Reproductive endocrinology of zoo-housed aardwolves

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Knowledge regarding the relationship between endocrine parameters and reproductive activity can offer important insights into how social and environmental factors influence the reproductive success of mammals. Although components of both the physical and social environment affect endocrine regulation of reproduction, less is understood about the potential role of interactions between different endocrine axes on reproductive activity. We evaluated temporal patterns of reproductive and adrenocortical steroids in two male and three female aardwolves (*Proteles cristata*) housed in captivity at Brookfield Zoo, Chicago, USA. We found seasonal variation in faecal androgens, estrogens, and progestagens, which provide support for previous observations of the aardwolf as a seasonal breeder. However, the timing of peak endocrine activity did not correspond to observations from wild populations. Our interpretation is that this discrepancy is caused by photoperiodic regulation of reproductive activity. We found a positive relationship between faecal androgens and faecal glucocorticoid metabolites in males and a positive relationship between faecal estrogens and faecal glucocorticoid metabolites in females when housed with conspecifics but not when housed alone. We also found a positive but asymptotic relationship between faecal progestagens and faecal glucocorticoid metabolites. We argue that these observations indicate a potential effect of reproductive endocrine activity on the hypothalamic-pituitary adrenal axis, which could result in interesting physiological trade-offs in male reproductive tactics and female pre-partum maternal investment because of the negative effects of long-term glucocorticoid elevation on reproductive performance. Finally, our results suggest that social and environmental factors interact in regulating many aspects of endocrine fluctuations in this mostly solitary species.

Key Words *aardwolf, hypothalamic-pituitary adrenal axis, seasonality, stress, reproductive timing*

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Introduction

Knowledge of how social and environmental factors influence animal reproductive physiology is important for our ability to understand the regulation of reproductive success. Reproductive activity in mammals is often either seasonal or non-seasonal, with subsequent different longitudinal patterns of reproductive hormones. In non-seasonal breeders, females exhibit ovulatory cycles and males are typically reproductively active throughout the year (Brown 2006). Contrarily, in seasonally breeding species reproductive hormones are elevated but highly variable during a defined period of time in both males and females (Monfort *et al.* 1989; Wingfield 1990; Monfort *et al.* 1997; Kraaijeveld-Smit *et al.* 2002; Kretzschmar *et al.* 2004; Hesterman *et al.* 2005; Dloniak *et al.* 2006b; Fanson *et al.* 2010a, b). The ultimate causes for seasonal breeding strategies are linked to temporal variation in resource abundance, whereas the most important proximate regulator of reproductive activity is photoperiod (Scott 1986; Goldman 1999), typically with the relative rate of change in daylight patterns being more important than the photoperiod per se (McAllan and Dickman 1985). However, social factors may also influence such environmental regulation of circannual hormonal rhythms (Scott 1986).

The physiological stress-response is primarily regulated by glucocorticoids mediated through the hypothalamic-pituitary adrenal (HPA) axis. Although there are well documented negative effects of persistent elevated glucocorticoid levels on reproduction (Sapolsky 2002), comparatively little attention has been given to the possible impact of reproductive endocrine activity on HPA activity (but see e.g., Rasmussen *et al.* 2008, Ganswindt *et al.* 2010). Although it is unlikely that there is a negative feedback of gonadal activity on the HPA axis, reproductive activity could act as a stressor since males often compete for mating opportunities through aggressive interactions and gestation and lactation are physiologically demanding periods for females (Goymann *et al.* 2001; Weingrill *et al.* 2004; Monclus *et al.* 2009). Because of the observed suppressive effects of elevated glucocorticoid levels on reproduction, a potential impact of reproductive activity on the HPA axis could lead to interesting physiological trade-offs.

The aardwolf (*Proteles cristata*) is a small hyaenid species (8-12kg) that lives in semi-arid regions of southern and eastern Africa. Aardwolves are solitary foragers but have been described as socially monogamous and show little sexual dimorphism. Data from wild populations suggest that they are strictly seasonal breeders (Richardson 1987). Aardwolves give birth to litters of 1-4 offspring during the rainy season, which coincides with time of peak food abundance in the wild. However, physiological data related to reproductive activity are lacking. Therefore, we used non-invasive monitoring of faecal hormone metabolites in zoo-housed aardwolves to provide basic information on longitudinal variation of reproductive hormones in males and females. We also quantified the effects of the social environment on reproductive endocrine activity as well as the relationships between reproductive and adrenocortical hormones.

Materials and methods

Study animals and animal housing

The study included five zoo bred aardwolves (two males, three females) housed at Brookfield Zoo, Chicago, Illinois, USA (Table 1). One of the males (Mutosi) and one of the females (Mwena) were the biological parents of the other three animals. The social groupings varied over time. At first, all animals were housed together. However, they were gradually separated due to increasing aggressive behaviour. The male Mutosi was given an epididectomy in 1998 in an effort to prevent potential inbreeding while allowing for group housing. The male Rafiki

was separated from the other animals at the age of six months (October 1997) and housed alone with no direct contact with other aardwolves until his death on 16 November 2010. The remaining group of Mutosi and the females Mwena, Safina and Mlia was separated on 20 February 2001 into a pair of Mwena and Mlia and another pair of Mutosi and Safina. On 10 June 2002, Mwena and Mlia were separated and on 29 May 2003, Safina was separated from Mutosi. These four animals were housed adjacent to one another (mean cage size: 4.3 x 3.9m; $n = 13$) and therefore could hear and smell one another regularly and occasionally see each other. Rafiki was housed separately in another area in the building (mean cage size: 3.8 x 2.8m, $n = 2$), but could likely still smell and hear the other aardwolves. However, he had no visual access to them. All aardwolves were fed 226 - 300g daily of a mix which consisted of 25% ground Hill's Feline Maintenance chow and 25% ground Marion Leafeater chow mixed with 50% water. Aardwolves were also fed 10-20 insects per day. To enable individual identification of faecal samples, each animal had their food marked with commercial food colour when housed together with conspecifics (Fuller *et al.* 2009). Table 1 shows life history data and arrangements of housing situation at Brookfield Zoo from birth until the end of the study period.

Sample collection and extraction protocol

A total of 1709 faecal samples were collected from July 2001 until April 2004. Details regarding sample sizes for each individual during the different housing situations are given in Table 1. Samples were collected by the zookeepers every 2-3 days and kept frozen until extraction at -20°C. Aliquots of 0.5g well-mixed, defrosted faecal material were extracted with 5.0 ml of 80% ethanol in distilled water by horizontal shaking for 14-18 hours at room temperature. After centrifuging for 15 min. at 1500 g, 1 ml of supernatant was added to 1 ml of assay buffer (0.1M phosphate buffered saline containing 1% BSA, pH 7.0), and the mixture stored frozen at -20 °C until assaying.

Enzyme immunoassays

Diluted faecal extracts were measured for immunoreactive androgen (fA), oestrogen (fE) and progestagen (fP) metabolites using previously described enzyme immunoassay systems from Coralie Munro's laboratory at the University of California, Davis, California, USA (Graham *et al.* 2001; deCatanzaro *et al.* 2003; Atsalis *et al.* 2004; Dloniak *et al.* 2004; Fanson *et al.* 2010a,b). We quantified fA using the testosterone polyclonal antibody R156/7, and cross-reactions of relevant steroids are given in deCatanzaro *et al.* (2003). Sensitivity of the assay (at 90% binding) was 0.04 ng/well. Intra-assay coefficients of variation (CV), determined by repeated measurements of low and high value quality controls ranged between 7.5% and 10.2%. The inter-assay CV was 19.6% and 23.8%, respectively. Recovery of exogenous hormone was 85.1 ± 5.8 % (mean ± SD, $n = 7$).

We used the estradiol-17β antibody R4972 to quantify fE. Cross-reactions of relevant steroids are given in deCatanzaro *et al.* (2003). Assay sensitivity at 90% binding was 0.39 ng/well. Intra-assay CV was 9.4% and 12.2% for low and high controls, respectively. Inter-assay CV was 16.7% and 18.9%, respectively. Recovery of exogenous hormone was 80.6 ± 2.8 % ($n = 7$).

We used the progesterone monoclonal antibody CL425 to quantify fP. Relevant cross reactivities are given in Graham *et al.* (2001). Sensitivity of the assay at 90% binding was 0.05 ng/well. Intra-assay CV for low and high value quality controls was 8.3% and 10.6%, respectively, and inter-assay CV was 18.7% and 22.6%. Recovery of exogenous hormone was 72.2 ± 4.3 % ($n = 7$).

We quantified faecal glucocorticoid metabolites (fGC) using a commercially available enzyme-immunoassay (Corticosterone, Assay Designs, Ann Arbor, MI), which successfully have been used on several similar species (e.g., river otter, *Lontra canadensis*, Rothschild *et al.* 2008, Canada lynx, *Lynx canadensis*, Fanson *et al.* 2012). The antibody cross-reacts with corticosterone (100%), deoxycorticosterone (28.6%), progesterone (1.7%), and less than 1% for other tested steroids. Assay sensitivity was 0.03 ng/well. Intra-assay CV was 12.3% and 13.7% for low and high controls, respectively, and inter-assay CV were 18.2% and 16.8%. Recovery of exogenous hormone was 82.0 ± 4.7 % (n = 7).

Samples were assayed in duplicate, and serial dilutions of extracted faecal samples gave displacement curves which were parallel to the respective standard curves in all assays. Data are expressed as µg per g wet faecal weight.

Biological validations of immunoassays

To evaluate the biological relevance of the fA assay, we contrasted baseline levels of fA in one male (Mutosi) and one female (Safina) to levels found during periods of mating activity. Since mating activity is typically regulated my testosterone in mammalian males, but not in females, contrasting correlations between mating activity and fA's in males and females can function as a biological validation for fA. The baseline fA value was calculated using an iterative process in which values that exceeded the mean plus 1.5 standard deviations (SD) were excluded. The average was then recalculated and the elimination process was repeated until no values exceeded the mean plus 1.5 SD (Brown *et al.,* 1994b).

To evaluate the biological relevance of the fE and fP assays, we similarly contrasted baseline concentrations, calculated as described above, to those found during and after periods of mating activity.

To evaluate the biological relevance of the fGC assay, we used samples from a previously described study on wild aardwolves, in which exogenous adrenocorticotropic hormone (ACTH) were injected to one male and one female to determine stress-related physiological responses using another enzyme immunoassay (Ganswindt et al. 2012). We used contrasts in fGC levels between samples collected prior to injection and within 20 hours after ACTH administration to verify the reliability of the fGC assay used.

Data analyses

We used mixed linear models to test for variation among months in reproductive hormone metabolites, as well as to test for the relationship between reproductive hormone metabolite and fGC concentrations. We created two separate pairs of models to test for monthly variation in reproductive hormone metabolite levels. First, we created two models that only included data collected during time periods when animals were housed alone, one for variation in fA concentrations in males and one for variation in fE concentrations in females. In these models we included data from both males (fA) and two of the females (Mwena and Mlia: fE) since we did not have data from the third female when she was housed alone. We used fA and fE concentrations as response variables, respectively, month as a fixed effect predictor and controlled for non-independence within individuals as well as temporal pseudoreplication by adding sample day blocked over each individual as random effects. Secondly, we created two models to test for possible interactions between housing arrangements and monthly variation in fA and fE concentrations. In these two models, we only included data from one male (Mutosi) and from one female (Mwena), because these two where the only individuals for which we had data from both when they had been housed alone and together with a conspecific. Similarly to the models described above, we used fA concentrations as response,

and added month, housing and a two way interaction as fixed effects. Since the models only included one animal, we only added sample day as random variable.

To test for possible effects of reproductive hormone metabolites on fGC concentrations, and whether or not these effects where influenced by the presence of conspecifics, we first created three models using fGC concentrations as the response variable and reproductive hormone metabolite concentrations (fA and fE, respectively) as fixed effect. In these models, we included both males when housed alone (for the model on fA), two of the females (Mweni and Mlia) when housed alone (fE) and in the last model data from one female housed together with a male (since we had no data for this female when she was housed alone). Secondly, we tested if housing influenced the effect of hormone metabolites on fGC by creating two models only including one male (Mutosi) and one female (Mwena), respectively. In these models we included faecal reproductive hormone metabolite concentrations, housing (for the model on fA this consisted of alone or housing together with a female, Safina; for the model on fE this consisted of housed alone or together with another female, Mlia) and the two-way interaction as fixed effects. We added similar random effect structures to all these models as described above.

We used a non-linear mixed-effects model to test for an asymptotic relationship between fGC and fP concentrations for data from one female (Safina). We tested an asymptotic rather than a linear relationship since visual inspection of the data suggested this to be a more appropriate representation of the relationship, and a likelihood ratio test confirmed that an asymptotic model provided a better fit to the data than a linear one (χ^2 = 247.69, df = 1, $p < 0.001$). The model used three parameters as fixed effects: the asymptotic value of fGC, the intercept of the function and the slope until the asymptote. Similarly to the linear models including only one individual, we added sample day as random effect.

All models where fitted to raw endocrine data, but a variance power function was used to account for heteroscedasticity (Pinheiro and Bates 2000). Statistical significance was set to 0.05 and all tests were two tailed. Statistical analyses were performed with the software R, version 2.15.1 for Linux (http://www.r-project.com) using functions in the user contributed package nlme (Pinheiro *et al.* 2012).

Results

Biological validations of immunoassays

In the male Mutosi, fA concentrations were elevated over 5 times above the baseline during February 2003 where frequent copulations with a female were observed (Figure 1a: baseline = 0.19 µg/g wet weight vs average 1.12 µg/g wet weight during February 2003). For the female Safina, fA concentrations were not as elevated above baseline concentrations during this mating period (Figure 1b: baseline = 0.13 µg/g wet weight vs average 0.39 µg/g wet weight during February 2003), but fE concentrations were (Figure 2a: baseline = 0.13 µg/g wet weight vs average 0.78 µg/g wet weight during February 2003). For the same female, fP concentrations were above baseline post elevated estrogen levels and observed copulations (Figure 2a: baseline = 0.30 µg/g wet weight vs average 19.0 µg/g wet weight during Mars/April 2003). For fGC concentrations, post injection samples had 2.5 times higher concentrations compared to pre sample average in the male (pre sample average = 0.29 µg/g wet weight vs post sample = 0.72 µg/g wet weight) and 2.1 times higher concentrations in the female (pre sample average =. 0.27 vs post sample average = 0.57 µg/g wet weight).

Variation in fA concentrations

When housed alone, fA concentrations for both males were significantly higher during February, March, June, July, and December compared to the nominate month January (Table 2; Fig 2a). However, for the male Mutosi, there was a significant interaction effect of month and housing on fA concentrations (Mutosi; $F = 2.07$, $df = 10,375$, $p = 0.026$; Fig. 2b), with elevated fA concentrations during October 2002 and during February 2003 when frequent copulations occurred (Fig. 1a). During this same period, there were no distinct fluctuations in fA concentrations in the female (Fig. 1b).

Variation in fE and fP concentrations

When housed alone, females had significantly higher fE concentrations in February compared to the nominate month January and lower levels in April, May, August, September, and November (Table 2; Fig 2c). However, as with one of the males, there was a significant interaction effect of month and housing on fE concentrations in the one female that was housed both alone and together with a conspecific (another female: $F = 10.49$, $df = 11391$, $p < 0.001$). There were more pronounced monthly fluctuations when housed with a male and drastically lower monthly fluctuations when housed with a female (Fig. 2d). In general, both fE and fP concentrations varied among the three females (Fig. 3), but the most drastic fluctuations were observed in the female that was housed together with a male, with elevated fE concentrations during a period with frequent copulations followed by a sharp increase in fP concentrations (Fig. 1c).

Relationships between fGC concentrations and reproductive hormone metabolite levels

When housed alone, fA concentrations had a positive effect on fGC concentrations for the two males ($\beta = 4.54$, $df = 445$, $t = 5.41$, $p < 0.001$; Fig 4a). In addition, the one male that was housed both alone and together with a female had higher fGC concentrations when he was together with the female compared to when he was alone ($\beta = 8.84$, $df = 419$, $t = 6.26$, $p < 0.001$). However, there was an almost significant interaction effect between fA concentration and housing arrangement for this male ($F = 3.34$, $df = 1,419$, $p = 0.068$), suggesting that fA had a stronger effect on fGC when he was housed together with a female than when he was alone ($\beta = 6.43$, $df = 419$, $t = 1.82$, $p = 0.068$; Fig 4b). Similarly, there was a positive effect of fE concentrations on fGC concentrations for females when housed alone ($\beta = 5.61$, $df = 674$, $t = 2.76$, $p = 0.006$; Fig 4c) and together with a male ($\beta = 62.38$, $df = 288$, $t = 8.00$, $p < 0.001$; fig 4d). For the one female that was housed both alone and with a female, there was a significant interaction effect of fE concentrations and housing on fGC concentrations with a more pronounced effect of fE concentrations on fGC concentrations when animals where housed with a female compared to when housed alone ($\beta = 115.71$, $df = 411$, $t = 12.04$, $p < 0.001$; Fig. 4c-d). All parameters in the non-linear model of the effects of fP concentrations on fGC concentrations were significant (Asymptote = 32.19, $t = 13.34$, $df = 287$, $p < 0.001$; Intercept = 2.89, $t = 4.35$, $df = 287$, $p < 0.001$; $\beta = 2.23$, $t = 17.58$, $df = 287$, $p < 0.001$, supporting an asymptotic relationship between fP and fGC concentrations in this female (Fig. 4c).

Discussion

This is the first study to present reproductive endocrine data for aardwolves, and the first study to present longitudinal data on glucocorticoid hormone metabolites for this species. Our results demonstrate that monitoring faecal hormone metabolites can be a useful non-invasive tool for assessing both gonadal and adrenocortical activity in this species. A previous study on wild aardwolves similarly validated a different EIA for glucocorticoid metabolites

(Ganswindt *et al.* 2012), which further lends support for the usefulness of non-invasive monitoring of endocrine parameters in aardwolves.

Temporal variation in reproductive hormone metabolites

Zoo-housed aardwolves exhibited monthly fluctuations in both male and female reproductive hormone metabolites. Although there were some variations in these monthly fluctuations, both within and between sexes, both males and females had elevated fA and fE concentrations during February when housed alone. However, these fluctuations where more pronounced in the male and the female that was housed together. Our interpretation of these observations is that here is an inherent seasonality in aardwolf reproductive physiology, but that gonadal activity has remained receptive to the presence of opposite sex conspecifics. For males in particular, an endocrine response to female oestrogen levels has also been found in many other carnivore species (Wingfield *et al.* 1990; Hesterman *et al.* 2005; Brown 2006; Dloniak *et al.* 2006b), and has been suggested to promote a close timing of mating activity to ovulation as a way of maximizing reproductive success (Kraaijeveld-Smit *et al.* 2002).

The timing of increased reproductive hormone metabolite concentrations differed from observations of wild aardwolf populations in African savannas, where mating activity has been recorded during May to July (Richardson 1987). In wild populations, drastic seasonal fluctuations in food supply linked to rainfall patterns are likely the ultimate cause for reproductive seasonality in the aardwolf. However, rainfall and food supply are unlikely proximate regulators of seasonal fluctuations in reproductive physiology. Since our observations of mating activity in February were done in the northern hemisphere, they correspond to the same season as field observations from the southern hemisphere. We therefore suggest that our observations of contrasting periods of mating activity between the northern and southern hemisphere points to a photoperiodic regulation of reproductive endocrine activity in this species.

There was some discrepancy in the timing of peak fA concentrations in the two males, with the male who was consistently housed in isolation showing some distinct elevations in fA concentrations in June and July. Numerous studies have demonstrated that captivity can adversely affect an animal's behaviour and physiology (reviewed in Lindburg and Fitch-Snyder 1994; Carlstead 1996; Estep and Dewsbury 1996), and lack of exposure to an appropriate social environment may disrupt the ability to correctly respond physiologically to environmental changes (Meier 1965; Bekoff 1972; Marchlewska-Koj 1997; Tilbrook *et al.* 2000; Blanchard *et al.* 2001; Lovic *et al.* 2006). We suspect that long term isolation caused one of the males to lose the ability to respond appropriately to environmental cues and hence time his peak in fA concentrations to periods of peak oestrus activity in females. This would further support that both environmental and social cues may be necessary for developing coordinated temporal fluctuations in gonadal activity between males and females in this species.

Pregnancy and pseudo-pregnancy in mammalian females are reflected by elevated progesterone levels caused by an increased production of progesterone first from the *corpus lutea* and later from the placenta (Schwarzenberger 1996; Monfort *et al.* 1997; Dloniak *et al.* 2006a; Schwarzenberger 2007; Van Meter *et al.* 2009; Fanson *et al.* 2010b). For felids, starting 1 to 2 days after ovulation, progestagen secretion from *corpora lutea* increases and concentrations remain elevated for 64-67 days in pregnant cats and approximately half that in not pregnant cats (Paape *et al.* 1975; Wildt *et al.* 1981; Tsutsui and Stabenfeldt 1993). We propose that the 300 fold increase in fP concentrations observed in one of the may be indicative of a pseudo-pregnancy since the male Mutosi was epidectomized and should have therefore been incapable of producing viable, fertile sperm. However, since we did not

observe distinct elevations in fP during the period of repeated mating activity, our data do not suggest that the aardwolf is be an induced ovulator.

Relationships between fGC concentrations and reproductive hormone metabolites

Due to the well documented negative effects of HPA activity on reproductive function (Dobson and Smith 2000), we regard it to be highly unlikely that our observations of positive associations between fGC concentrations and reproductive hormone metabolites were caused a positive effect of fGC on gonadal activity. Therefore, we suggest that our results points to possible effects of reproductive hormones on the activity of the HPA axis, and that reproductive activity could function as a physiological stressor. In females, fGC concentrations showed a stronger relationship with reproductive hormone metabolites when animals where housed with a conspecific than when kept alone. The observed asymptotic relationship between fP and fGC concentrations suggests that ovulatory state may also function as a physiological stressor. Since long-term elevations of glucocorticoids can compromise reproductive function, including terminating pregnancies (Sapolsky 2002), a positive association between reproductive endocrine parameters and glucocorticoids could lead to interesting physiological trade-offs. However, observations from wild populations suggest that aardwolves mostly live solitarily and mainly interact with conspecifics during the breeding season (Richardson 1987b). Therefore, these results may partly be confounded by the forced social situation that showed variation in faecal glucocorticoid and reproductive hormone levels when housed with a conspecific.

Conclusions

To conclude, we suggest that our study provided physiological support for the aardwolf as a seasonal breeder, and that this seasonality may be regulated by photoperiod. We also found a positive relationship between reproductive and adrenocortical endocrine activity, and we argue that this association is caused by a physiological stress response to reproductive activity. Such a HPA response to gonadal activity could result in interesting physiological trade-offs in male reproductive tactics and female pre-partum maternal investment. Finally, our results suggest that social and environmental factors interact in regulating reproductive activity in this mostly solitary species.

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Table 1 Life history data of five zoo-housed aardwolves, including variation in housing organisation with conspecifics during the data collection period, as well as sample sizes during each housing arrangement.

			Housing Organisation, Dates and Sample Sizes					
			Alone		With Female		With Male	
Animal ID	Sex	Born	Period	N	Period	N	Period	N
Mutosi[a]	Male	16/05/1992	29/5/2003-9/4/2004	129	18/7/2001-28/5/2003[c]	294		
Rafiki[b]	Male	30/04/1997	18/7/2001-9/4/2004	319				
Mwena[a]	Female	14/05/1993	11/6/2002-9/4/2004	259	18/7/2001-10/6/2002[d]	156		
Safina[b]	Female	30/04/1997					18/7/2001-28/5/2003[f]	290
Mlia[b]	Female	06/01/1998	11/6/2002-9/4/2004	262	18/7/2001-10/6/2002[e]			

[a] unrelated pair
[b] offspring of Mutosi and Mwena
[c] housed together with Safina
[d] housed together with Mlia
[e] housed together with Mwena, no samples collected
[f] housed together with Mutosi

Table 2 Summary of mixed-linear model parameters of fA for two males and fE for two females. Beta values indicates the difference between the nominate month January and consecutive months. Data are from when both the males and the females were housed alone.

Sex	Month	β	SE β	d*f*	t	p-value
Males	Feb	0.132	0.063	435	2.08	0.038
	Mar	0.167	0.056	435	3.03	0.003
	April	0.008	0.040	435	0.20	0.842
	May	0.050	0.053	435	0.95	0.343
	June	0.154	0.071	435	2.16	0.031
	July	0.387	0.123	435	3.14	0.002
	Aug	0.097	0.056	435	1.71	0.089
	Sep	0.033	0.044	435	0.76	0.449
	Oct	-0.044	0.034	435	-1.30	0.195
	Nov	0.102	0.057	435	1.77	0.077
	Dec	0.122	0.062	435	1.97	0.050
Females	Feb	0.049	0.019	664	2.59	0.010
	Mar	0.013	0.014	664	0.97	0.333
	April	-0.035	0.011	664	-3.14	0.002
	May	-0.045	0.012	664	-3.90	<0.001
	June	-0.012	0.017	664	-0.72	0.473
	July	-0.015	0.011	664	-1.37	0.171
	Aug	-0.025	0.010	664	-2.45	0.014
	Sep	-0.025	0.010	664	-2.37	0.018
	Oct	-0.016	0.011	664	-1.44	0.151
	Nov	-0.023	0.011	664	-2.11	0.035
	Dec	-0.015	0.011	664	-1.35	0.176

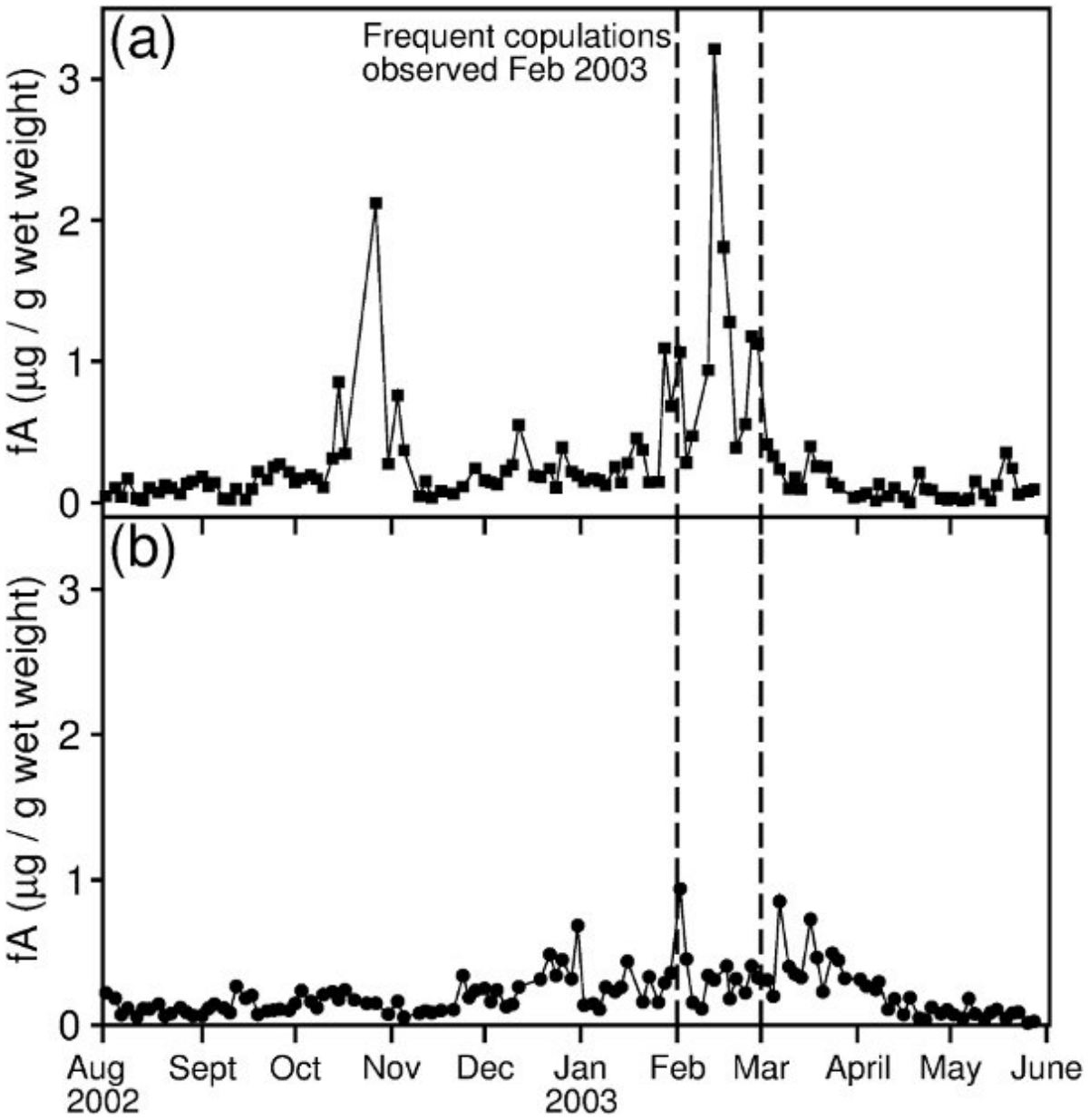

Fig. 1 Longitudinal profiles of fA concentrations in a male (a) and female (b) aardwolf over a 10 month period encompassing observed mating during February 2003

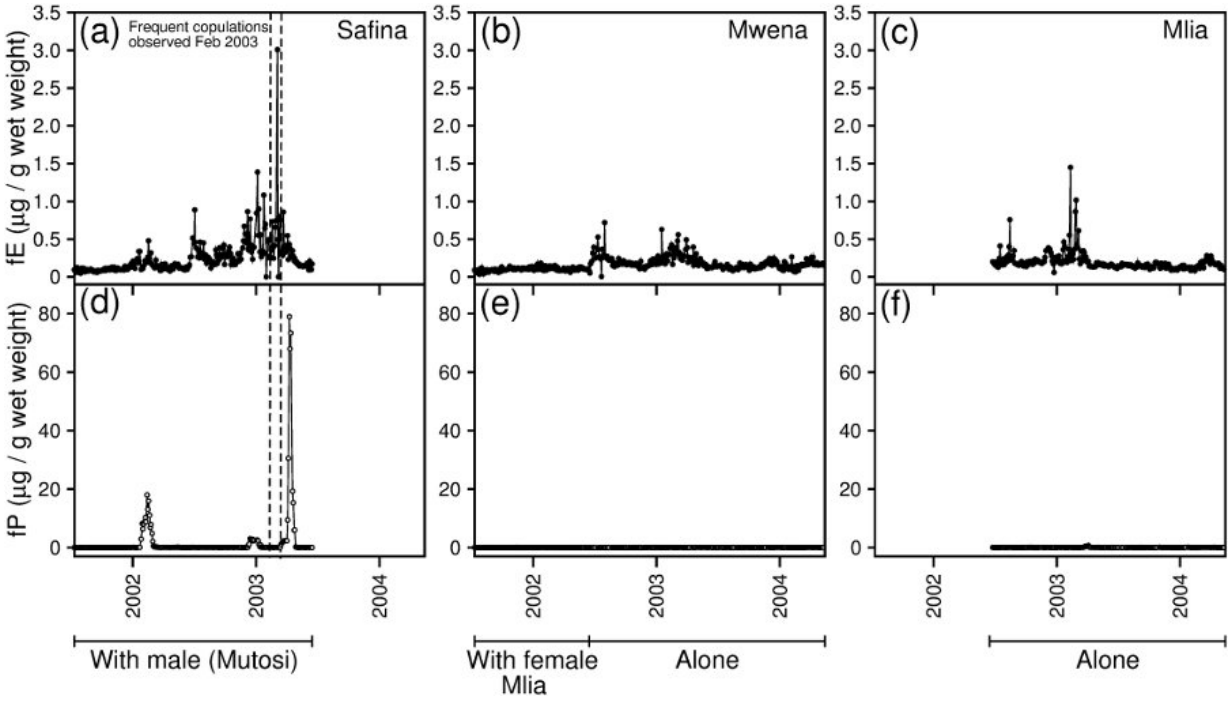

Fig. 2 Longitudinal profiles of fE (a-c) and fP (d-f) concentrations for three aardwolf females during the study period (Safina: a,d; Mwena: b, e; Mlia: c,f)

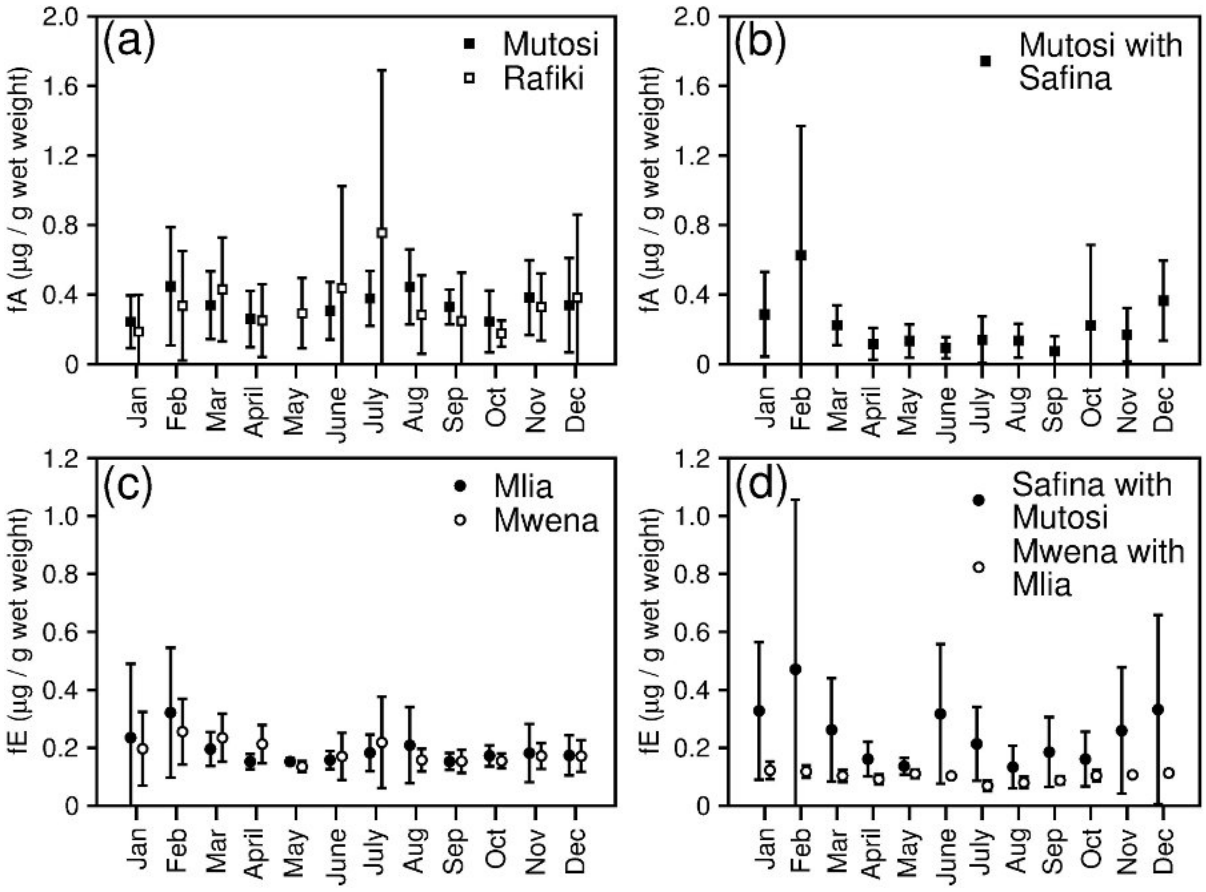

Fig. 3 Monthly variation (mean ± 1sd) in fA concentrations for two male aardwolves when housed alone (a), one male aardwolf housed together with a female (b) and monthly variation in fE concentrations for two female aardwolves when housed alone (c) and when housed together with a male (Safina with Mutosi) and a female (Mwena with Mlia) (d)

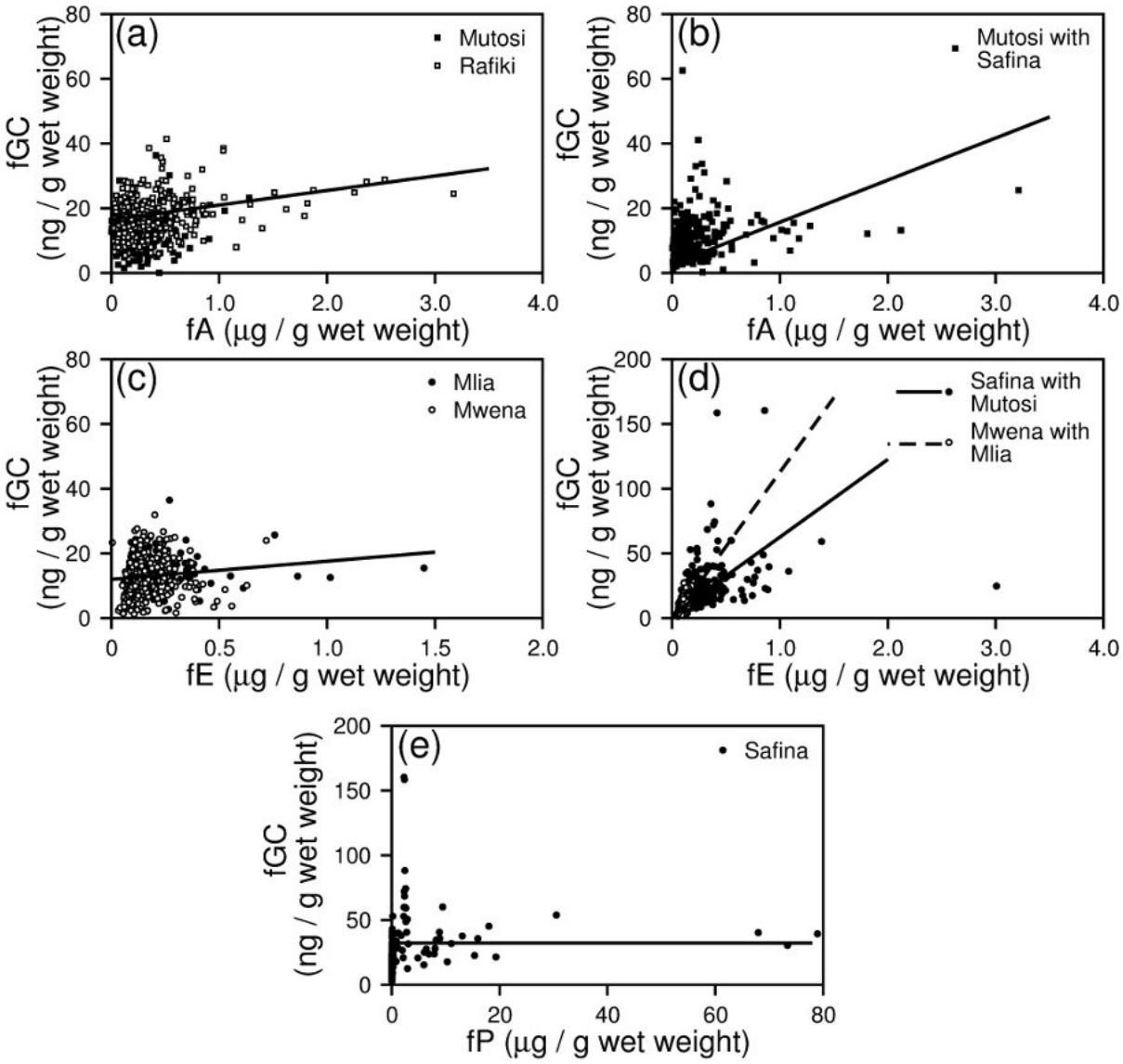

Fig. 4 Relationships between fA and fGC concentrations in two male aardwolves when housed alone (a) and one male housed together with a female (b), relationships between fE and fGC concentrations in two female aardwolves when housed alone (c) and when housed with a male (Safina with Mutosi) and a female (Mwena with Mlia; d), and the relationship between fP and fGC concentrations in one aardwolf female (e). Regression lines represent parameters from linear (a-d) and non-linear (e) mixed models