caught individuals. Average individual steroid concentrations were calculated per 12 h from 0 to 24 hours post treatment and for 24 h periods for the rest of the experimental days.

IBM SPSS 24 (IBM Corp., 2016) was used for all statistical analyses. The distribution of the data in all datasets was evaluated using Shapiro-Wilk tests and homogeneity was tested using Levene's tests. All parametric analyses were conducted on log-transformed data. Urinary cortisol, fGCM and fAM concentrations of captive individuals were compared between the sexes and the nine experimental

times before and after the ACTH challenge using generalized mixed models (GLMM) with individual as random factor. Sex and sampling time as well as the interaction of sex and sampling time were included as independent factors in both GLMMs. The analysis of urinary cortisol was fitted with a gamma distribution with log-link function and faecal steroid metabolites were analysed using linear distributions. To compare fGCM and fAM concentrations between young and adult females and males (mole-rats from Tswalu only), we used a generalized linear model (GZLM, gamma distribution with log-link function) or a general linear model (GLM), respectively. Age, sex and the interaction of age and sex were included as independent variables in the two models. Comparisons between the sexes and status (wild-caught breeding, wild-caught non-breeding and captive) of Damaraland mole-rats were performed using GLMs with sex, status and the interaction of sex and status as independent variables. Least significant difference pairwise comparisons (LSD) followed all significant interaction terms. *P*-values of ≤ 0.05 were considered to be significant and results are presented as mean ± standard error (SE).

3. Results

3.1. Urinary cortisol and fGCM concentrations in response to ACTH in captive mole-rats

Urinary cortisol concentrations of captive Damaraland mole-rats increased shortly after the ACTH injection. Already one hour after the challenge, concentrations were on average 874 ± 243% above baseline values. The highest individual urinary cortisol concentrations were measured at 12 and 24 hours post injection (LSD: $P \leq 0.005$, GLMM: $F_{8,178} = 63.64$, $P < 0.001$; Fig. 1). Thereafter, urinary cortisol concentrations decreased rapidly (Fig. 1), but returned to values similar to baseline only after about 120 h (at 96 h $P \leq 0.02$ compared to values before the ACTH challenge). Overall there was no significant difference in urinary cortisol concentrations between female (36.6 ± 4.6 ng/mg creatinine) and male Damaraland mole-rats (47.1 ± 6.0 ng/mg creatinine; GLMM: $F_{1,178} = 2.05$, $P = 0.15$) and both females and males responded with a distinct increase in urinary cortisol to the ACTH challenge. However, there were some time-related differences in the response (GLMM: $F_{8,178} = 3.28$, $P = 0.002$), with males showing higher cortisol concentrations shortly after the challenge, which was significant at 48 h after ACTH injection (LSD: $P = 0.03$; Fig. 1). In contrast, urinary cortisol was higher in females than in males at 72

h (LSD: *P* = 0.04), with concentrations decreasing much more abruptly in males (Fig. 1).

Overall mean fGCM concentrations did not differ between female (107.4 ± 11.3 ng/g DW) and male (140.4 ± 13.7 ng/g DW) Damaraland mole-rats (GLMM: $F_{1,9}$ = 0.64, P = 0.45). In contrast, faecal GCM concentrations were significantly elevated after the injection of ACTH (GLMM: $F_{8,81}$ = 3.16, P = 0.004). At 12 h post ACTH administration, fGCM concentrations were overall already 173 ± 20% above baseline (LSD: *P* ≤ 0.01; Fig.2) and remained significantly elevated until 48 h post treatment (LSD: *P* ≤ 0.015; Fig.2). Faecal GCM concentrations started to decrease 72 h after treatment and returned to baseline at 96 and 120 h after treatment (Fig.2). The interaction between sex and time was not significant (GLMM: $F_{8,81}$ = 0.66, P = 0.73) and as such, responses of fGCM concentrations to ACTH treatment appeared to be similar in females and males.

3.2. Faecal AM concentrations in response to ACTH in captive mole-rats

There was no difference in the mean fAM concentrations between captive females and males (GLMM: $F_{1,9}$ = 0.47, *P* = 0.51). The observed difference between the sampling times (GLMM: $F_{8,72}$ = 4.92, *P* < 0.001) was due to fAM concentrations decreasing throughout the entire experimental period, but not in response to the ACTH injection (Fig. 3). Although fAM concentrations steadily decreased, concentrations differed significantly only between the lowest concentrations at the end of the experimental period (96 and 120 h) and the highest concentrations between -24 h and 48 h (LSD: *P* ≤ 0.02). The interaction between time and sex was not significant (GLMM: $F_{7,72}$ = 1.81, *P* = 0.10).

3.3. Age-effects on faecal GCM and AM concentrations in wild-caught mole-rats

Mean fGCM concentrations were significantly higher in adult (56.8±4.2 ng/g DW) than young (41.8±5.0 ng/g DW) mole-rats (GZLM: Wald χ^2≤4.73, *df*=1, *P*=0.03). Although there was no overall differences between females and males (GZLM: Wald χ^2≤0.06, *df*=1, *P*=0.80), the interaction of age and sex was significant (GZLM: Wald χ^2≤10.74, n=1, *P*=0.001). The highest fGCM concentrations were measured in adult males, being significantly higher than fGCM concentrations measured in adult females (LSD: *P*=0.006) and young males (LSD: *P*<0.001; Fig. 4a). Faecal GCM concentrations were lower in young males than in young females, although this was

not quite significant (LSD: *P*=0.055; Fig. 4A). Adult and young female mole-rats showed no difference in fGCM concentrations (LSD: *P*=0.48).

Faecal AM concentrations were significantly higher in adult (0.48±0.03 µg/g DW) than young mole-rats (0.31±0.04 µg/g DW; GLM: $F_{1,45} = 4.43$, $P = 0.04$), which was primarily the result of higher concentrations in adult males compared to young males (LSD: *P*=0.04), despite a non-significant interaction of age and sex (GLM: $F_{1,45} = 0.47$, $P = 0.50$). Concentrations were also higher in adult males than in adult females (Fig. 4B), but this was not significant (LSD: *P*=0.12). There was no significant difference in fAM concentrations between adult and young females (*P*=0.35) or between young males and young females (*P*=0.85). There was no difference in average fAM concentrations between females and males (GLM: $F_{1,45} = 1.0$, $P = 0.32$).

3.4. Faecal GCM and AM concentrations in wild-caught and captive Damaraland mole-rats

Due to the significant differences in fGCM and fAM between young and adult individuals, only adult individuals were considered for the comparisons between breeding, non-breeding and captive individuals. Mean fGCM concentrations were not significantly different between the sexes (GLM: $F_{1,50} = 3.23$, $P = 0.08$) or affected by status (GLM: $F_{2,50} = 2.67$, $P = 0.08$). However, the interaction of status and sex was significant (GLM: $F_{2,50} = 4.05$, $P = 0.02$) and fGCM concentrations were highest in wild-caught breeding males. They were significantly higher compared to wild-caught breeding females (queens; LSD: $P = 0.001$) and wild-caught non-breeding males (LSD: $P = 0.007$; Fig. 5). Faecal GCM concentrations were similar in wild-caught non-breeding and breeding females (LSD: $P = 0.47$). Both captive females and males exhibited similar and intermediate fGCM concentrations compared to the wild-caught individuals and fGCM concentrations were found to be significantly lower only in wild-caught breeding females compared to captive females (LSD: $P = 0.025$; Fig. 5).

Mean fAM concentrations did not differ between females and males (GLM: $F_{1,50} = 1.46$, $P = 0.23$). Captive individuals had on average 131% higher fAM concentrations than any of the wild-caught mole-rats (LSD: $P \leq 0.001$, GLM (status): $F_{2,50} = 9.02$, $P < 0.001$; Fig. 6). The interaction between sex and status was not significant (GLM: $F_{2,50} = 0.34$, $P = 0.71$), but mean fAM concentrations of breeding males were 62% higher than those of queens. This was, however, not significant

(LSD: $P = 0.15$; Fig. 6). There was no difference in fAM concentrations between non-breeding and breeding females or non-breeding and breeding males (LSD: $P \geq 0.47$).

4. Discussion

One of the most widely used methods for validating assays for quantifying glucocorticoids or its metabolites in mammals is a pharmacological challenge with synthetic ACTH (Touma and Palme, 2005). ACTH regulates the release of glucocorticoids from the adrenal cortex (Axelrod and Reisine, 1984; Engelmann et al., 2004) and as such an increase in glucocorticoids and related metabolites measured in faeces is expected with its injection. In Damaraland mole-rats, both urinary cortisol and fGCM concentrations peaked within 12 h to 48 h after an ACTH injection. This confirms that the two assays (commercial cortisol RIA and an 11-oxoaetiocholanolone I EIA) are suitable for non-invasively monitoring adrenal activity in this species.

The effect of ACTH on glucocorticoid secretion was similar between the sexes, but it is important to note that urinary cortisol concentrations took two days longer to return to baseline levels in females than in males, which may suggest that females sustain their glucocorticoid response for longer than males. However, the same was not found for fGCM concentrations, which may be due to the more cumulative hormonal signal found in faeces. In addition, peaks in fGCM concentrations following ACTH administration were less pronounced in females than in males. Steroid hormones may be metabolised and excreted differently between sexes of the same species, resulting in differences in metabolite composition within the faeces (Touma et al., 2003, 2004). Further, a number of studies demonstrated sex-related differences in fGCM concentrations in response to ACTH administration (Ganswindt et al., 2012, 2014; LeRoux et al., 2016). For example, glucocorticoid levels in females can be affected by the oestrus cycle. In rats, glucocorticoid levels are similar in males and oestrus females, but much higher in females at other stages of the oestrous cycle (Atkinson and Waddell, 1997). Nevertheless, Damaraland mole-rats did not show an obvious overall sex-related difference in the response to ACTH. The small differences in signal strength and duration found for fGCM might be related to sex-related differences in the metabolic breakdown of glucocorticoids. In mice, males excrete a different set of glucocorticoid metabolites than females, resulting in quantitative differences in the immunoreactivity detected (Touma et al., 2003). Thus,

caution should be used when comparing absolute fGCM values between females and males. Nevertheless, the assays utilised turned out to be suitable for reliably quantifying urinary cortisol and fGCM concentrations in male and female Damaraland mole-rats and the differences between the sexes were small.

Some androgen and glucocorticoid metabolites show structural similarities, which can result in an EIA for the one to pick up concentrations of the other, especially if the steroid concentrations are very high (Ganswindt et al., 2003; Pribbenow et al., 2016, 2015). We, therefore, investigated if our androgen analysis could be compromised by measuring an increased signal in response to ACTH. No increase in fAM concentration in response to ACTH was found, in fact fAM concentrations decreased continuously throughout the seven day sampling period, suggesting that our testosterone EIA does not measure major quantities of metabolites of glucocorticoid origin in the faeces of Damaraland mole-rats. However, further studies would have to examine the actual nature and relative abundance of GCMs in Damaraland mole-rat faeces as well as the degree of antibody cross-reactivity to confirm that statement. Nonetheless, the fact that fAM concentrations did not rise after ACTH administration and that we can determine significantly higher fAM concentrations in adult males compared to young ones (wild-caught individuals) clearly indicates the suitability of the testosterone EIA to reliably measure fAM concentrations in Damaraland mole-rats.

Interestingly, very few differences in fAM concentrations were detected between female and male Damaraland mole-rats. At the end of the ACTH challenge, fAM concentrations were markedly lower in females than in males, but initial and overall average concentrations did not vary significantly between the sexes. Similarly, few differences in fAM concentrations were found between the sexes in the wild-caught individuals. The comparisons of fAM concentrations between adult males and females were not significant, although there was a trend for adult wild-caught males to have higher fAM concentrations than adult wild-caught females. The exceptional social structure of the Damaraland mole-rat may be responsible for the similarities in fAM concentration in males and females. There is ample evidence that moderate to high concentrations of androgens are present in female Damaraland mole-rats (Clarke et al., 2001; Lutermann et al., 2013). Lutermann et al. (2013) found that androgen concentrations vary in females of this species dependent on the season, with exceptionally high levels during the wet season when disperal opportunities are

also high and when non-breeding females may challenge the breeding monopoly of the breeding female. Elevated androgen concentrations were also observed in dominant and pregnant female meerkat, which were even above those found for males (Davies et al., 2016). Increased androgen concentrations during pregnancy were not found in female Damaraland mole-rats (Lutermann et al. 2013), but the sample size in this study was small and further studies may be needed to determine if a similar masculinization can be found in Damaraland mole-rats.

Although no study has compared androgen concentrations of males between the seasons, a similar pattern could be conceivable for males with androgen concentrations being relatively low during the dry season. Dispersal opportunities for both sexes are low in the dry season (Young et al., 2010) and the lack of outside breeding opportunities for subordinates gives no incentive to challenge the breeding monopoly of breeding individuals. Furthermore, breeding males do not have the breeding monopoly, as do females, in Damaraland mole-rats. Breeding and non-breeding males have similar urinary androgen concentrations and non-breeding males show spermatogenesis suggesting that reproductive suppression of males is regulated behaviourally and through incest avoidance (Bennett, 1994). As such non-breeding males are found to breed with non-related females from other colonies (Burland et al., 2004). However, access to other colonies, or single females is limited during the dry season when the soil is harder to dig in because of low soil moisture (Lovegrove, 1989; Molteno and Bennett, 2002; Young et al., 2010). During this period, elevated androgen concentrations may not be advantagous for males because of a lack of breeding opportunities and could explain the relatively low and uniform androgen concentrations found in the wild-caught breeding and non-breeding males. Further support comes from studies on other cooperative breeders, which also found similar androgen concentrations in dominant and subordinate males (Creel et al., 1992; Moss et al., 2001).

In contrast to fAM concentrations, we found larger variation in fGCM concentrations in the wild-caught individuals. Especially breeding males appeared to have much higher fGCM levels than females, or indeed young males. The reason is unclear, but may have to do with the breeding status, as both young and non-breeding males have low fGCM concentrations. In contrast, females, especially reproductively active ones (queens), show very low fGCM concentrations, which is in line with previous findings (Clarke et al., 2001; Young et al., 2010).

Overall, however, we found no indication in our study for higher glucocorticoid levels in dominant breeding individuals as has been reported for other cooperative breeding species (Carlson et al., 2004; Creel et al., 1997; Sands and Creel, 2004). The relatively similar glucocorticoid concentrations in non-breeding and breeding wild-caught mole-rats of both sexes may be attributable to the very stable colony structure during the dry season, which as discussed above, may also explain the relatively uniform androgen concentrations. The soil is very compacted during the dry season making digging for these subterranean rodents very difficult, resulting in reduced food availability (Molteno and Bennett, 2002). Due to these ecological constraints, it is more advantageous for all colony members to remain in a more stable social setting reflected in relatively low variations of steroid concentrations between the sexes and non-breeding and breeding individuals as observed here. Nevertheless, glucocorticoid concentrations can vary due to a large number of other factors and in meerkats, for example, glucocorticoid concentrations are increased in subordinates and dominants during pregnancy (Barrette et al., 2012) and they are higher in subordinate males when juvenile care is increased (Carlson et al., 2004). Unfortunately, we were not able to analyze effects of juvenile care on subordinate glucocorticoid levels because of the small sample size. Effects of juvenile care, such as the number of offspring present in a colony, could have influenced our results and would be an interesting opportunity for future studies. Further studies of physiological determinants of cooperative breeding should take into account differences in ecological constraints that could affect the stability of a cooperative breeding system and also the intrinsic factors of a colony.

In contrast to the wild-caught individuals, the captive individuals were singly housed without the suppression of the breeding individuals and ample food supply. Female Damaraland mole-rats show increases in reproductive activity as soon as they are separated from the queen (Clarke et al., 2001; Molteno and Bennett, 2000). Males, on the other hand, experience increased breeding opportunities with unrelated females when removed from their natal colony (Burland et al., 2004) and as such also increased reproductive activity. During this transition from a non-breeding to a breeding status, both females and males experience great morphological changes and increases in body size (Young and Bennett, 2013). An increase in reproductive activity due to removal from the natal colony and queen may explain the comparatively high fAM concentrations in our captive individuals. The relatively low

fGCM concentrations of captive individuals especially in comparison to wild-caught breeding males was surprising, but could be attributed to the long time (> 6 months) that the animals were housed in captivity before the start of the experiment. In addition, some of the differences between wild-caught and captive individuals seen could be explained by the differences in diet. Fibre content, for example, can have an influence on the faecal hormone metabolite concentrations measured (reviewed in Goymann, 2012) and the natural diet of mole-rats has usually a higher fibre content (Bennett and Jarvis, 1995) than the sweet potatos fed in the laboratory. However, further studies are required to better understand the effects of diet on the measurement of faecal hormone metabolites in mole-rats and other species.

The study demonstrates that both the 11-oxoaetiocholanolone I EIA and the testosterone EIA are suitable for monitoring faecal glucocorticoid and faecal androgen metabolite concentrations in Damaraland mole-rats, respectively. This adds a new method, which will enable the feedback free measurement of respective hormone metabolites in this species and eliminates some of the constraints imposed by urine and blood sampling. In addition, we have shown that fGCM and fAM concentrations are comparable in non-breeding and breeding Damaraland mole-rats of both sexes when ecological constraints on dispersal are severe and colony structures are stable.

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Figure legends

Fig. 1. Concentrations (ng/mg creatinine) of urinary glucocorticoid metabolites (A, B) and % response to baseline values (average over the first 48h; C, D) 48 h before and 120 h after a challenge with synthetic ACTH. A and C show responses in female and B and D in male Damaraland mole-rats. Data presented as mean ± standard error.

Fig. 2. Concentrations (ng/g dry weight) of faecal glucocorticoid metabolites (fGCM; A, B) and % response to baseline values (C, D) of female (A, C) and male (B, D) Damaraland mole-rats. fGCM and % response values (mean ± standard error) are shown for 48 h before and 120 h after a challenge with synthetic ACTH.

Fig. 3. Concentrations (µg /g dry weight) of faecal androgen metabolites (fAM; A, B) and % response to baseline values (C, D) of female (A, C) and male (B, D) Damaraland mole-rats. fAM and % response values (mean ± standard error) are shown for 48 h before and 120 h after a challenge with synthetic ACTH.

Fig. 4. Faecal glucocorticoid (fGCM in ng/g DW; A) and androgen (fAM in µg/g DW; B) metabolite concentrations of adult and young subordinate Damaraland mole-rats. Data presented as box-plots. The boxes show the median value and the upper and lower quartile values; the whiskers show the 10th and 90th percentiles of the values and the dots represent outliers. Sample sizes (n) are included and asterisks are *$P \leq 0.05$ and *** $P \leq 0.001$.

Fig. 5. Faecal glucocorticoid metabolite (fGCM) concentrations (ng/g DW) measured in wild-caught and captive Damaraland mole-rats. fGCM values (means) are presented separately for males and females and breeding (queen and males) and non-breeding wild-caught mole-rats. Bars are mean fGCM concentrations and sample sizes (n) are included for each of the six groups. *$P \leq 0.05$ and *** $P \leq 0.001$.

Fig. 6. Faecal androgen metabolite (fAM) concentrations (µg/g DW) of wild-caught (breeding and non-breeding) and captive Damaraland mole-rats. Values (means) are shown separately for males and females and sample sizes (n) are included for each of the six groups. *** $P \leq 0.001$.

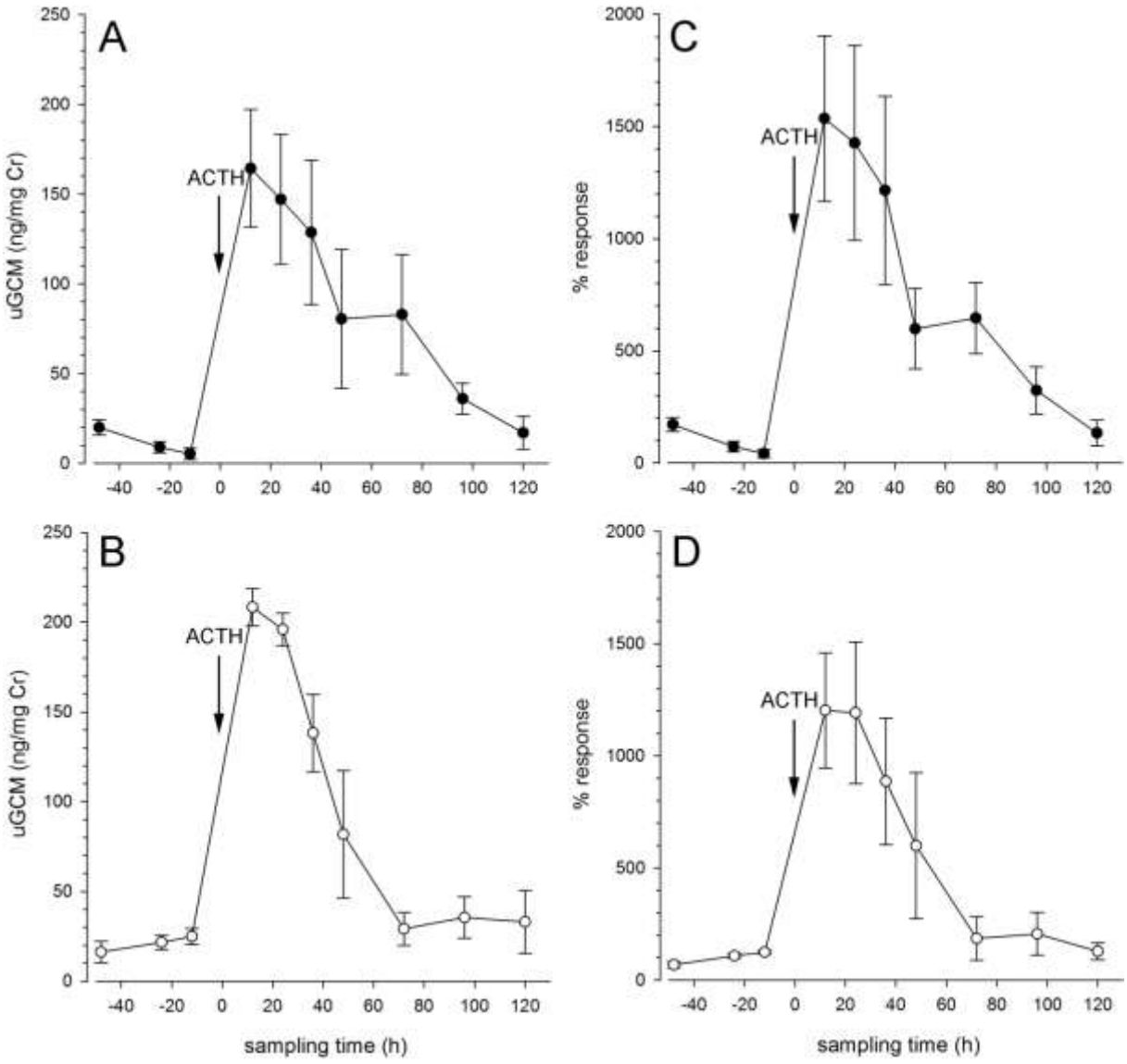
A
B
C
D
ACTH
uGCM (ng/mg Cr)
% response
sampling time (h)
250
200
150
100
50
0
2000
1500
1000
500
-40
-20
0
20
40
60
80
100
120

Figure 1

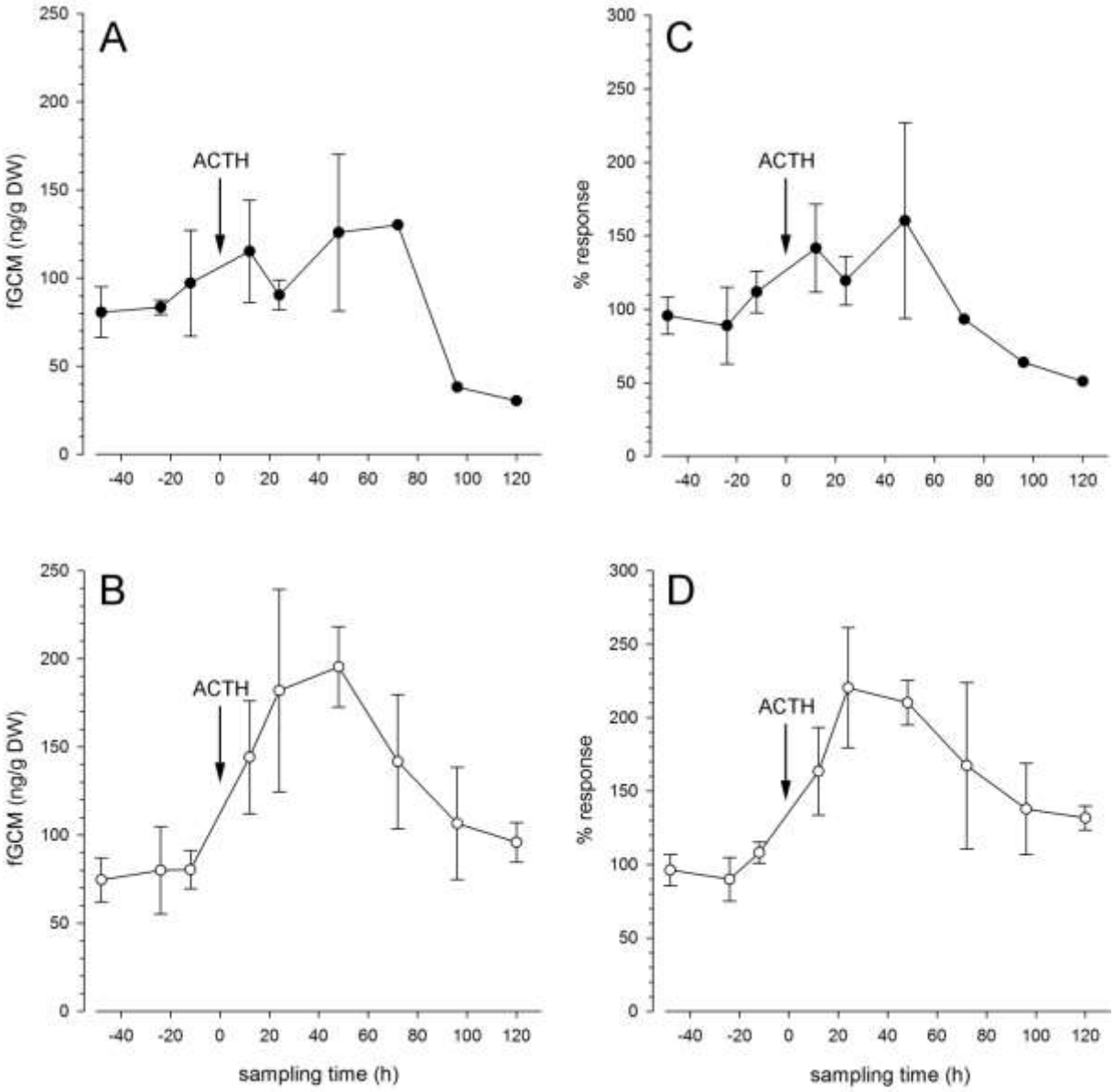
A
B
C
D
ACTH
fGCM (ng/g DW)
% response
sampling time (h)
0
50
100
150
200
250
300
-40
-20
0
20
40
60
80
100
120

Figure 2

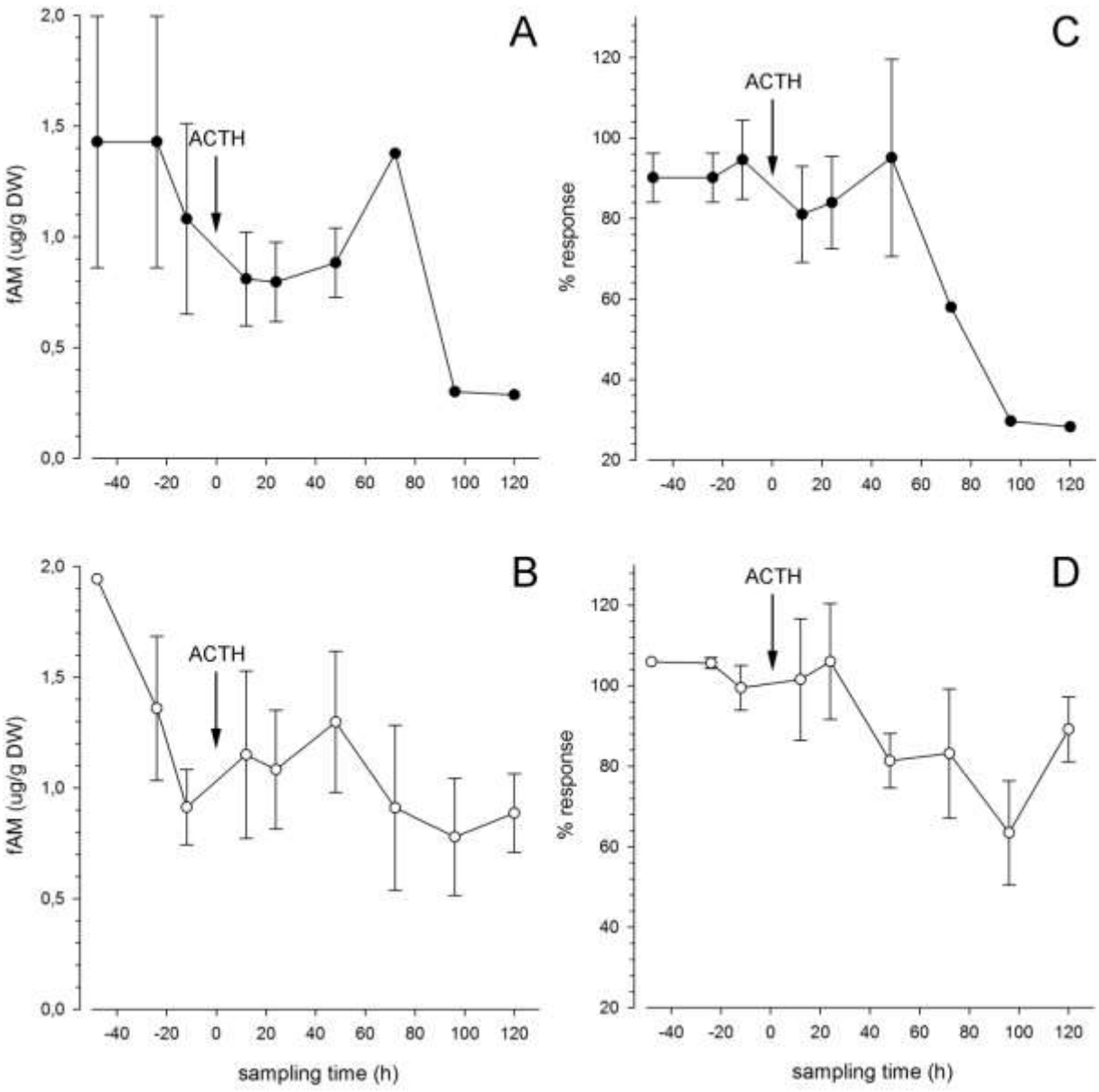
A
B
C
D
ACTH
fAM (ug/g DW)
% response
sampling time (h)
2,0
1,5
1,0
0,5
0,0
120
100
80
60
40
20
-40
-20
0
20
40
60
80
100
120

Figure 3

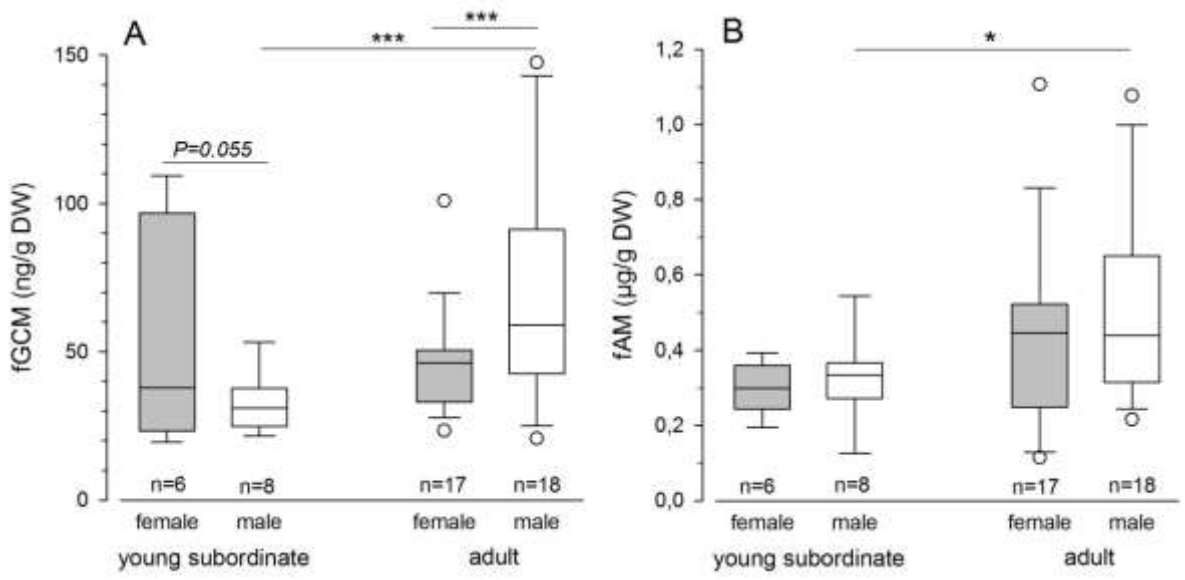
A
B
fGCM (ng/g DW)
fAM (µg/g DW)
P=0.055
***
***
*
n=6
n=8
n=17
n=18
female
male
female
male
young subordinate
adult
150
100
50
0
1,2
1,0
0,8
0,6
0,4
0,2
0,0

Figure 4

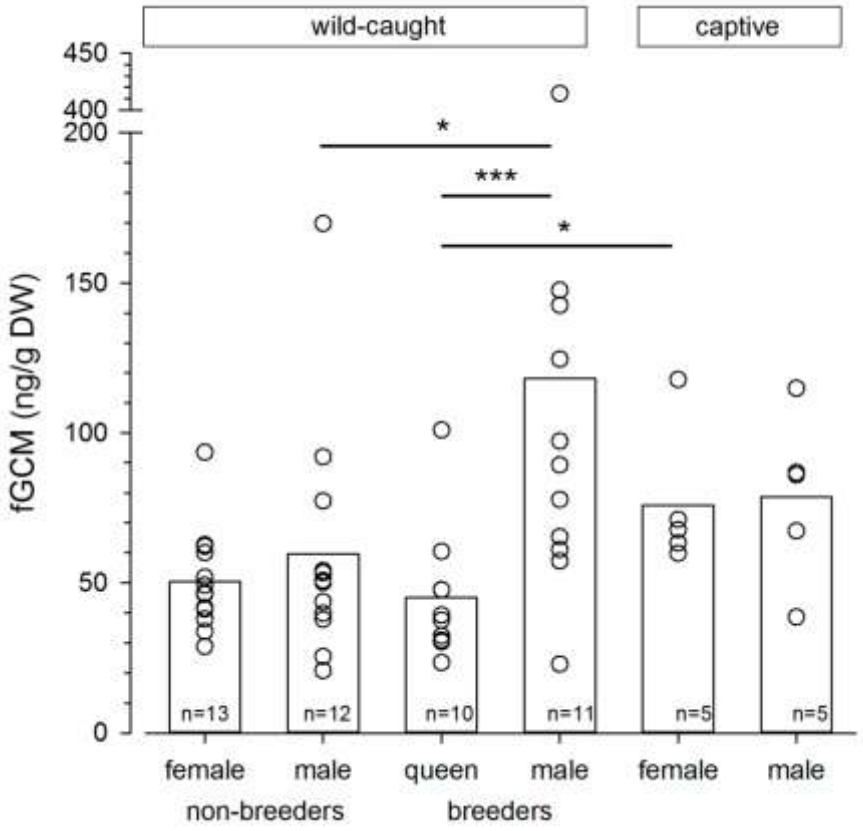
wild-caught
captive
450
400
200
150
100
50
0
fGCM (ng/g DW)
*
***
*
n=13
n=12
n=10
n=11
n=5
n=5
female
male
queen
male
female
male
non-breeders
breeders

Figure 5

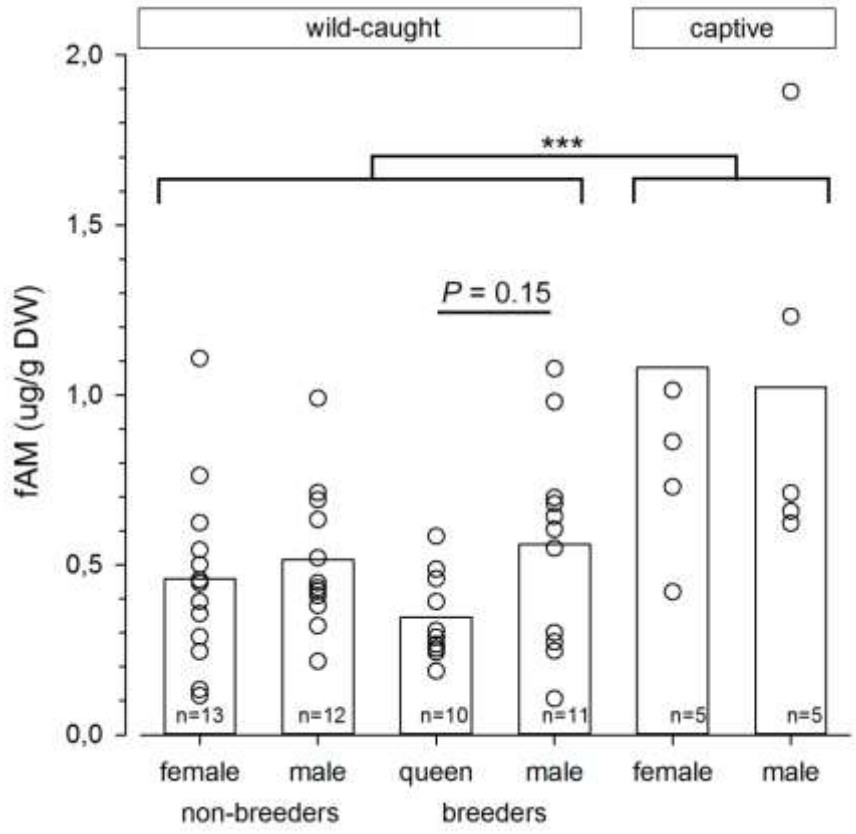
wild-caught
captive
2,0
1,5
1,0
0,5
0,0
fAM (ug/g DW)
***
P = 0.15
n=13
n=12
n=10
n=11
n=5
n=5
female
male
queen
male
female
male
non-breeders
breeders

Figure 6