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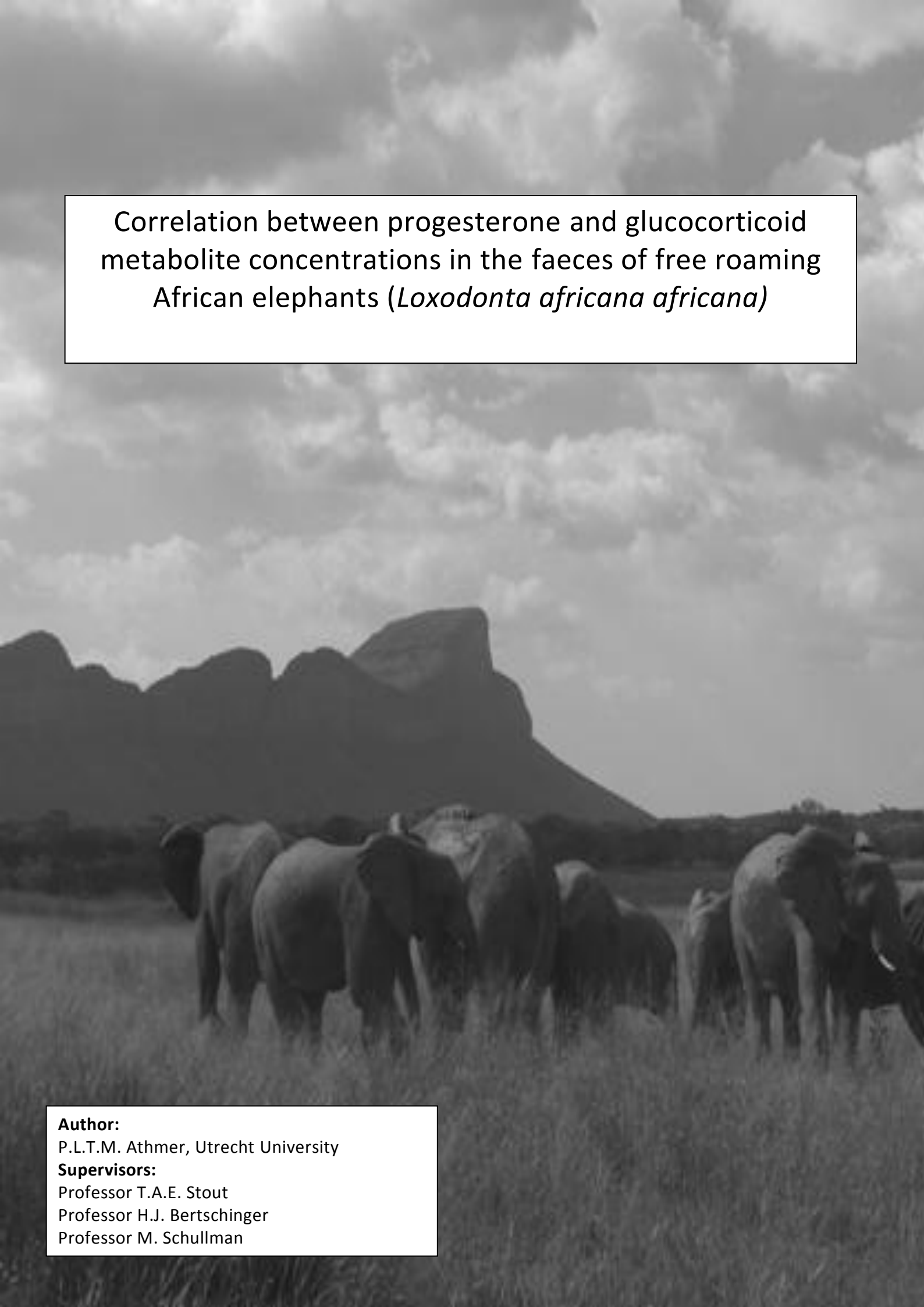
Correlation between progesterone and glucocorticoid metabolite concentrations in the faeces of free roaming African elephants (*Loxodonta africana africana)*



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Abstract

Elephant populations in Southern Africa have been increasing in number following their protective status as endangered species by CITES in 1989, to the extent that elephant populations have to be regulated. Elephant population control can be done in two basic ways: decreasing their number (i.e. culling or relocation) or preventing further increase by reduction of their reproductive rate (i.e. contraception). Culling elephants is an effective but controversial method due to public disapproval, while translocation is a less suitable method due to various practical problems. Contraceptive methods should ideally meet certain prerequisites based on efficacy, minimal animal handling, reversibility, safety for use in pregnant animals, minimal health side effects, no passage through the food chain, minimal effects on behaviour and low costs. Contraceptive methods as surgical sterilization, mechanical and hormonal contraception do not meet all of these prerequisites. Immunocontraception has become a point of interest with the antigens zona pellucida (ZP) and gonadotrophin-releasing hormone (GnRH) most intensively studied. GnRH is a neuropeptide produced by the hypothalamus and plays a crucial role in hypothalamic-pituitary-ovarian axis involved in ovulation. By vaccinating against GnRH, antibodies are thought to bind to GnRH, thereby blocking an important step in the ovulation process. The ultimate purpose of GnRH vaccination is to induce infertility by returning treated animals to a (temporary) pharmalogical pre-pubertal state. The eventual aim of this study was to test a commercial GnRH vaccine, Improvac®, for its effect on female African elephants. In this current pilot study we measured the progestagen and glucocorticoids metabolite levels in the faeces of two African elephant cows during an entire oestrus cycle (20 weeks) prior to GnRH vaccination. The faecal concentrations were estimated using Enzyme Immuno Assays (EIA) for 5α pregnane-3-ol-20-one (progesterone metabolite) and 3α,11-Oxo-cortisol (glucocorticoid metabolite). The aim by doing so was to provide a baseline for faecal progestagen and glucocorticoid concentrations in cycling non-pregnant African elephant cows and investigate correlations between reproductive status (progestagen) and stress (glucocorticoids) using a non-invasive sampling method.

Although the number of samples analysed in this study was limited, it did demonstrate that faecal progesterone concentrations could be used to demonstrate luteal activity, making it of considerable use for assessing the effects of GnRH vaccination. Faecal glucocorticoids levels did not appear to be significantly influenced by stage of cycle. A complete database of glucocorticoids levels during the long term studies could be helpful to determine whether GnRH vaccination is detrimental to welfare.

Contents

1. Introduction

1.1 Loxodonta africana africana

The African elephant is classified under the genus *Loxodonta* of the family Elephantidae. There are two subspecies of African elephant, the savannah (or bush) elephant (*Loxodonta africana africana*) and the smaller forest elephant (*Loxodonta africana cyclotis).* The larger *L. africana africana* mostly inhabits the northern, eastern and southern countries of the African continent whereas *L. africana cyclotis* lives in central and western Africa (Roca *et al.* 2001).

Elephants are known to be highly social animals, and live in matriarchal herds. A herd generally consists of 2 to 10 (related) adult females and their immature offspring, thus forming a family group. The family group is headed by the matriarch, generally the oldest female in a family (Laws *et al.* 1969; Douglas-Hamilton 1972; McComb *et al.* 2000). Elephants live in a so called fission-fusion formation, where large social groups are not stable, but wherein family groups can divide into smaller groups or temporarily aggregate with other family groups to form 'bond groups' (Archie *et al.* 2008; Douglas-Hamilton 1972).

In the dry season elephant herds are smaller and family groups join-up less frequently with other family groups than in the wet season (Douglas-Hamilton 1972; Moss 1983). In addition, when male calves reach puberty at around 12-14 years of age, they are excluded from the family group (Poole 1996a&b) and either join another family, live alone or assemble with other young adult males to form a 'bachelor' group (Poole 1996a). Although males are sexually mature when they leave their natal group (Laws 1969), they do not normally compete for access to oestrous females until several years after becoming independent (Laws 1970).

1.2 African elephant populations in Southern Africa

Since elephants became listed as an endangered species by CITES in 1989 they have benefited from extensive protection in Southern Africa. The result of establishing wildlife parks to protect elephants, e.g. by measures to prevent poaching, has been a steady increase in numbers within several game reserves. As early as 1967 it was decided that the Kruger National Park (KNP) would have to

cull or relocate elephants to prevent excess elephant numbers leading to ecological damage (Van Aarde *et al.* 1999; Whyte *et al.* 1999). Subsequently, culling was performed annually until 1994, with the aim of maintaining the population in KNP at around 8000 elephants (Van Aarde *et al.* 1999). In 1995, the culling was stopped because of concerns raised about the ethics of culling by pressure groups and the western public (Cumming *et al.* 1997; Fayrer-Hosken *et al.* 1997, 1999; Van Aarde *et al.* 1999). The effect of the ban on culling in the KNP (and other parks) has been a steady rise in elephant numbers, leading to local overpopulation (Fayrer-Hosken *et al.* 2000; Blanc *et al.* 2003). Most 'free-ranging' African elephants (70%) now live in protected areas, which correspond with approximately 16% of their original distribution range (Blanc *et al.* 2003).

1.3 Consequences of African Elephant overpopulation in Southern Africa

Elephant densities beyond a certain threshold leads to destruction of the natural habitat (Cumming *et al.* 1997; Fayrer-Hosken *et al.* 1999, 2000; Whyte *et al.* 1999) and can threaten the survival of other species (Cumming *et al.* 1997; Whyte *et al.* 1999). Furthermore, overly large elephant populations are more likely to come into conflict with human populations living close to the elephant's habitat (e.g. as a result of crop raiding).

1.4 Methods for controlling elephant populations

Regulating the size of elephant populations can be performed in two basic ways: decreasing the number (i.e. culling or relocation) (Kirkpatrick and Rutberg 2001), or preventing further increase by reducing the reproductive rate (i.e. contraception) (Patton *et al.* 2007).

Reducing reproduction rates using contraceptive techniques is currently considered a better option for small parks where a medium to long-term effect is desired (Kirkpatrick and Rutberg 2001). Contraceptive techniques have, ideally, to meet certain prerequisites, such as: a contraceptive efficacy of at least 90%, minimal handling of animals during application, reversibility of contraception, safety for use in pregnant animals, absence of significant health side effects, no passage of contraceptive agents through the food chain, minimal effects on

individual and social behaviour and low costs (Kirkpatrick and Turner 1991; Kirkpatrick and Rutberg 2001).

1.4.1 Culling

Culling elephants is a rapid and effective method of reducing their numbers. Culling was performed in the Kruger National Park between 1967 and 1994 (Van Aarde *et al.* 1999), but was stopped because of public disapproval (Cumming *et al.* 1997; Fayrer-Hosken *et al.* 1999, 2000; Van Aarde *et al.* 1999). Moreover, since many areas African wildlife parks are dependent on the income derived from non-consumptive tourism (Breytenbach 2001), culling is considered an effective but controversial method for controlling elephant populations.

1.4.2 Translocation

Translocation involves the moving of entire elephant herds from one park to another; while it has been done in the past it does involve a number of significant practical problems (Whyte *et al.* 1999). Translocation of large numbers of elephants is difficult and impractical for logistical and financial reasons. Moreover, as a result of a translocation programme from the KNP during 1994-1996 most areas in Southern Africa large and safe enough to house elephant herds are already occupied (Delsink *et al.* 2006).

1.4.3 Surgical sterilization

Surgical sterilization is highly effective but irreversible for all practical purposes. In females, ovariectomy or ovariohysterectomy can be performed but not without considerable surgical and anaesthetic risks, while the resulting infertility is irreversible. Males can be castrated or vasectomized, where the latter is theoretically reversible. The advantage of targeting male animals is that a small proportion of bulls is responsible for the vast majority of matings; theoretically it would therefore be possible to target only the large dominant bulls. However, castration will remove the source of testosterone responsible for reproductive dominance such that the mating role will be subsumed by younger smaller males (Patton *et al.* 2007). In addition, because in contrast to other land mammals, elephants are testicond (i.e.

the testis are internal) both castration and vasectomy are expensive, invasive, require full immobilization and are not suitable for large numbers of animals.

1.4.4 Mechanical contraception

By placing a non-medicated copper intrauterine device (IUD) into the uterus, mechanical contraception has been achieved in feral horses (Daels and Hughes 1995). Although reversible, this method is less suitable for large elephant populations because of the need to immobilize the elephant cows. In addition, difficulties with placing an IUD into the elephant's uterus can be expected as a result of the long narrow vagina. In an experiment with Brahman cattle, the contraceptive effects of an IUD were poor and there was a high risk of uterine perforation (Fordyce *et al.* 2001).

1.4.5 Hormonal contraception

Various hormones have been described to be effective contraceptives, including reproductive steroid hormones (e.g. progestins) administered orally, or as remotely delivered implants or depot preparation. However, progestins are not recommended for use in pregnant animals because of the risk of prolonged gestation, stillbirth, and abortion in some species. Implant introduction can also necessitate complete immobilization of the animal to be treated and can be expensive, making them less suitable for use in elephants (Bertschinger *et al.* 2008). A disadvantage of feeding progestins to wildlife is that it can never be assured that the food will be eaten only by the target animals. The risk of progestins entering the food chain is thus real (Patton *et al.* 2007).

GnRH agonists administered as implants or depot-injections have also shown to be successful contraceptives in several animal species. Large doses cause a down-regulation of GnRH receptors, and thereby inhibit LH release. However, treatment of female animals with GnRH agonists can trigger an initial surge of gonadotropins that induce cause oestrous behaviour and ovulation, such that the female may be fertile for an initial short period after administration (Patton *et al.* 2007).

1.4.6 Immunocontraception

Of the contraceptive techniques currently available, immunocontraception appears to best meet the prerequisites listed above, and has been subject to further investigations in several animal species. Vaccination against porcine zona pellucida is the most intensively studied immunocontraceptive technique and has proven to be safe and reliable in horse and ungulate species (Perdok *et al.* 2007).

1.5 The reproductive cycle of the African elephant

The oestrous cycle of the female African elephant has an overall length of 15-16 weeks, with a luteal phase of 8-11 weeks and a follicular phase of 4-6 weeks. The African elephant has a unique feature of two LH peaks during the follicular phase of the cycle. The second LH peak, approximately 3 weeks after the first, is associated with ovulation. During the post-ovulatory period, several corpora lutea (CL) appear to be functional for approximately 10 weeks if conception does not occur. During this time an average of 6-8 CL produce progesterone, measurable mainly as the 5α-reduced compounds, such as 5α-pregnane-3-ol-20-one (Hodges 1998).

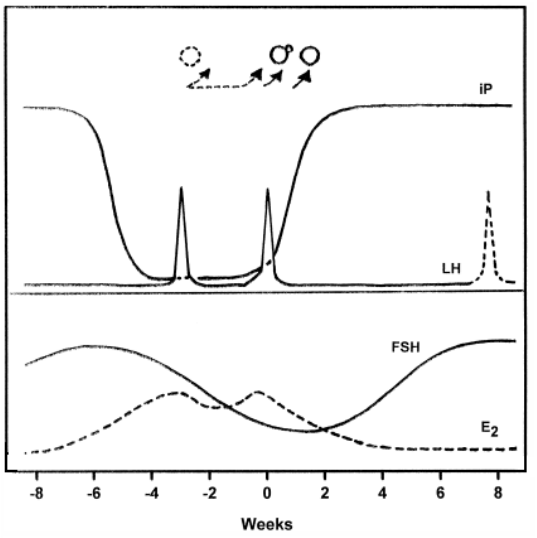

Figure 1. The oestrous cycle of the female elephant. Week 0 marks the presumed time of ovulation (Hodges 1998).

1.6 Immunocontraception following GnRH vaccination

As with disease prevention, immunocontraception is based on the principle of stimulating the immune system to produce antibodies against molecules

(antigens) delivered in the vaccine (Fayrer-Hosken *et al.* 2000; Kirkpatrick and Rutberg 2001).

The antigens that have been most extensively studied for immunocontraception are zona pellucida (ZP) and gonadotrophin-releasing hormone (GnRH) (Perdok *et al.* 2007). In the long term, GnRH may become the antigen of choice. This neuropeptide produced by the hypothalamus stimulates the FSH and LH secretion required for oestrous cyclity by gonadotropes in the anterior pituitary gland.

In mammals, the follicular period is initiated after luteolysis has resulted in an abrupt reduction in circulating progesterone concentrations. The fall in progesterone leads to a cessation of its negative feedback on GnRH secretion by the hypothalamus. This results in production and secretion of higher amplitudes and frequencies of GnRH which, in turn, results in increased release of LH and FSH from the pituitary gland such that follicular development is promoted. The growing follicles produce increasing amounts of oestrogen which, in turn, stimulates the hypothalamic surge centre triggering a burst of GnRH secretion and thereby a surge of LH release that will induce ovulation (Fig 1).

Antibodies produced against GnRH following vaccination, are thought to bind to GnRH in the hypothalamic-pituitary blood vessels and thereby prevent it from binding to the GnRH receptors on the gonadotrope cells. As a result the secretion of FSH and LH fails and follicle development and ovulation will cease.

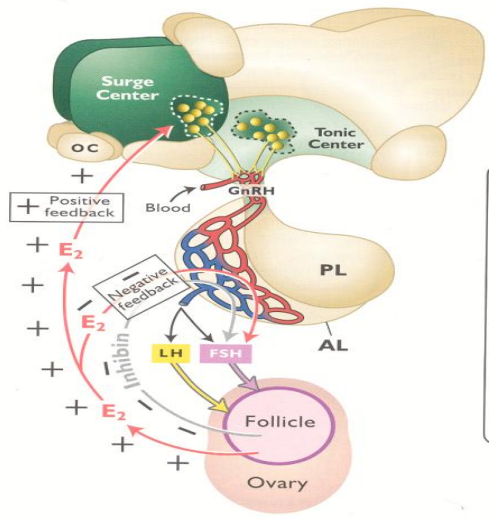

Figure 2. The Hypothalamic-Pituitary-Ovarian Axis (Senger et al.).

The ultimate effect of GnRH vaccination is that treated animals return to a (temporary) pharmacological pre-pubertal state, and become infertile (Stout and Colenbrander 2004). As with any vaccination, the adjuvant used for GnRH vaccination is important for the success of vaccination and duration of effect (Stout and Colenbrander 2004). The adjuvant enhances the efficacy of vaccination by stimulating the immune system to produce more antibodies against GnRH. A disadvantage of an adjuvant with strong immune inducing capacity is that they are often more "aggressive" and provoke a more severe injection site reaction (Harrenstien *et al.* 2004). This can lead to swelling and abscessation or even systemic reactions such as fever and anaphylactic shock.

The eventual aim of this study was to test a commercial GnRH vaccine, Improvac® (Pfizer Animal Health, Sandton, South Africa) for its effects in female elephants. The vaccine was designed for reducing "boar taint" in fattening pigs and has previously proven to be effective for suppressing testosterone production and aggressive behaviour, without local or systemic side-effects, in semi-domesticated African elephants bulls (Bertschinger, pers. comm.). The eventual plan is to test its efficacy and short and medium term side-effects in free-ranging African elephant cows.

The current study was designed as preparation for the GnRH vaccination study and concentrated on measuring correlations between reproductive state and stress in terms of faecal concentrations of glucocorticoids. Making elephant cows return to a pharmacological pre-pubertal state by vaccinating against GnRH could have either a negative or a positive effect on stress levels within the matriarchal herds. Adverse reactions to vaccination may also be measurable as acute or more prolonged rises in stress hormones or changes in behavioural patterns.

In this study we measured the progestagen and glucocorticoid metabolite levels in the faeces of African elephant cows in a 20 week period prior to vaccination. The study was therefore intended as a pilot study to investigate "normal" progestagen and glucocorticoid concentrations and correlations in the faeces of normal, cycling, non-pregnant African elephant cows. Measurements were made on faeces because this material can be collected non-invasively from free-roaming / wild animals.

1.7 Stress as a physiological mechanism

Stress responses in animals have been studied intensively at the behavioural and physiological levels. Research into metabolic, immunological and neuro-endocrine mechanisms makes it possible to describe the stress reaction in physiological terms (Balm 1999). A multitude of hormones (e.g., ACTH, glucocorticoids, prolactin, etc.) is involved in the stress response (Matteri *et al.* 2000). The adrenal glands play an important role in the endocrine reaction to stress because they are involved both in the hypothalamic–pituitary–adrenocortical axis and the symphatho-adrenomedullary system (Moberg 2000). Exciting or stressful situations trigger a response by the adrenal glands, leading to an increase in glucocorticoid and/or catecholamine secretion. These increases have an important role in the defence against stressful conditions, and high levels of stress hormone are therefore not inherently bad (Moberg 2000). Glucocorticoid concentrations have also been shown to be elevated in response to mating, copulation and hunting (Broom and Johnson 1993). During short-term stress, glucocorticoids mobilise energy to improve fitness (Raynaert *et al.* 1976) and may change an animal's behaviour (Korte *et al.* 1993). Long periods of severe stress, as indicated by prolonged periods of high cortisol concentrations can cause immunosuppression, atrophy of tissues (Munck *et al.* 1984) and reduction in fertility (Liptrap 1993; Dobson and Smith 1995).

1.8 Measuring stress hormone concentrations in the African elephant

Stress hormone concentrations in elephants can be measured in blood collected from an ear vein. However, because this is an invasive procedure it is not applicable to wild animals without interfering with their natural behavior. Furthermore it is not considered an accurate method of measuring stress in captive animals because of the handling required can itself induce a stress response.

Estimates of adrenal function in African elephant cows have been performed by measuring cortisol levels in urine (Brown *et al.* 1995; Schmid *et al.* 2001). Although a useful non-invasive technique in captive animals, however, urine collection is impractical in free-ranging wild animals. Another widely used and accepted method for measuring stress levels in wild animals is to measure glucocorticoid

metabolite concentrations in faeces. Faecal collection is non-invasive and should not involve stress caused by capturing or interfering with normal behaviour (Millspaugh *et al.* 2004). It is therefore considered a highly suitable sampling method for wild animal research.

Various studies have shown that faecal glucocorticoid assays reliably detect endogenous changes in adrenal activity. For example, a rise in cortisol metabolite concentrations in the faeces of African elephant cows was induced by ACTH challenge (Foley *et al.* 2001; Wasser *et al.* 2000).

In the current study, glucocorticoid concentrations in solvent extracted faecal samples were measured using an enzyme immunoassay (EIA) for 3α,11oxo-cortisol metabolites (3α,11oxo-CM), which was previously shown to provide reliable information on adrenal endocrine function in male African elephants (Ganswindt *et al.* 2003).

In addition, faecal progesterone metabolite concentrations were measured using a Progesterone Enzyme Immunoassay (PEIA), previously validated for determining 5α pregnane-3-ol-20-one concentrations in the faeces of African elephants. High-performance liquid chromatography (HPLC) analysis was carried out on the samples according to the method described by Heistermann *et al.* (1997).

1.9 Stress and stress hormone concentrations in the African elephant

Although various researchers have tried to estimate the levels of stress and discomfort using behavioural characteristics, animal behaviour studies are never truly unbiased. By measuring faecal glucocorticoid metabolite concentrations, it is theoretically easier to measure stress in an unbiased fashion. Measuring faecal glucocorticoid levels as an indicator of stress was previously used to monitor the long term effects of culling and other management practices (Foley *et al.* 2001). Stress hormone concentrations were found to be influenced by factors such as season, dominance rank within the family group and group size. Glucocorticoid metabolite concentrations were highest during the dry season, particularly if it followed a relatively dry rainy season (Foley *et al.* 2001).

In addition, glucocorticoid metabolite levels were higher in larger groups, but only during the dry season, and higher in low ranking females at least in a large group consisting of 16 elephants; this study also included pregnant females (Foley *et al.* 2001). Progesterone and glucocorticoid metabolite concentrations have also been reported to be positively correlated. During the dry season, the concentrations of progesterone were lower in pregnant elephants, whereas the glucocorticoid levels increased (Foley *et al.* 2001). In these studies, glucocortocoid concentrations in faeces were measured per gram of dry weight, such that dietary influences should not be responsible for seasonal changes (Wasser *et al.* 1993). Moreover, progesterone and glucocorticoid levels shouldn't change in opposite directions if a dietary effect of seasonal change was responsible (Foley *et al.* 2001).

1.10 Stress and stress hormone concentrations during the reproductive cycle of the African elephant

The behaviour of female mammals changes during the oestrous cycle. In the female African elephant, oestrous behaviour has been described to include 5 behavioural characteristics: Wariness, the oestrous walk, the chase, mounting and consort behaviour (Moss 1983). Wariness during oestrus consists of the female being noticeably alert and wary of males. A female carries her head high, eyes opened wide and directs her gaze at other elephants. When a bull makes an attempt to approach her, she moves away quickly. The 'oestrous walk' consists of a female avoiding an approach from adult bulls repeatedly, and then moving away from her family group with a bull. Multiple bulls may follow the cow in this situation. The oestrous walk differs from a normal walk. During an oestrous walk the female's head is held high and/or turned to one side as she looks back over her shoulder at the following male. The walk itself is characterised by a greater stride-length and speed than that of an undisturbed elephant. Sometimes the female's tail is held up and out from her body. If the bull stops, the female will stop as well, usually keeping at least 20 meters ahead and turning back slightly to keep him in sight. An oestrous walk may develop into a chase, the latter being distinguished by the intensity of male pursuit. The bull looks like he wants to

catch the female, both animals run and in most cases the bull has an erection. The female almost invariably stops running if the bull is able to touch her with his trunk. Once the female has stopped, the bull will almost always make an attempt to mount her. Sometimes his attempt will result in the female setting off again. A bull usually mounts a female after a chase or at the very least after a brief period walking closely behind the female. The bull stays mounted for about 45 seconds and intromission usually lasts about 40 seconds. The female either stands still or turns slightly during copulation. When the male dismounts the female usually stands still, but she may also run off (Moss 1983).

The behaviour of walking away from a bull that is trying to approach could be considered stressful or at least as an interference with the preferred behaviour of the cow. The elevated alertness and running during the chase could also be considered stressful, as could the act of being mounted. If so, glucocorticoid metabolite concentrations might be expected to be negatively correlated with progesterone metabolite concentrations, since progesterone is low during oestrus. The duration of oestrus in elephants has been estimated, on the basis of behavioural signs as 2-6 days, with a maximum of 10 days (Moss 1983).

During gestation however, progesterone and glucocorticoid metabolite concentrations have been shown to be positively correlated (t= 4.22; p< 0.0001) (Foley *et al.* 2001).

2. Aim of the study and hypothesis

2.1 Aim of the study

In respect of the need for an effective and safe method of elephant population control, GnRH vaccination looks promising. In this study we measured progestagen and glucocorticoid metabolite levels in the faeces of two free roaming female African elephants during an entire oestrous cycle (20 weeks), prior to GnRH vaccination. The aim was to provide a baseline for faecal progestagen and glucocorticoid levels in non-pregnant female African elephants, using a non-invasive sample collection method. These baseline levels could subsequently be compared to those after vaccination to assess the influence of GnRH vaccination on reproductive status and stress. In addition, we aimed to determine whether faecal glucocorticoid metabolite concentrations correlate with reproductive cycle stage, as indicated by faecal progesterone metabolite concentrations. The progesterone and glucocorticoid metabolite concentrations were estimated using Enzyme Immuno Assays (EIA) for 5α pregnane-3-ol-20-one (progesterone metabolite) and 3α,11-Oxo-cortisol (glucocorticoid metabolite).

2.2 Hypothesis

The aim of the study was thus to determine whether the faecal glucocorticoid metabolite concentrations correlated with the oestrus cycle stage in free-roaming African elephants *(Loxodonta africana africana)*. As a working hypothesis it was proposed that faecal 3α,11-Oxo-cortisol metabolite concentrations would not correlate (either negatively or positively) with faecal 5α-pregnane-3-ol-20-one concentrations or stage of the oestrous cycle.

Overall, it was expected that faecal progesterone concentrations would be low because the samples were to be collected during the dry season, while glucocorticoid levels would be relatively high (Foley *et al.* 2001).

3. Experimental set up and procedures

3.1 Long term studies

This research was part of a longer study to monitor the effect of GnRH vaccination on the oestrous cycle of free-ranging elephant cows as determined by faecal progesterone metabolite concentrations. This research was performed by Gabriela Benavides Valades between May 2009 and July 2010 in Entabeni Private Game Reserve, Limpopo, South Africa.

3.2 Experimental animals

The elephant cows used in this study were African elephants *(Loxodonta africana africana)* in a herd located at Entabeni Private Game Reserve (EPGR) in the Waterberg region, Limpopo, South Africa. EPGR is a 13 000 hectare malaria-free reserve containing 5 different ecosystems: the upper escarpment (5000 hectare) is predominantly grassland, while the south of the park consists of the warmer sandy wetlands of the lower escarpment (8000 hectare). The samples were collected during the dry season (May-August 2009).

The herd in the lower escarpment was moved to EPGR in July 2008. It consists of ten elephants, including nine non–pregnant females and a five year old male calf. Two female elephants, numbers 1 and 2, were both still nursing. Elephant number 9 was fitted with a VHF radio collar to enable location of the elephants. Table 1 shows the characteristics of the herd on the lower escarpment.

Number	Sex	Name	Age (years)	Size	Characteristics	Use in trial
1	♀	Mum	31	Large	Matriarch Nursing nr 7, TE*	Yes
2	♀	Scara	25	Large	Nursing nr 6, TE*	Yes
3	♀	Holy	16	Large		No
4	♀	Gigi	11	Medium		No
5	♀	Neat Ears	7	Medium		No
6	♀	Gigi	5	Small	Suckling from nr 2	No
7	♀	Scruffy	10	Medium	Suckling from nr 1	No
8	♀	Trunky	31	Large		No
9	♀	Colleen	31	Large	Fitted with VHF collar. Nursing nr 10, TE*	No
10	♂	Calvin	5	Small	Suckling from nr 9	No

*Table 1: Elephant herd on lower escarpment at EPGR. *TE= teats enlarged by nursing.*

3.3 Identification of the elephants

All the elephants were identified during a four week observation prior to faecal sample collection. By making photos and drawings of all the elephants, an identikit was built up (Appendix I). Physical characteristics such as shape, size and distinctive marks on the ears, trunk, tail and tusks, as well as body size were used for this purpose. After the four weeks, we were able to identify individual elephants from a distance. During the identification period the elephants were conditioned to follow the research vehicle by offering them food (BOSKOS®, Wildblocks® and Lucerne) in an area with less dense vegetation. The elephants remained at the feeding area for an average period of 1-2 hours, during which they could be observed from the research vehicle.

3.4 Collection of faecal samples

Faecal samples were collected from identified cows as often as could practically and safely be achieved; this was an average of 1-2 times a week from each individual cow.

Using telemetry, the location of elephant number 9's VHF radio collar could be determined. Due to the conditioning to food and the research vehicle, the elephants became familiar with following the vehicle and could be lead to a less dense vegetated area where the food was spread out over an area large enough to enable observation of the elephants to see where each defecated. The faecal samples were collected once the elephants had finished feeding and had moved off to a safe distance.

Faecal samples were collected from the centre of the freshest bolus and homogenized by hand, using a rubber glove. By homogenizing the sample, it was assured that the hormone concentrations were even distributed throughout the faecal sample (Wasser *et al.* 1996). The sample was put in a glass vial, and sealed with a rubber stop. Each sample contained approximately 5-10 gram faeces and was immediately frozen in a mobile freezer. The samples were then stored at – 20 ˚C until extraction and analysis in the laboratory at Pretoria University.

3.5 Laboratory analysis of faecal samples

All collected faecal samples were lyophilized. Following lyophilisation, dried samples were gently pulverized by hand, thereby separating the faecal powder from any undigested food content. Next, 0.05 g of faecal powder was mixed for 15 minutes with 3ml methanol (80%). The resulting suspension was then centrifuged for 10 minutes at 3,300 g. The supernatant (aliquot) was harvested for further analysis.

All faecal samples were analysed for progestagen and glucocorticoid concentrations using an EIA previously validated elephant faecal samples. The samples were analysed using the following procedure:

- Pipet 50 µl aliquots of standards (range: 1.02-250 pg), quality controls, and diluted faecal extracts in duplicate into micro-titre plate wells.
    - Add 50 µl of 5α-pregnan-3α-ol-20-one-3-HS-peroxidase label and rabbit anti-5α-pregnan-3α-ol-20-one-3-HS-BSA antibody (progestagen concentrations)
    - Or add 50 µl of rabbit anti-5β-androstane-3α-ol-11-one-17-CMO-BSA label (glucocorticoid concentrations)
- Incubate plates overnight at 4°C.
- Wash four times, add 150 µl (20 ng) of streptavidin-peroxidase to each well.
- Incubate in the dark for 30 min, wash plates, add 150 µl perioxidase substrate solution
- Incubate plates for 30-60 min.
- Terminate reaction by adding 50 µl of 4N H_2SO_4
- Measure light absorption using a spectrophotometer

Samples were run in duplicate. The optical density was measured for its absorbance at 492 nm, using an 8-channel microplate reader (Labsystems iEMS MF).

The extraction efficiency for determining 5α-preganane concentrations, determined previously by the recovery of [^{3}H]of 5α pregnane-3-ol-20-one from randomly selected faecal samples, was 83.8 ± 5.9% (mean ± SD; *n*=50) (Fieβ et al., 1999).

Sensitivity of the assay used for progesterone metabolite concentrations at 90% binding was 3 pg per well, and the inter- and intra-assay coefficients of variation ranged between 4.2% and 11.3%. Sensitivity of the assay used for the glucocorticoid concentrations at 90% binding was 3 pg/well, and the intra- and interassay coefficients of variation ranged between 2.4 and 11.9% (Ganswindt *et al.* 2003).

By measuring all hormone concentrations per gram of dry weight diet-related changes in hormone excretion rates were accounted for (Wasser *et al.* 1993).

3.6 Statistical analyses

All statistical analyses were performed using SPSS (SPSS 16.0 for Windows, SPSS Inc. Chicago, IL, USA) and Microsoft Office Excel (Microsoft Office Excel for Windows, Microsoft Corporation).

4. Results

The concentrations of progesterone (gestagen) and glucocorticoid (GCM) metabolites in the faecal samples of the two elephant cows monitored are shown in µg per g faeces dry weight (µg/g DW).

Cow 1, the matriarch of the herd was an estimated 30 years old. She showed peak faecal progesterone metabolite concentrations during weeks 5 (14.35 µg/g DW), 8 (5.24 µg/g DW) and 20 (8.44 µg/g DW) (Figure 3 and table 2). In week 1, weeks 9-15 and at the end of week 20, the progestagen concentrations in the faeces were below 1.00 µg/g DW. The values between week 2 (2.65 µg/g DW) and week 8 (5.24 µg/g DW) were all in excess of 1.00µg/g, as were the values between weeks 17 (1.02 µg/g DW) and week 20 (1.23 µg/g DW); with the exception of the second half of week 20.

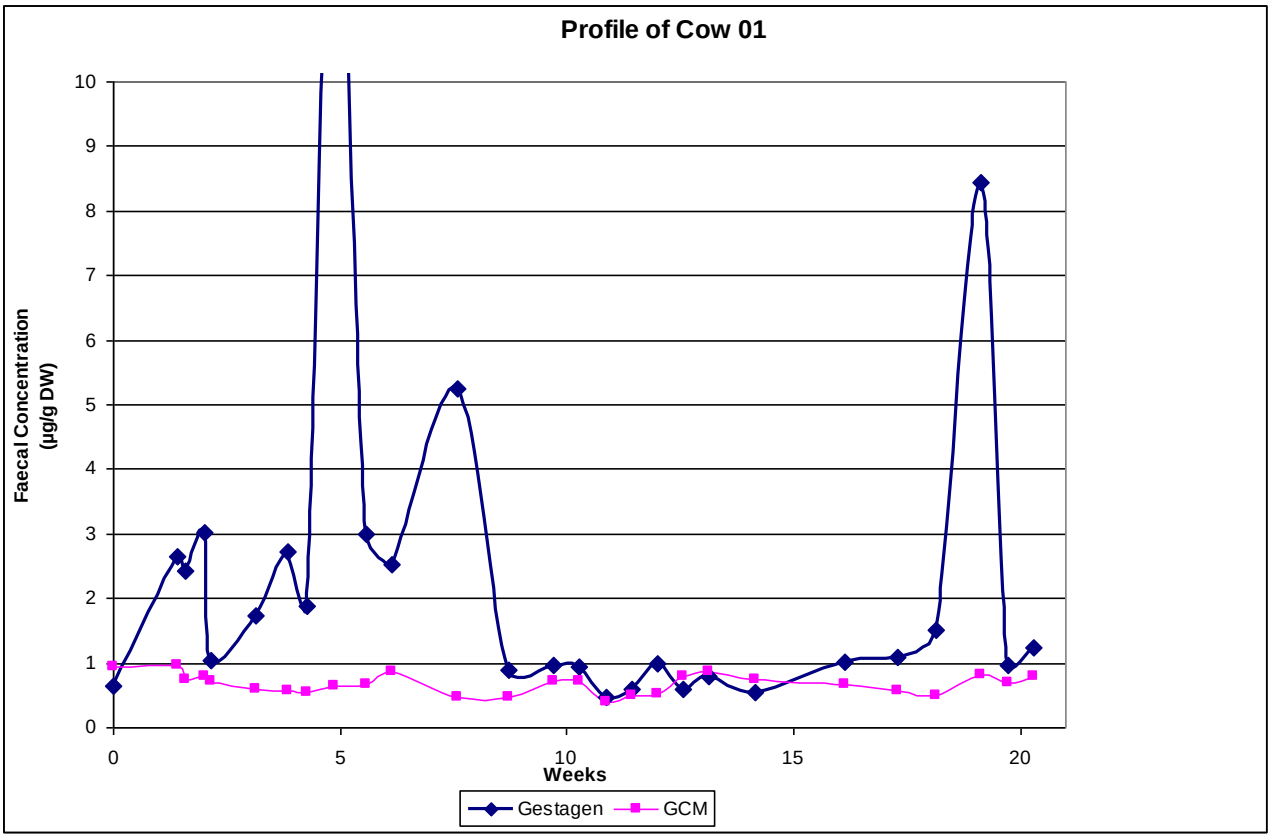

Figure 3. Gestagen and Glucocorticoid metabolite (GCM) profile of cow 1. 27 samples were analysed.

The glucocorticoid metabolite concentrations of Cow 1 were irregular and showed no obvious link with progesterone metabolite concentrations, as shown in Figures 3 and 4. The mean glucocorticoid metabolite concentration was 0.68 µg/g DW, with minimum and maximum concentrations 0.39 µg/g DW and 0.97 µg/g DW, respectively, and a standard deviation of 0.15 µg/g DW (n=27). Pearson's correlation coefficient for the relationship between progesterone and glucocorticoid metabolite concentrations was 0.017, confirming the absence of a significant correlation (p= 0.935 > 0.05, n=27).

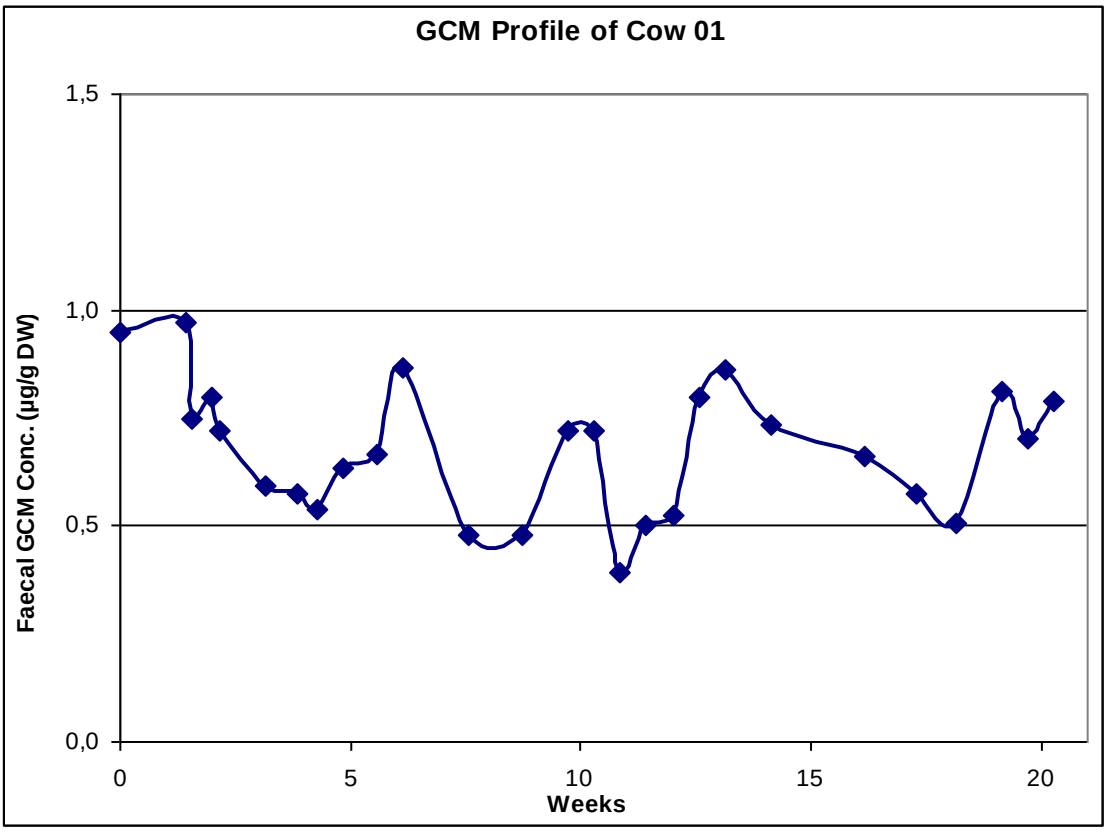

Figure 4. Glucocorticoid metabolite (GCM) profile of cow 1. 27 samples were analysed.

The second cow (cow 02) was an estimated 24 years old. For this animal, several peaks in the faecal 5α-P-3OH concentrations were detected (Figure 5 and Table 2). The values in week 1 were both below 1.00 µg/g DW. In week 2, both concentrations were above 1.00µg/g DW (2.59 µg/g DW and 1.14 µg/g DW). In the second half of week 4 until week 12 the gestagen concentrations in the faeces exceeded 1.00µg/g DW. From week 13 until week 19, the concentrations were generally low (<1.00 µg/g DW), with the exception of week 14 (1.15 µg/g DW).

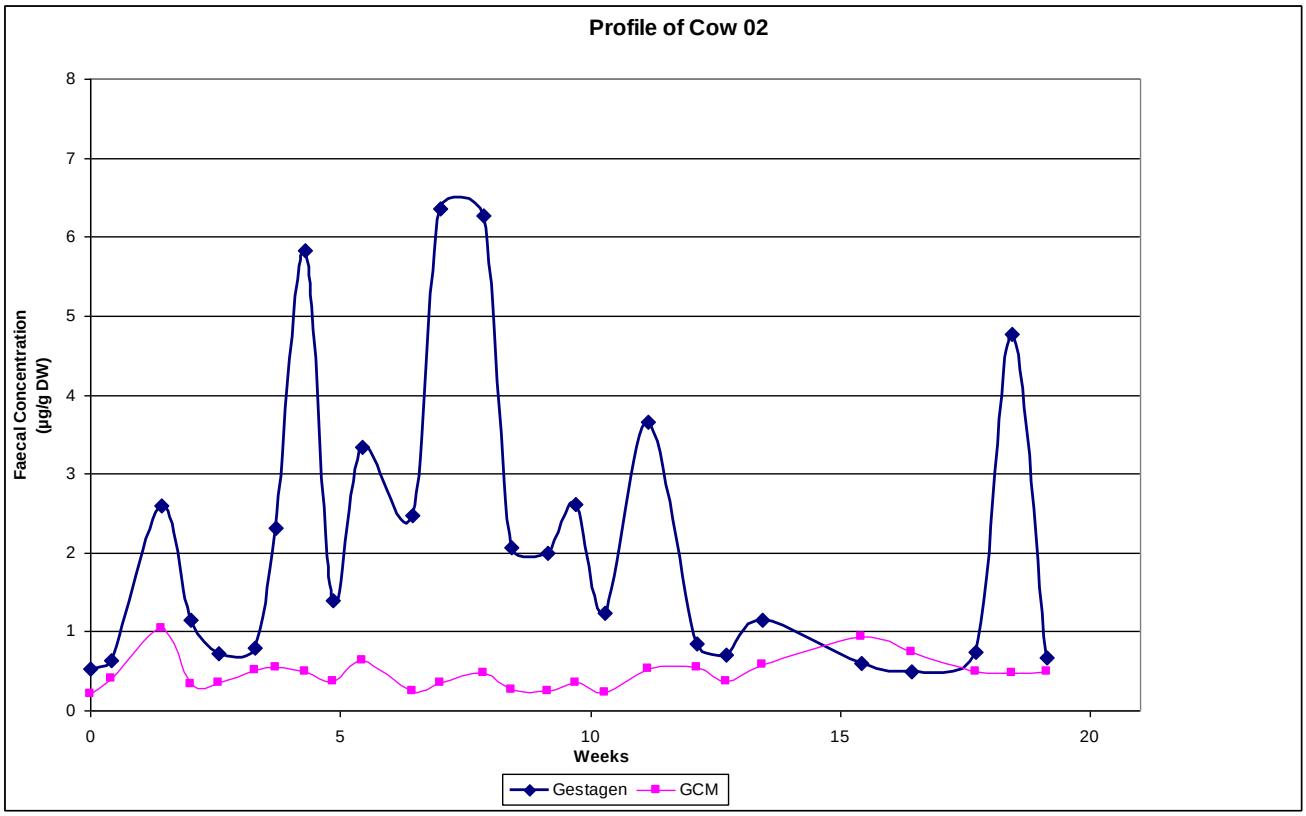

Figure 5. Gestagen and Glucocorticoid metabolite (GCM) profile of cow 2. 26 samples were analysed.

The glucocorticoid metabolite profile for Cow 2 also showed little obvious pattern over time, as shown in Figures 5 and 6. The mean glucocorticoid metabolite concentration was 0.47 µg/g DW, with minimum and maximum concentrations of 0.22 µg/g DW and 1.03 µg/g DW, respectively, and a standard deviation of 0.20 µg/g DW (n=26). Figure 5 does however suggest a possible correlation between glucocorticoid metabolite and progesterone metabolite concentrations. However, overall the Pearson's correlation coefficient (-0.021) suggested no significant correlation (p= 0.917 > 0.05, n=26). Because the samples (n=4) taken from the second half of week 13 (in Table 2 week 13.43) to week 17 (in table 2 week 17.71), appeared to be negatively correlated these four measurements were re-analyzed using the Pearson's correlation test. The outcome (-0.544) also failed to reach statistical significance (p= 0.456 > 0.05, n=4).

Analysing all samples (n=53) for a correlation between faecal progesterone and glucocorticoid metabolites also yielded a negligible correlation (0.016) that did not achieve significance (p= 0.908 > 0.05, n=53).

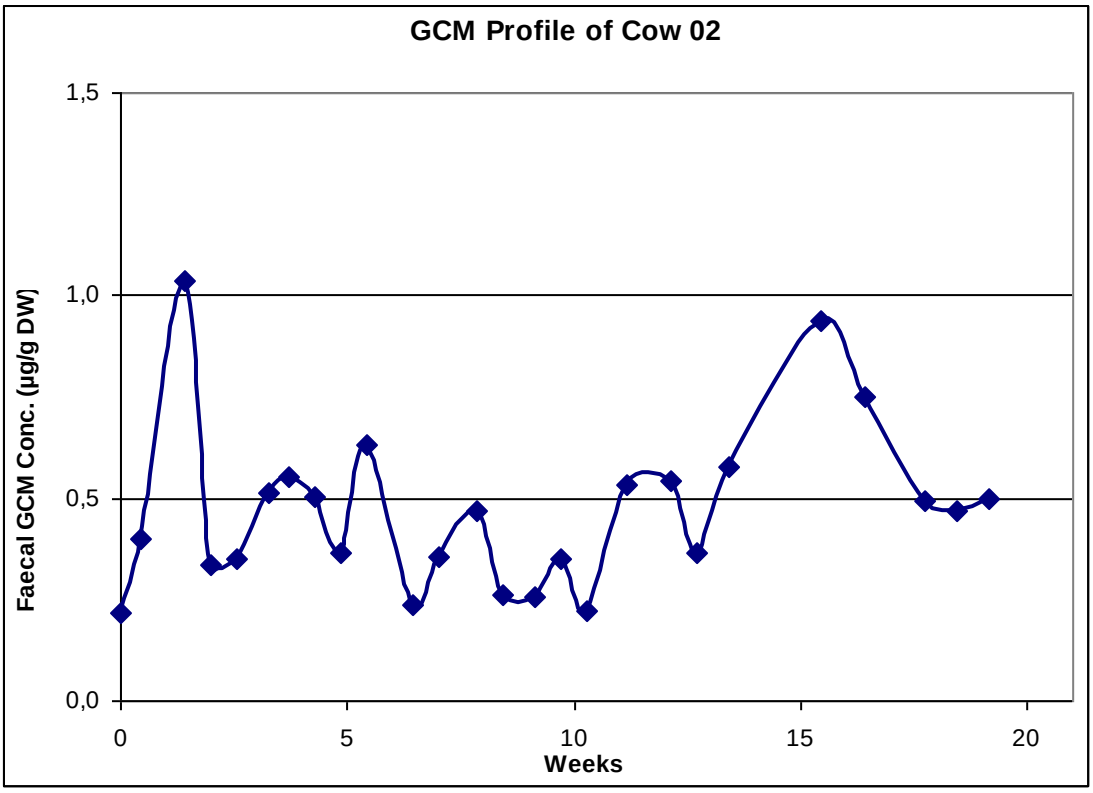

Figure 6. Glucocorticoid metabolite (GCM) profile of cow 2. 26 samples were analysed.

Cow 1 Weeks	Gestagen conc. (µg/g DW)	GCM conc. (µg/g DW)	Cow 2 Weeks	Gestagen conc. (µg/g DW)	GCM conc. (µg/g DW)
0	**0,65**	**0,95**	0	**0,53**	**0,22**
1,43	**2,65**	**0,97**	0,43	**0,63**	**0,40**
1,57	**2,43**	**0,75**	1,43	**2,59**	**1,03**
2,00	**3,02**	**0,80**	2,00	**1,14**	**0,34**
2,14	**1,04**	**0,72**	2,57	**0,73**	**0,35**
3,14	**1,73**	**0,59**	3,29	**0,80**	**0,52**
3,86	**2,72**	**0,58**	3,71	**2,32**	**0,55**
4,29	**1,89**	**0,54**	4,29	**5,82**	**0,50**
4,86	**14,35**	**0,63**	4,86	**1,39**	**0,37**
5,57	**3,01**	**0,66**	5,43	**3,33**	**0,63**
6,14	**2,53**	**0,87**	6,43	**2,47**	**0,24**
7,57	**5,24**	**0,48**	7,00	**6,35**	**0,35**
8,71	**0,90**	**0,48**	7,86	**6,27**	**0,47**
9,71	**0,96**	**0,72**	8,43	**2,07**	**0,26**
10,29	**0,95**	**0,72**	9,14	**2,00**	**0,26**
10,86	**0,48**	**0,39**	9,71	**2,62**	**0,35**
11,43	**0,58**	**0,50**	10,29	**1,23**	**0,22**
12,00	**0,99**	**0,52**	11,14	**3,66**	**0,53**
12,57	**0,60**	**0,80**	12,14	**0,85**	**0,54**
13,14	**0,79**	**0,86**	12,71	**0,70**	**0,37**
14,14	**0,53**	**0,74**	13,43	**1,15**	**0,58**
16,14	**1,02**	**0,66**	15,43	**0,61**	**0,94**
17,29	**1,08**	**0,57**	16,43	**0,50**	**0,75**
18,14	**1,51**	**0,51**	17,71	**0,74**	**0,49**
19,14	**8,44**	**0,81**	18,43	**4,77**	**0,47**
19,71	**0,98**	**0,70**	19,14	**0,67**	**0,50**
20,29	**1,23**	**0,79**			

Table 2. Progesterone (Gestagen) and Glucocorticoid (GCM) metabolite concentration (µg/g DW) profiles for cows 1 and 2. Days of sampling differ between cow 1 and 2.

5. Discussion

This study examined faecal progesterone and glucocorticoid metabolite concentrations in two fertile non-pregnant elephant cows over a 20 week period. The progesterone profile of cow 1 can be interpreted as displaying a 9.5 week luteal phase (weeks 2-11; progestagen concentrations above 0.9 µg/g DW), and a 4.5 week follicular phase (weeks 11.5-15; progestagen concentrations below 0.79 µg/g DW). For cow 2, it was more difficult to propose a definitive reproductive profile in terms of a luteal and a follicular phase; although it is clear that luteal activity was present during weeks 3.5-11.5 while weeks 13.5-18 appear to represent the follicular phase, the onset and end of the luteal phase were more difficult to discern.

As seen in table 3, the correlations between the faecal progesterone and glucocorticoid metabolite concentrations were low (1 indicates a 100% correlation). Even when we tried to make the sample size bigger (analyzing all samples; n=53), there appeared to be a very low correlation between the two hormones, knowing 0.016 (p= 0,908 > 0.05; n=53).

	Correlation	***p***	**n**
Cow 1	0.017	0.935	27
Cow 2	- 0.021	0.917	26
All samples	0.016	0.908	53
Cow 2 (week 0 – 13.43)	0.174	0.45	21
Cow 2 (week 13.43 - week 17.71)	- 0.544	0.456	4

Table 3. Correlations between Gestagen and GCM analysed using Pearson's test.

However, in the presumed oestrus period of cow two (weeks 13.43 to 17.71) there was a reasonable correlation (-0.544) between faecal progesterone and glucocorticoid metabolite concentrations, although this did not reach statistical significance (p= 0.456).

The overall pattern of faecal progesterone metabolite concentrations differed from those described by Fieβ *et al.* (1999) in that the low follicular phase values were lower than they recorded (1.5–2.5 μg/g) , even though the maximum luteal phase values (3–10 μg/g) were comparable. The influence of the season in which the samples were collected may explain this difference (Foley *et al.* 2001). The mean faecal glucocorticoid levels were expected to be higher than in previous studies because the samples were collected in the dry season (Foley *et al.* 2001). Nevertheless, the recorded values were lower than concentrations previously reported in pregnant elephants (Foley *et al.* 2001). There may thus be an effect of pregnancy state, although we are unable to prove this since no pregnant elephants were monitored in the present study. Neither is it clear whether the fact that both elephants in the current study were nursing a calf would have affected the glucocorticoid levels.

The results of this study do not support the hypothesis that faecal glucocorticoid and progesterone metabolite levels in the faeces of non-pregnant fertile female African elephants are correlated, or indeed that oestrus cycle stage significantly affects glucocorticoid/stress levels. However, it is impossible to draw firm conclusions on the basis of one cycle / 20 weeks in two individuals.

There are also factors of the present study that could have affected apparent stress levels / glucocorticoid levels in the faeces; the regular feeding and observation presumably interfered with preferred behavior and migration routes. Stressful events and stress reactions in the herd (e.g. profound secretion of temporal glands, extreme flight behavior and excessive trumpeting) were documented, but also didn't clearly correlate with high glucocorticoid levels founding the faeces. Besides this, it is difficult to know how well the faecal glucocorticoid levels represent the occurrence of stressful events. Other possible sources of error include variations in duration of time between defaecation and sampling and differences in sample collection, and of course the low number of samples and animals examined.

6. Conclusion

Although the number of samples analysed in this study was limited, in terms of the longer term study on the effects of GnRH vaccination, it did demonstrate that faecal progesterone concentrations could be used to demonstrate luteal activity and give an impression of cylicity in free-roaming African elephants and should, therefore, be of considerable use for assessing the effects of contraceptive treatments on ovarian activity and the maintenance of infertility. Measuring faecal glucocorticoid levels should be useful for measuring disturbances in adrenal functioning and / or stress and did not appear to be significantly influenced by stage of cycle. Although relationships between faecal glucocorticoid concentrations and human interference in the form of GnRH vaccination may be difficult to interpret (Millspaugh *et al.* 2004), a complete database of the glucocorticoid concentrations of all elephants during long term studies may eventually help determine whether such a treatment is detrimental to welfare. Analyzing more samples from more elephants should give a clearer indication with respect to the existence of a correlation between faecal progesterone and glucocorticoid concentrations.

Wildlife fertility control programs are crucial in Southern Africa, but subject to considerable ethical debate (Kirkpatrick 2007). It is therefore of great value to monitor GnRH vaccination not only in terms of contraceptive efficacy but also in terms of how it may affect individual and group behaviour, welfare and population structure in African elephants.

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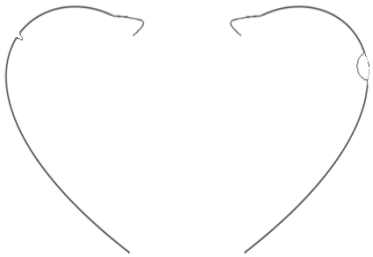
9. Appendix I Identikit of elephant cows number 1 and 2



Elephant No. **1**

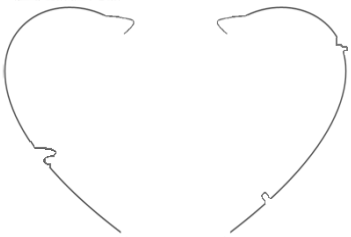
♀ "Mum"

Matriarch

Age: 30 years

Size: Large

Teats enlarged by nursing



Elephant No. **2**

♀

"Scara"

Age: 24 years

Size: Large

Teats enlarged by nursing No. 6