Fecal 20-oxo-pregnane concentrations in free-ranging African elephants *(Loxodonta africana)* treated with porcine zona pellucida vaccine

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Abstract

Due to overpopulation of African elephants in South Africa and the consequent threat to biodiversity, the need for a method of population control has become evident. In this regard, the potential use of the porcine zona pellucida (pZP) vaccine as an effective means for population control is explored. While potential effects of pZP treatment on social behaviour of African elephants have been investigated, no examination of the influence of pZP vaccination on the endocrine correlates in treated females has been undertaken. In this study, ovarian activity of free-ranging, pZP-treated African elephant females was monitored non-invasively for one year at Thornybush Private Nature Reserve, South Africa, by measuring faecal 5α-pregnan-3β-ol-20-on concentrations via enzyme immunoassay. Simultaneously, behavioural observations were made to record the occurrence of estrous behaviour for comparison. Each elephant under study showed 5α-pregnan-3β-ol-20-on concentrations rising above baseline at some period during the study indicating luteal activity. Average 5α-pregnan-3β-ol-20-on concentrations were 1.61 ± 0.46 μg/g (mean ± SD). Within sampled females, 42.9% exhibited estrous cycles within the range reported for captive African elephants, 14.3% had irregular cycles, and 42.9% did not appear to be cycling. Average estrous cycle duration was 14.72 ± 0.85 weeks. Estrous behaviour coincided with the onset of the luteal phase and a subsequent rise in 5α-pregnan-3β-ol-20-on concentrations. Average 5α-pregnan-3β-ol-20-on levels positively correlated with rainfall. No association between average individual 5α-pregnan-3β-ol-20-on concentrations or cyclicity status with age, or parity were detected. Earlier determination of efficacy was made indicating reproductive control was established 22 months post-treatment. Results indicate the presence of ovarian activity amongst pZP-treated female African elephants in two years following initial immunization. Further study should now be aimed toward investigating the long term effects of pZP vaccination on the reproductive function of female African elephants.

Keywords: pZP-immunocontraception; elephant; estrous cycle; progestagen; faeces; enzyme-immunoassay

1. Introduction

Rapidly expanding elephant populations in the Republic of South Africa has led to widespread concern over the resulting destruction of habitat and consequent threats to biodiversity [1-4]. Currently, various approaches have been explored in regards to managing elephant numbers, all with different advantages and disadvantages, including culling [5,6], translocation [5,7], range expansion [5,7,8], sterilization [9,10] and contraception [10,11]. In this regard, the use of the porcine zona pellucida (pZP) vaccine for immunocontraception of African elephant cows is a fairly recent development. The method has shown promise as an effective and acceptable means for management [12,13]. Zona pellucida glycoproteins isolated from pig ovaries have been shown to be homologous to those of the African elephant [1,14]. After treatment intramuscularly, the vaccine triggers anti-zona pellucida antibodies which bind to the zona pellucida capsule including sperm receptor sites and so prevent fertilization from taking place [1,15]. Since the initial studies, pZP-immunocontraception has been implemented in 7 private game reserves where it has been shown to be 100% effective in over 100 cows treated [10]. Nevertheless, pZP vaccination was not fully embraced primarily due to a lack of knowledge regarding potential adverse changes in behaviour. However, Delsink *et al*. [11,16] found no significant change in core and total range use, matriarchal status, cow/calf interaction, herd fragmentation/isolation, musth occurrence, bull hierarchy, and no aberrant or unusual behaviour witnessed among the herds. Furthermore, concerns were raised as to the potential effect of the pZP vaccine on luteal function, but to date, there has been no attempt to examine endocrine correlates associated with pZP vaccine use in African elephants.

In the female African elephant (*Loxodonta africana*), estrous cycle length is reported to range between 13 to 17 weeks [17-25]. The cycle is divided into a 4 to 6 week follicular phase after which an increase in progesterone levels, believed to trigger ovulation, marks the onset of an 8 to 11 week luteal phase [17,20-22]. Progesterone metabolites have been widely accepted as the major luteal and circulating progestagens in the African elephant and are thus considered to be a reliable indicator for monitoring ovarian function [19,20,22]. However, the knowledge acquired regarding the reproductive endocrinology of the African elephant is predominantly from studies on captive individuals. Amongst wild populations, the information gleaned regarding estrous cycles has been inconclusive due to the common occurrence of pregnancy or lactation among females [26-28].

Monitoring luteal endocrine function via faecal hormone analysis has made it possible to examine the reproductive biology of wild and captive animals [1,18]. Faecal samples can be easily collected and non-invasive methods have already been developed and successfully tested to enable the measurement of progestagen metabolites in elephant faeces [22,29]. These techniques allow novel exploration of the pZP vaccination effect on the frequency and duration of estrous cycles as well as integration of behavioural and endocrine mechanisms which will aid in a better understanding of the elephant's reproductive physiology.

In this publication, we aim to provide detailed information regarding the 20-oxo-pregnane concentrations in free-ranging African elephant (*Loxodonta africana*) treated with pZP vaccine. Such information will promote a better understanding of the underlying impact of utilizing pZP immunocontraception on ovarian function of treated elephant cows.

2. Materials and methods

1.1 Study site and animals

The study was carried out at Thornybush Private Nature Reserve (TPNR) (24°23' to 24°33'S, 31°05' to 31°13'E) in the Limpopo Province of South Africa. The area consisted of moderately dense tree savannah comprised predominantly of *Acacia* and *Combretum* species. Annual rainfall fluctuates greatly from year to year with a mean of 601 mm falling within the months of October to April with the remainder of the year being predominantly dry [30]. Therefore, seasons were defined as wet (October-April) and dry (May-September) [30]. Field work took place during a drought where rainfall recorded in both 2007 (405.4 mm) and 2008 (333 mm) fell well below the average. The TPNR population consists of 29 elephant cows and 11 bulls, two of which were independent adult bulls. Nineteen of the 40 elephants were females of breeding age [31] and were thus included in the study analysis. Identification kits, incorporating tusk shapes and sizes, ear markings and ear venation patterns, were made for all individuals in the TPNR population prior to the study, which allowed for individual recognition (Ahlers, unpublished data;11]. Age distribution was determined at the beginning of the study using a combination of known dates of birth, and rough estimates on shoulder height and age correlation as compared to known adults in the population [32]. Age groups were defined as adult (12+ years; n=10), or sub-adult (6-11 years; n=9) (Table 1). The management of TPNR were aiming to achieve zero population growth; therefore no untreated control animals could be used in this study. To include a control untreated population would have required locating another population within the same environmental and social conditions and without any mature bulls present to ensure no pregnant or lactating cows which at the time of the study was not attainable.

1.2 pZP treatments

From 2005 onwards, all cows of reproductive age [31] at TPNR received three initial immunizations in order to build up pZP antibody titre concentrations in 2005 [16]. These were delivered remotely from helicopter using methods modified from Delsink *et al*. [33]. The vaccination protocol for the TPNR population was as previously described by Bertschinger [34]. Briefly, a primary dose consisting of 400 µg pZP protein in 1 mL PBS plus 0.5 mL of Freund's modified complete adjuvant was administered in May 2005. This was followed by two boosters of 200 µg pZP protein each in 1 mL PBS plus 0.5 mL of Freund's incomplete adjuvant administered in June 2005 and August 2005 respectively. The TPNR breeding population received its first annual booster of 200 µg pZP protein in 1 mL PBS plus 0.5 mL of Freund's incomplete adjuvant in September 2006 and an equivalent booster in September 2007.

1.3 Behavioural data and faecal sample collection

Elephants were tracked daily from March 2007 to February 2008 by means of radio contact from field guides giving last reported location of herd, as well as fresh footprints and dung. Behavioural observations were recorded regularly (a minimum of 3 times weekly per individual) for an average of 3.5 hours/day using focal animal sampling [35-37]. Estrous behaviour, as described by Moss [38], observed in any individuals was recorded.

During each observation session, approximately 50 g of faeces was collected from the study animal shortly after it had defecated and moved away. Using rubber gloves, well homogenized aliquots of each faecal sample were taken and stored in glass vials [18,39]. Samples were placed on ice and frozen at the end of the work day (max. 6 hours after collection), and stored at –20^{o}C until hormone analysis.

The total time spent in the field with behavioural observations and faecal sample collections was 984 h distributed over 234 days. A total of 732 faecal samples were collected over the study period. Mean sample number collected per individual was 38 (min-max: 16-52).

1.4 Faecal sample processing and 5α-pregnan-3β-ol-20-on *immunoassay*

Faecal samples were lyophilized, pulverized and sieved through a nylon mesh to remove fibrous material [18,22,40-42]. Approximately 50 mg of faecal powder was extracted with 3 mL 80% ethanol in water [42]. The mixture was vortexed for 15 minutes prior to centrifugation at 1500 g_n for 10 minutes. The resulting supernatant fluid was transferred to Eppendorf tubes for hormone analysis [29,40,43]. The extracts were measured for immunoreactive progesterone metabolites using an enzyme immunoassay (EIA) for 5α-pregnan-3β-ol-20-on which has been shown to provide reliable information on reproductive steroid hormone pattern in elephant cows [44]. EIAs were performed following Ganswindt *et al.* [40]. Sensitivity of the assay at 90% binding was 3 pg per well. Inter- and intra-assay coefficients of variation ranged between 8.0% and 17.6% for the progestagen measurements. To adjust for water content variations, faecal hormone concentrations were expressed as mass/g dry weight.

1.5 Statistical analysis

Of the 19 elephant females sampled during the study period, five cows were excluded from all analysis due to low sample size (n<30; table 2), because interpretation of luteal and follicular phases was rendered unreliable. For the remaining focal animals, 5α-pregnan-3β-ol-20-on concentrations were expressed as μg/g dried faeces and plotted against time (weeks) for each pZP-treated cow. Baseline values of 5α-pregnan-3β-ol-20-on concentrations were ascertained for each female using an iterative process as described by Brown *et al.* [45,46]. The average was subsequently recalculated and the elimination process was repeated until there were no values greater than the mean plus 2 standard deviations (SD). The remaining values yielded the baseline 5α-pregnan-3β-ol-20-on concentration. Ovarian cycle length and periodicity was then determined according to the procedure described by Brown *et al*. [45,47]. Phase length estimates as well as ovarian cycle length estimates are given as mean ± SD. Females were categorized as having an irregular cycle when overall cycle length exceeded or fell short of the reported norm of 13 to 17 weeks [10,17-25]. The non-cycling category was reserved for females who had no identifiable pattern in 5α-pregnan-3β-ol-20-on concentrations throughout the study period [46]. Due to small sample sizes, irregularly cycling females were combined with non-cycling females for statistical analysis comparing cyclicity status to baseline 5α-pregnan-3β-ol-20-on levels and age. Individuals were categorized as demonstrating periods of anestrus if they had a follicular phase lasting longer than twice the duration of an average normal follicular phase (≥ 10 weeks) [25,47]. Determination of pregnancy within treated cows was based on luteal phase lengths persisting longer than 3-5 months [21,22]. Occurrence of behavioural estrus was compared with the time of increasing progesterone metabolite concentrations as behavioural estrus has been reported to last from 2 to 6 days [10]. Increases in progestagen concentrations following the end of the follicular phase confirm that ovulation has taken place with the formation of an active corpus luteum [21].

To determine the effect of age, reproductive status, and seasonal influences on faecal 5α-pregnan-3β-ol-20-on concentration, individual averages were first tested for normality using Shapiro-Wilks ($p>0.05$ = normal). Normally distributed data were analysed using Student’s t-test and data failing the normality test were analysed using Wilcoxon Rank Sum Test (with continuity correction of 0.5). Spearman Rank Correlation was used to assess the effects of seasonal rainfall on progestagen concentrations. A two-tailed Fisher's Exact Test was used to test cyclicity status correlation to age, parity and lactational status [48,49]. Statistical significance was assumed when $P < 0.05$. Statistical analyses were performed using the software programs OpenStat [50] and KyPlot (Version 2.0 beta 13 1997). Data are presented as means ± SD.

2. Results

3.1 Luteal activity

In all females (n=14) where more than 30 faecal samples were collected, 5α-pregnan-3β-ol-20-on concentrations exceeded individual baseline levels more than once during the study period, indicating luteal activity (Fig. 1). All remaining cows (Table 2) also showed signs of luteal activity with elevations in 5α-pregnan-3β-ol-20-on levels mainly occurring in the second wet season of 2008.

- Insert Table 1 & 2-

3.2 Ovarian cyclic pattern

In eight of the 14 elephant cows a cyclic pattern in 5α-pregnan-3β-ol-20-on levels could be identified. Of these eight individuals, two cows showed an irregular cyclic pattern lasting longer than the reported maximum of 17 weeks (17.43 and 20.43 weeks, respectively), and for the remaining six females at least one complete estrus cycle could be identified (Figs. 1b & 1c) Acyclic periods lasting 18.68 ± 5.94 weeks (range = 13.14 to 24.57 weeks) were detected in four females.

- Insert Figure 1-

Within the 5α-pregnan-3β-ol-20-on profiles of regular cycling females (n = 6) a total of 9 complete estrous cycles were identified (Table 3). For four elephant cows one cycle, falling within the normal 13-17 week estrous cycle length, were identified. For the remaining two females, two and three complete estrous cycles were identified, respectively. Mean estrous cycle length determined from faecal 5α-pregnan-3β-ol-20-on measurements was 14.72 ± 0.85 weeks with a luteal phase of 8.89 ± 1.38 weeks and a follicular phase of 5.82 ± 1.44 weeks (Table 3). Three of the cycling females showed longer than expected follicular phase lengths while one cow had a shorter than expected luteal phase. For the six cycling elephant cows, baseline 5α-pregnan-3β-ol-20-on concentrations ranged between 1.04 ± 0.40 and 1.43 ± 0.52 µg/g DW (Table 3). 5α-pregnan-3β-ol-20-on concentrations during peak luteal phase ranged from 2.71 to 9.96 µg/g. The highest concentrations were found in the two females that showed irregular cycle lengths (Table 3). No difference in baseline 5α-pregnan-3β-ol-20-on levels were found between cycling and non-cycling females ($t = 2.57$, $n = 6$, $P > 0.05$).

- Insert Table 3-

3.3 Effect of age on ovarian cycles

Of the nine adult cows monitored five showed a regular and one an irregular cyclic pattern. The remaining three were acyclic according to the 5α-pregnan-3β-ol-20-on analyses (Table 1). One each of the sub-adult cows had a regular or an irregular cyclic pattern, respectively, while the remaining three were acyclic. Age and the occurrence of estrous cycles was not significantly correlated (Fisher's exact test; $P = 0.30$) and no significant difference could be found between average 5α-pregnan-3β-ol-20-on concentrations of adult and non-adult individuals ($t = 1.13$, $n = 14$, $p > 0.5$).

3.4 Effect of calving interval on ovarian cycles

All of the adult cows considered in the study (n = 9) had calved at least once (1 - 4 calves) prior to pZP treatment with the most recent dates of parturition occurring in August 2006 (Table 1). The faecal 5α-pregnan-3β-ol-20-on measurements revealed that no females were pregnant at the time of the study. All adult females except one (Mandy) were lactating for the duration of the study.

Three of six cows that had calved ≤18 months (range 6-14 months) failed to show an ovarian cycle while three had a regular cyclic pattern. All three cows that had calved more than 18 months (range 24-36 months) prior to commencement of the study were cyclic, although one of these had an irregular pattern (Table 1).

3.5 Effect of season on 5α-pregnan-3β-ol-20-on concentrations

Average 5α-pregnan-3β-ol-20-on concentrations obtained from individual cows during the wet season (1.98 ± 0.58 µg/g DW) were significantly higher ($t = 3.22$, $n = 12$, $P < 0.01$) than mean 5α-pregnan-3β-ol-20-on concentrations obtained from females during the dry season (1.25 ± 0.44 µg/g DW). Overall mean monthly 5α-pregnan-3β-ol-20-on concentrations were significantly correlated with total amount of monthly rainfall ($t = 2.00$, $n = 12$, $p < 0.05$) (Figure 2).

- Insert Figure 2-

3.6 Relationship of observed estrus and faecal 5α-pregnan-3β-ol-20-on concentrations

Estrous behaviour was noted on three occasions during the study and in each case coincided with the onset of a luteal phase as indicated by a rise in 5α-pregnan-3β-ol-20-on levels above baseline.

4 Discussion

This study is the first to examine the effects of pZP treatment on faecal progestagen concentrations in female African elephants while further demonstrating the usefulness of non-invasive faecal endocrine monitoring in assessing reproductive status in wild populations of African elephants [18,22,26,29]. The results generated in this study underline earlier efficacy of pZP vaccination [10] with regards to managing elephant numbers by detecting no pregnant focal animals 22 months after treatment. Observed estrous behaviour was associated with a rise in progestagen concentration indicating the initiation of the luteal phase as confirmed by Hodges [21] and Brown [23] in untreated African elephant females. Concerning seasonality, the generated endocrine correlates in pZP-treated individuals all remained in agreement with findings in literature regarding untreated populations [26,29]. Seasonal influences on reproductive steroid hormones have been widely reported in a variety of species [51-53] as well as African elephants [26,29]. As a consequence of low rainfall experienced during the dry season, quality and availability of food and water decline [26]. These dry season conditions result in a decline in body condition and have been linked to lowered progestagen concentrations, periods of anestrus, silent heats, and subsequently reduced fertility [26,29]. The period of study took place during a drought where rainfall in both 2007 (405.4 mm) and 2008 (333 mm) fell below the average 601 mm reported for the study area [30]. The sub-optimal conditions arising from the poor rainfall during the study period likely resulted in nutritional stress in the Thornybush elephant population which in turn could explain the low frequency of cyclic patterns in their progestagen concentrations. Overall average monthly progestagen concentrations closely followed rainfall patterns (Fig 2) and further verified past studies that indicated availability of water, food and body condition influence reproduction [26,29].

Our knowledge regarding reproductive endocrine function in free-ranging elephants is limited and information gleaned from captive populations cannot be transferred one-on-one without limitations due to inconsistencies in the environmental and social set-up. Therefore, further investigation of cycling patterns in both free-ranging untreated and treated populations of African elephant is necessary to evaluate the effect of pZP on the reproductive cycle. Our data demonstrate ongoing luteal activity in all elephants treated with pZP vaccine at TPNR, challenging current speculations that pZP treatment may alter ovarian function by destroying oocytes or follicular cells as has been proposed in dogs [54]. Furthermore, our results demonstrate that in the two years following pZP treatment at TPNR, estrous cycles are present amongst nearly half of treated individuals, indicating ovarian functionality. Previous studies already documented alternation between cyclic and non-cyclic periods as well as erratic progestagen secretion in untreated African elephant populations [27,55]. Furthermore, most data on wild populations have been unable to determine continuous cyclicity status, as in the majority of cases, the subjects already are or become pregnant during the course of the study [26-28]. Interpreting causes for irregular or non-cyclic patterns of progestagen secretion in African elephants is fraught with complexity given a vast variety of potential influences such as an individual's social status, body condition, climatic and seasonal influences [26,29,55]. Regarding the potential influence of the pZP treatment, episodic ovulatory failure amongst pZP-treated feral horses has also been reported but could not be irrefutably linked to pZP treatment alone and estrous cycle characteristics were consistent with untreated mares [56]. Additionally, seasonal and social influences on hormone activity also complicate analysis as both factors have an impact on body condition thus affecting reproductive fitness [29,55]. Thus it is difficult to conclude the effect of pZP treatment on elephant reproductive function as the exact effects of each of the aforementioned factors on reproduction in untreated populations is not yet fully understood.

In conclusion, the absence of an indefinite period of anestrus within the study population is encouraging as it demonstrates that pZP treatment unlikely interferes with reproductive function in the African elephant. Future study should be geared towards monitoring pZP-treated females alongside a comparable untreated, free-ranging, control group and for a longer duration (we suggest a minimum study period of two years) to minimize external influences on reproduction as well as ascertain the long-term effects of pZP vaccination on free-ranging African elephant populations.

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Table 1. Age, calving history, reproductive status, and baseline 5α-pregnan-3β-ol-20-on concentrations ± SD of pZP-treated African elephant cows with individual faecal samples sizes >30 at Thornybush Private Nature Reserve, South Africa.

Cow	Age (Years)	Age class	Calves (n)	Last calf (Months)	Samples (n)	Reproductive status	Baseline 5α-pregan-3β-ol-20-on (μg/g DW)
Hook	30-35	Adult	3	*36	52	Cyclic	1.06 ± 0.44
Flo	25-30	Adult	3	*36	42	Irregular cycling	1.20 ± 0.51
Kombela	25-30	Adult	3	8	31	Non-cyclic	0.86 ± 0.49
Mandy	25-30	Adult	2	12	47	Cyclic	1.19 ± 0.36
One Tusk	25-30	Adult	3	7	51	Cyclic	1.04 ± 0.40
Thembisa	25-30	Adult	4	6	35	Cyclic	1.43 ± 0.52
Madam M	20-25	Adult	3	6	33	Non-cyclic	1.00 ± 0.54
Dana	12-15	Adult	1	24	41	Cyclic	1.22 ± 0.54
Khala	12-15	Adult	2	14	45	Non-cyclic	1.70 ± 0.58
Hannah	9-12	Sub-adult	0	-	42	Irregular cycling	2.19 ± 0.80
No Tusks	9-12	Sub-adult	0	-	42	Non-cyclic	0.71 ± 0.22
Rex	9-12	Sub-adult	0	-	37	Cyclic	1.14 ± 0.45
Suka	9-12	Sub-adult	0	-	52	Non-cyclic	1.29 ± 0.56
Ziggy	8-10	Sub-adult	0	-	46	Non-cyclic	0.94 ± 0.18

Table 2. Age, calving history, and 5α-pregnan-3β-ol-20-on concentrations of pZP-treated African elephant cows with individual faecal samples sizes <30 at Thornybush Private Nature Reserve, South Africa.

Cow	Age (Years)	Age class	Calves (n)	Last calf (Months)	Samples (n)	5α-pregnan-3β-ol-20-on (µg/g DW)		
						Mean	Min	Max
Umkhonto	25-30	Adult	3	24	29	1.73	0.41	5.45
Skew	9-12	Sub-adult	0	-	28	1.09	0.33	2.82
Nkanu	6-9	Sub-adult	0	-	25	1.47	0.70	3.27
Ulwazi	6-9	Sub-adult	0	-	16	1.70	0.72	4.34
Zula	6-9	Sub-adult	0	-	25	1.22	0.39	7.14

Table 3. Number of cycles, phase lengths and baseline of faecal 5α-pregnan-3β-ol-20-on concentrations ± SD in cycling pZP-treated African elephant females at Thornybush Private Nature Reserve, South Africa.

Cycling cows	No. of cycles found	Luteal phase (weeks)	Luteal phase 5α-pregnan-3β-ol-20-on (µg/g DW)	Follicular phase (weeks)	Follicular phase 5α-pregnan-3β-ol-20-on (µg/g DW)	Cycle length (weeks)
Dana	1	8.29	2.08 ± 0.47	8.00	0.91 ± 0.18	16.29
Hook	2	8.72 ± 1.01	2.32 ± 0.99	5.43 ± 1.82	0.94 ± 0.36	14.14 ± 0.81
Mandy	3	10.33 ± 0.30	1.91 ± 1.07	4.81 ± 0.36	0.94 ± 0.25	15.14 ± 0.38
One Tusk	1	8.14	2.27 ± 1.11	5.71	0.90 ± 0.32	13.86
Rex	1	8.86	2.03 ± 0.61	5.43	0.89 ± 0.20	14.29
Thembisa	1	6.29	2.11 ± 0.01	8.00	0.96 ± 0.41	14.29
Mean ± SD		8.89 ± 1.38	2.07 ± 0.95	5.82 ± 1.44	0.95 ± 0.31	14.72 ± 0.85

Figure 1. Faecal 5α-pregnan-3β-ol-20-on concentrations (●) of three adult African elephant females (No Tusks, Hannah, and Mandy) treated with pZP vaccine depicting a) no cyclic pattern, b) irregular cyclic pattern, and c) cyclic estrous pattern. Dotted lines represent onset of successive luteal phases and two-way arrows represent the length of one complete estrous cycle. Solid line illustrates baseline 5α-pregnan-3β-ol-20-on concentrations.

a)

b)

c)

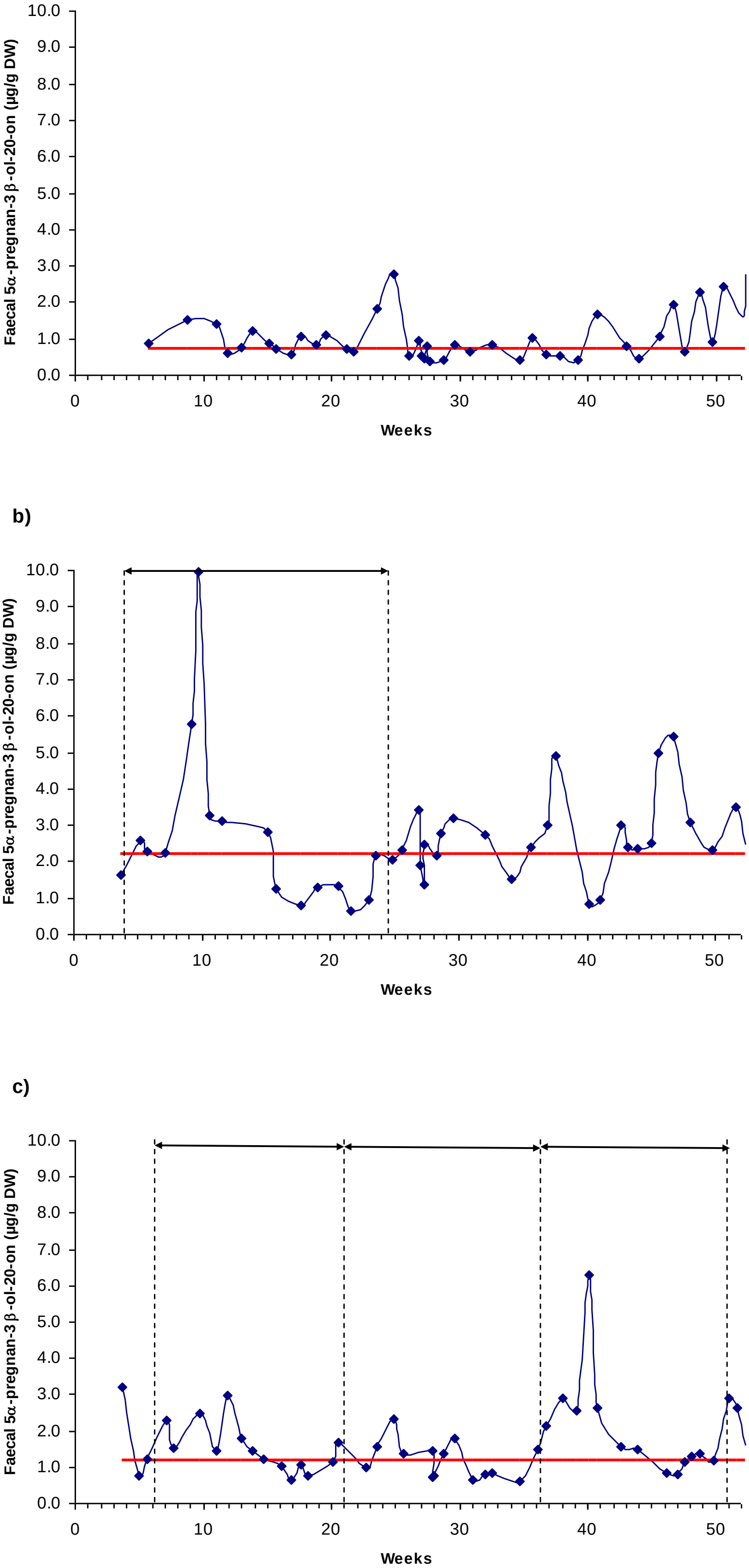
Faecal 5α-pregnan-3β-ol-20-on (µg/g DW)
Weeks
0
10
20
30
40
50
0.0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0

Figure 2. Mean monthly rainfall (horizontal bars) ± SE and overall mean monthly 5α-pregnan-3β-ol-20-on concentrations (●) for 14 pZP-treated African elephant females from March 2007 to February 2008.