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Induction of anoestrus in free-ranging African elephant (*Loxodonta africana*) cows using a gonadotrophin-releasing hormone vaccine

By

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DECLARATION

I, Gabriela Benavides Valades, do hereby declare that the research presented in this dissertation, was conceived and executed by myself, and apart from the normal guidance from my supervisor, I have received no assistance.

Neither the substance, nor any part of this dissertation has been submitted in the past, or is to be submitted for a degree at this University or any other University.

This dissertation is presented in partial fulfillment of the requirements for the degree MSc. in Production Animal Studies.

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Signed _____

Gabriela Benavides Valades

Date _____

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LIST OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

5α-DHP	5α-pregnane-3,20-dione
5α-P-3α-OH	5α-pregnane-3 α -ol-20-one or 3α-hydroxy-5α-pregnan-20-one
AMH	Anti-Müllerian hormone
CITES:	Convention on International Trade in Endangered Species
CL:	Corpus luteum
DW:	Dried weight
EIA:	Enzyme immunoassay
EPGR:	Entabeni Private Game Reserve
FPM:	Faecal progestin metabolites
FSH:	Follicular stimulating hormone
GnRF:	Gonadotrophin releasing factor
GnRH:	Gonadotrophin releasing hormone
HPLC:	High-performance liquid chromatography
ICI:	Inter-calving interval
ILP:	Inter-luteal phase
IUCN:	International Union of Conservation for Nature
IUDs:	Intrauterine devices
LH:	Luteinizing hormone
LP:	Luteal phase
NT:	Near threatened
pZP:	Porcine zona pellucida
RIA:	Radioimmunoassay
RnRF:	Relative nuclear roundness factor
SD:	Standard deviation

SEM: Standard error of the mean

ZP: Zona pellucida

ABSTRACT

The GnRH vaccine may offer an alternative to the current immunocontraceptive method in elephant cows which uses native porcine *zona pellucida* proteins derived from abattoir slaughtered pigs as the immunogen, greatly limiting its availability. The pZP vaccine is stored at -20 °C and must be mixed with an adjuvant before use. The GnRH vaccine Improvac® is commercially available, already contains the adjuvant and can be stored at 4 °C.

The aim of this study was to evaluate the efficacy of the gonadotrophin releasing hormone (GnRH) vaccine Improvac® (Pfizer Animal Health, Sandton, South Africa) in the induction of anoestrus in elephant cows. The Improvac® was administered to eight adult, female, healthy, free-ranging elephants, located in Entabeni Private Game Reserve in the Limpopo Province, South Africa. Another four cows were left untreated and served as controls.

The monitoring of the experimental population was conducted over a twelve-month observation period via non-invasive faecal steroid analysis. Progesterone metabolites in extracted samples were measured by Enzyme Immunoassay (EIA) to determine luteal activity and thus the effect of the GnRH vaccine on the endocrine correlates. This study started with a three-month control period prior to vaccination when faeces were collected from each study animal, as soon as possible after defecation to ensure positive identification of the individual with its sample. The three-month period was followed by the immunization protocol. The elephants all received a primary, followed by a booster vaccination dose five weeks later. Each dose of 3 ml contained 600 µg of RnRF-protein conjugate; both treatments were applied via remote delivery. Monitoring continued until the end of the twelve-month observation period. Observations of oestrous behaviour during the twelve-month period were also recorded.

The results showed no statistical difference between treated and control females. There was, however, marked individual variation in response to GnRH immunization. This was possibly influenced by physiological and environmental factors such as age, where the youngest cows showed a better response in terms of reduced progestagen secretion; as well as season, where progestagen levels increased 1.3 times during the rainy season compared to the dry season. There was no association between average progestagen concentration and social hierarchy ranking. A high percentage (86.48%) of behaviours that could be related to oestrus coincided with the onset of the luteal phase and a subsequent rise in progestagen concentrations.

All the females (treated and control) showed some evidence of ovarian cyclicity during the study, although 75% of the cycles did not fall within the normal 13-17 week oestrous cycle range reported, suggesting that abnormal cycles are a common reproduction irregularity inherent to non-pregnant wild African elephants.

Further research to determine the optimal vaccination protocol is indicated in order to obtain consistent responses to the vaccine that will provide an efficient and safe contraceptive for use in female African elephants.

CHAPTER 1

INTRODUCTION

The African elephant is widely distributed throughout Africa south of the Sahara (Blanc *et al.* 2003). In central and west Africa, elephant populations remain very threatened by poaching, habitat loss, and civil wars (van Aarde & Jackson 2006). In southern Africa, many years of investment in conservation has lead to a high density of elephants and an increasing population (Slotow *et al.* 2005). The over-abundance of elephants and space constraint can deleteriously affect the environment, reducing food and water availability for elephants and other wildlife species. Elephants that roam beyond conservation areas cause human-elephant conflict (Pimm & van Aarde 2001; van Aarde & Jackson 2007).

Several options are being currently considered in southern Africa to mitigate the overpopulation problem. This includes culling, translocation to under-populated areas, range expansion through establishment of cross border protected areas and protection of migration corridors, and fertility control (Delsink *et al.* 2007). Among the available fertility control options, immunocontraception seems to be the most acceptable as a viable means of population management for controlling small and confined populations of elephants (Delsink *et al.* 2006).

The use of gonadotrophin-releasing hormone (GnRH) vaccine as an immunocontraceptive has been reported to be safe and effective in several domestic and wildlife species (Miller *et al.* 2000; Delves & Roitt 2005; Killian *et al.* 2006). In male elephants the GnRH vaccine has proven to be a safe alternative to suppress aggressive behaviour and musth (De Nys 2005; De Nys *et al*. 2010). In elephant cows it offers another option to the current immunocontraceptive method which uses native porcine *zona pellucida* (pZP) proteins. The GnRH vaccine offers the advantages of being freely available, ready to use, easy to store, as well as being reversible once antibody titres decline (Miller *et al.* 2000; Delves & Roitt 2005; Killian *et al.* 2006). In long-term studies pZP contraception has also been associated in other species with permanent ovarian damage which will be an undesirable effect on elephants (Kirkpatrick *et al.* 1997; Perdok *et al*. 2007).

This was a twelve-month study to determine the efficacy of a GnRH-contraceptive vaccine in inducing anoestrus in eight African elephant cows in the Entabeni Private Game Reserve, Limpopo Province, South Africa. The reproductive status of the cows was monitored through non-invasive faecal progesterone metabolite evaluation and analyzed by means of EIA.

The objectives of this study were three-fold:

1. To monitor the reproductive status and oestrous cycle of twelve elephants cows through non-invasive faecal progesterone metabolite evaluation and behavioural observations.

2. To administer a GnRH contraceptive vaccine by means of remote delivery to eight of these cows.

3. To monitor the effects of the GnRH contraceptive vaccine on the oestrous cycle of cows through faecal progesterone metabolite evaluation.

CHAPTER 2

LITERATURE REVIEW

2.1 History of the African elephants

The African elephant *Loxodonta africana* appeared during the Pleistocene and used to inhabit most of sub-Saharan Africa. At present their distribution is restricted to one third of the continent, mainly associated with average rainfall of the region. In North Africa they became extinct about 1400 years ago and in Eritrea they were exterminated by the beginning of the last century (Spinage 1994).

The African elephant is the largest living terrestrial animal (Macdonald 2001). There are two subspecies of African elephants recognized: the savanna or bush elephant, *Loxodonta africana africana*, and the forest elephant, *Loxodonta africana cyclotis*. Savanna elephants are larger than forest elephants. The savanna elephant is found mostly in eastern, southern and West Africa. It occurs in a wide variety of habitats, such as forests, mopane and miombo woodlands, Sahelian scrub, and even deserts (Blanc *et al.* 2003).

Elephants are herd animals, living in a well structured and complex society, dominated by females comprising an older female, known as the matriarch and her mature daughters and their offspring (Moss and Poole 1983; Estes 1993; Macdonald 2001). Such a group is referred to as a breeding unit or family unit, and family units often join up with other bands of females forming “bond groups” (Burnie 2001).

Male elephants leave their natal group at puberty and live alone or in bachelor herds; being non-territorial, mating success depends on size, weapons, rank and the behavioural and physiological state called *musth.* Musth is a post-pubertal phenomenon, characterized by increased testosterone levels, aggression and heightened sexual activity designed to enhance its mating success with the very scarce females on oestrus (Sukumar 2003; Hollister-Smith *et al.* 2007). Growth continues into old age; older bulls are therefore larger and the biggest tuskers (Poole 1989; Estes 1993; Macdonald 2001; Hollister-Smith *et al.* 2007).

For millennia when human population pressures were low in Africa, elephants and man co-existed without any problems. The demand for ivory started with the European colonization of Africa and increased until the 19th century (Sugg & Kreuter 1994), causing a catastrophic decline of 80% of the elephant population by the 1970’s and 1980’s as high demand for ivory made poaching as profitable as drug dealing (Estes 1993).

The worldwide concern over the elephants' predicament resulted in most major importing countries banning trade in ivory. This led to a dramatic drop in the price of raw ivory in 1990 causing a decline in the poaching of elephants in East Africa (Estes 1993). The African elephant is classified as Near Threatened (NT) on the IUCN Red List (IUCN 2008), and listed in Appendix II in the Convention on Migratory Species (Convention on Migratory Species 2006). Also listed in CITES Appendix I in 1989, but the populations of Botswana (1997), Namibia (1997), South Africa (2000) and Zimbabwe (1997) have since been transferred back to Appendix II (CITES 2008).

Elephants continue to roam the African continent, but remain under threat from poaching and habitat loss in the face of expanding human populations.

2.2 Current status in southern Africa

With the banning of the ivory trade, effective protection from illegal hunting and human encroachment, elephants were forced to move, concentrate and remain in protected areas, where they were provided with a permanent water supply and, under these conditions, the African savanna elephants started to recover in numbers (Hanks 2006). This has mainly been in the southern part of Africa where 70% of Africa's elephants are currently found (Fayrer-Hosken *et al.* 2000; Blanc *et al.* 2003). In southern Africa, the population is increasing at 4 – 5% *per annum* (Slotow *et al.* 2005). Most of these populations are fragmented and are mostly confined to formal fenced protected areas, which correspond to 16% of their original distributional range (Nowak 1999; Blanc *et al.* 2003). These factors have a pronounced effect on the natural movements of elephants (van Aarde & Jackson 2007). Under these circumstances, controlling local elephant numbers has become an inevitable management necessity (van Aarde & Jackson 2007).

2.3 Effects of over-abundance of elephants

The space constraint in this herd animal will result in limiting natural behaviour associated with group dynamics, which will possibly result in inbreeding, overpopulation, and inter-aggression (Patton *et al.* 2007). As regarding for their habitat, the high densities will possibly cause habitat degradation with a consequent negative impact on the many species that share the same environment, potential spread of pathogenic infectious diseases, conflict with humans communities that border conservation areas, and the possibility of population crashes affecting over-abundant fauna or wildlife populations near urban areas (Pimm & van Aarde 2001; Cooper & Larsen 2006).

2.4 Reproduction of African elephants

Loxodonta africana is a long-lived species with a prolonged period of sexual immaturity and a relatively slow rate of reproduction (Moss 2001). The elephant is polygynous and sexually dimorphic, as a fully-grown male will weigh twice as much as a fully-grown female (Sukumar 2003). The sex ratio in the adult population is biased towards females, as males suffer higher death rates being more susceptible to nutritional stress, diseases and male-male competition (Sukumar 2003).

2.4.1 Puberty

Puberty is considered in females to be the onset of the first oestrous cycle, evidenced by the development of a large follicle; and sexual maturity is the age at first ovulation, evidenced by the presence of at least one *corpus luteum* (CL) (Sukumar 2003). There may be a difference of 2-4 years between puberty and sexually mature female elephants. In wild African elephant females, puberty is reached at about 10 to 12 years old, sexual maturity varies between 11 to 14 years of age (Sukumar 2003; Bertschinger *et al.* 2008; Lueders *et al.* 2010), mainly associated with level of nutrition, density, local climate, and social structure, ranging from 7 to 22 years (Hanks 1972; Laws *et al.* 1975*;* Sukumar 2003; Delsink *et al.* 2006). The age at first parturition ranges from 9 to 18 years (Owen-Smith 1988; Garaï 2005).

In males, puberty is considered to occur when sperm production begins (Whyte 1996) at around 12 to 15 years of age (Laws & Parker 1968; Garaï 2005), and this occurs later than for female elephants. They only begin competing with older males for oestrus females when they are between 20 and 25 years of age, showing both sexually active and inactive periods; and when they reach 30 years of age, males have usually exhibited their first musth period and will reach their prime at about 45 years old (Poole 1987; Poole 1996).

2.4.2 Oestrous cycle, oestrus and mating behaviour

Elephants are considered to be non-seasonal, polyestric and uniparous breeders (Hildebrandt *et al.* 2010); although there is a peak in conception rates and births during the rainy season, when resources are abundant (Poole 1987; Estes 1993; Foley *et al.* 2001; Wittemeyer *et al*. 2007a).

The oestrous cycle length on elephants has been reported to last from 13 to 17 weeks (First reports, Plotka et al. 1988). This consists of a luteal phase which lasts 6-12 weeks, followed by the inter-luteal

or follicular phase ranging from 4-6 weeks (Plotka *et al.* 1988; Heistermann *et al.* 1997; Schwarzenberger *et al.* 1997; Hodges 1998; Fieβ *et al.* 1999; Brown 2000; Ortolani *et al.* 2005; Brown 2006; Bertschinger *et al.* 2008a; Hildebrandt *et al.* 2010). Elephants are a monovular species, meaning that a single follicle is ovulated, however multiple non-ovulatory CL are present in every cycle. Usually a fertile cycle will result in pregnancy, thus consecutive non-conceptional cycles are a rare feature in free-ranging cows (Rasmussen and Schulte 1998; Hildebrandt *et al.* 2010).

The reproductive biology of female elephants is not fully understood. Hodges (1998) proposed a model of the ovarian cycle, which has been updated by Brown (2000) (Figure 1). This model proposes that during the luteal phase, the elevated levels of progestins from the ovary inhibit follicular development and LH release. Follicular activity resumes when progestins decrease and FSH from the pituitary gland increase at the beginning of the inter-luteal phase, which recruits follicles and initiates two successive waves of follicular development, each about three weeks in duration, which culminates in a distinct LH peak. The follicles that develop during the first wave regress and form a CL after the first or anovulatory LH surge and become steroidogenically active later in the cycle. The second follicular wave results in the formation of one large Graafian follicle that ovulates about 24 hours after the second or ovulatory LH surge. The elevated oestrogens, which have low concentrations on elephants, may trigger the LH surges during each follicular wave. The Graafian follicle may also secrete small amounts of progestins in the period leading up to ovulation. After ovulation, the progestin levels rise along with the maturation of the CL, followed by a gradual rise in FSH that peaks at the end of the luteal phase. Inhibin produced by the follicles is negatively correlated to FSH secretion, reaching maximum values during the mid-luteal phase.

The behavioural oestrus and associated oestrus signs have been reported to last from 2 to 8 days in which the female can be mounted by different males (Moss 1983; Western & Lindsay 1984). Subordinate bulls may attempt to mate around the time of the first LH surge, but the more experienced, high-ranking and musth bulls only mate 2-3 days up to and including the day of the second LH peak in the mid-oestrus when ovulation and conception occurs (Poole 1987; Poole 1996; Lueders *et al.* 2010). Mating would be dependant on male dominance hierarchies and female choice of mates (Sukumar 2003).

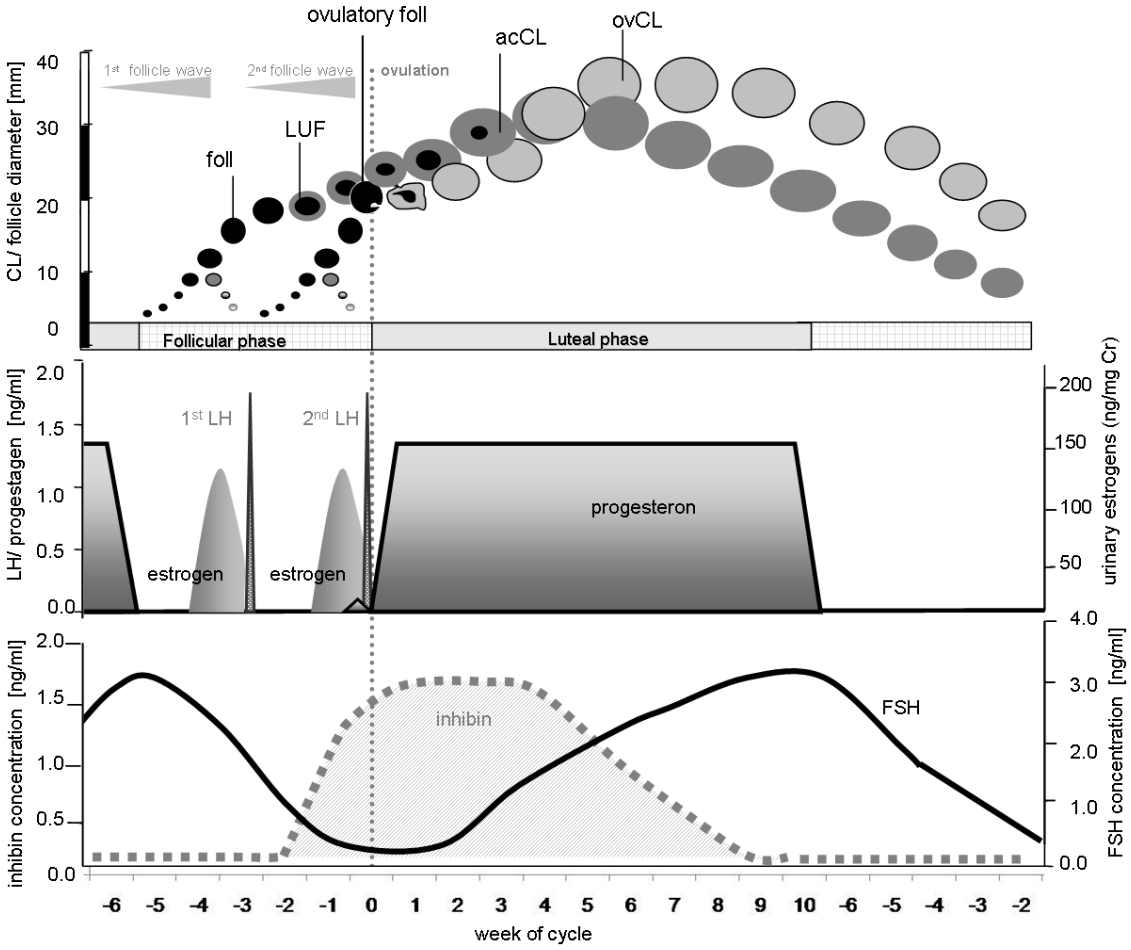

Figure 1. Scheme of events during the oestrous cycle: ovarian activity, development of follicles (foll), luteinizing follicles (LUF), accessory CLs (acCL) and ovulatory CL (ovCL), in relationship to steroid hormone (progestagens, oestrogen), inhibin and gonandotrophin (luteinizing hormone [LH] and follicle stimulating hormone [FSH]) secretion (Lueders & Hildebrandt 2010).

Oestrus females attract males by displaying distinct behaviour, auditory and chemical signals (Poole 1996). Oestrus behaviour has been described by Moss (1983) in four different stages:

1. *Wariness:* A cow is noticeably alert and wary of approaching bulls, quickly moving out of their way and not tolerating attempts to test her reproductive condition. She carries her head high and gazes towards other bulls following her (Moss 1983). Females may advertise their status by slapping the tip of their tail against the urogenital region and holding it high in the air for a while (Sukumar 2003).

2. *Oestrus walk:* In response to approaching bulls, an oestrus female may walk back and forth; she arches her back, tail raised and head held at an angle. When males arrive she increases vocalizations (Moss 1983).

3. *The chase:* An oestrus cow may increase her pace to a run and may be pursued by one or more bulls. The cow usually describes a wide arch-shaped trail, which may last hours, before returning to her group. However, she may stop if a bull succeeds in touching her, and the bull will attempt to mount her (Moss 1983).

4. *Consortship:* After the male is able to touch the female with his trunk, he places his trunk on her back, resting his head and tusks on her rump, and stands in his rear legs. The female remains still during intromission, which lasts about 40 seconds. An individual cow and a large bull may maintain physical proximity for a short period. The large bull chases away other bulls that approach the pair, while the cow avoids other bulls by moving toward her partner (Moss 1983).

Females in oestrus will perform auditory signals, calling loudly and frequently prior to ovulation, using low-frequency acoustic signals with a range of up to 8 km, which attract the bulls over long distances (Leong *et al.* 2003). A female elephant also advertises her condition through chemical signals, releasing pheromones through urine or vaginal secretions (Sukumar 2003; Hildebrandt *et al.* 2010). An elephant bull may regularly test the urogenital orifice or the urine of a female, but the frequency of flehmen responses will increase about 10-fold when a cow is in oestrus (Sukumar 2003). Other indications of oestrus include reddening of the clitoris, which can be noticed at the vestibular opening when the cow is urinating or relaxed (Hildebrandt *et al.* 2010).

2.4.3 Inter-calving interval

The inter-calving interval (ICI) is the mean interval between successive calves, and it takes into account the duration of pregnancy (22 months in African elephants) and the length of lactational anoestrus (Hanks 1972). It has been considered to be the most important parameter influencing the growth rate of an elephant population (Hanks & McIntosh 1973; Bertschinger *et al.* 2008). As with the age of maturity, the ICI can also be expected to vary with environmental conditions and density, increasing with high-density and/or nutritionally stressed populations (Moss 2001; Sukumar 2003), which will potentially slow the population growth rate (Laws *et al*. 1975). ICI from several

populations throughout Africa ranged from 2.9 to 9.1 years (Eltringham, 1982; Moss 2001), the more typical ICI is four years, from which females move into a synchronous wave pattern with birth peaks every four years under optimal conditions (Moss 2001; Sukumar 2003; Garaï 2005). The sex ratio of calves at birth is 50:50 (Hanks 1972; Poole 1996; Moss 2001).

Birth interval will be extended with the survival of the calf over two years of age (Moss 2001) and also, the ICI will be 2-5 months longer on average if the calf is a male than when the cow has a female, possibly because of the higher investment needed for nurturing a male calf (Lee & Moss 1986; Sukumar 2003).

2.4.4 Lactational anoestrus

After successful parturition, a cow would be in lactational anoestrus for about 2 to 2.9 years before resuming oestrous cyclicity (Whyte 1996; Sukumar 2003; Garaï 2005). This is likely mediated by high levels of prolactin which accompany lactation which suppresses GnRH and/or LH (Friesen 1977). This period is characterized by low progestagen concentrations (Hodges 1998; Brown 2000). Under normal conditions fertile cows would lactate from one calf to the next (Bertschinger *et al.* 2008), although there are some reports of cows lactating with a calf for up to eight years (Laws 1969; Poole 1996).

Some reported factors that may shorten the duration of the lactational anoestrus for up to 8 weeks are: retained placenta, reduced milk production, calf mortality, and premature weaning (Brown 2000).

2.4.5 Reproductive senescence

Elephant females seem to be able to reproduce until the end of their lifespan, with a reproductive peak at about 20 years; although there is a sharp decline in fecundity after 50 years of age (Owen-Smith 1988; Moss 2001). Reproductive senescence or complete cessation of reproduction has been reported in some individuals possibly as a consequence of a combination of uterine defects and a reduction in oocyte numbers (Hanks 1972).

It has been proposed for other long-lived species, such as whales and humans that the cessation of ovulation late in life could be related to the length of time needed for the last offspring to reach puberty, but clear documentation in elephants is lacking (Moss 2001; Asa 2010).

2.5 Methods of population control

In a fenced environment where the population growth exceeds the habitat´s carrying capacity, some form of population management is indicated. Some aspects that must be taken into account when considering methods of population control is the effect of density on age of sexual maturity, ICI and incidence of anoestrus (Bertschinger *et al.* 2008). The traditional control methods used in elephants include culling, translocation and fertility control (Delsink *et al.* 2007; van Aarde & Jackson 2007).

2.5.1 Culling

Culling is the traditional method that has been used to control overpopulation of elephants. It involves the shooting of entire family groups, including infants, juveniles, and adults simultaneously (Hutchins 2005; Slotow *et al.* 2008). Culling is an effective but controversial control strategy and has now become ethically unacceptable to the general public (Delsink *et al.* 2006). Elephants are intelligent and empathetic mammals and understand the concept of death. Family members that are 'missed' during a cull may result in severe psychological long-term stress for these survivors (Delsink *et al.* 2007; Gobush *et al.* 2007).

Culling has also a marked effect on population density, and although it decreases numbers temporarily, when it stops it leaves more resources available for the remaining individuals which then grow and breed at a maximal rate (Caughley 1983; van Aarde *et al.* 1999; Whyte *et al.* 2003; van Aarde & Jackson 2007). This can be supported by the findings of Laws (1969) and Hanks (1972) who showed that reproductive parameters were affected by population density and resource availability, in which parameters such as ICI, age at first ovulation and percentage of anoestrus cows (≥25 years and not lactating) were shortened at lower densities, which resulted in a faster population increase.

Culling should only been considered as a management program if high elephant densities persist for two consecutive years, as the populations often decline the following year without intervention when competition for resources limits growth rates. Culling should consequently be regarded as the last resort for controlling over-population (van Aarde *et al.* 1999).

2.5.2 Translocation

The movement of individual elephants or breeding units from one area to another is both costly and complicated because of the size and weight of the species (Fischer & Lindenmayer 2000; Grobler *et al.* 2008). Translocation of large numbers of elephants is unrealistic and impractical and there are very few areas large enough to receive them and allow population growth (Delsink *et al.* 2006; Delsink *et al.* 2007). Translocation may also enhance growth rates by decreasing population densities and altering reproductive parameters similar to the post-culling effect (van Aarde & Jackson 2007); e.g. relocated populations tend to present onset of puberty at an earlier age (7-8 years old) compared to normal parameters (10-12 years old) (Delsink *et al.* 2006).

There is a potential for an undesirable impact when translocations are from larger to smaller reserves (Garaï *et al.* 2004). In addition to the problems of transportation, reintroduced elephants may exhibit behavioural abnormalities due to disruption of their social structure (Garaï *et al.* 2004; Bradshaw *et al.* 2005). e.g. the two herds at Entabeni Safari Conservancy were originally translocated from Kruger National Park plus the Lower Escarpment herd was relocated again from Shambala Private Reserve, these resulted in both herds being extremely nervous, running away from game drive vehicles and avoiding open spaces; together with these behavioural changes, the Lower Escarpment herd lost one cow after injury during transport from Shambala to Entabeni, plus it has one cow that doesn´t seem to belong to any family unit (Benavides, personal observation).

2.5.3 Fertility control

Fertility control has been achieved through different contraceptive methods in different species. The ideal contraceptive method should:

- be highly effective,
- be safe,
- be inexpensive,
- have a prolonged duration of action,
- be potentially reversible,
- be easily accessible,
- require infrequent administration,
- be compatible with private use,
- be deliverable from a distance,

- cause no changes to social behaviours and group integrity,
- not pass through the food chain, and
- be safe during pregnancy

(Kirkpatrick & Turner 1996; Perdok *et al.* 2007; Bertschinger *et al.* 2008a)

2.5.3.1 Contraceptive methods used in wildlife

The different contraceptive methods used in zoo and wildlife management include surgical sterilization, mechanical contraception, hormonal methods, and immunocontraception (Patton *et al.* 2007).

- *Surgical sterilization* is, for practical purposes, irreversible but highly effective. Techniques in males comprise vasectomy and castration. A reversible vasectomy, in which the end of the *vas deferens* is not tied, potentially allows for reversal using microsurgical techniques. In females the techniques include the removal of gonads (ovariectomy), removal of the entire genital tract (ovariohysterectomy), and ligation of the oviducts (salpingectomy). Reproductive and territorial behaviour and secondary sexual characteristics will be altered by removal of the gonads (Asa 2005; Patton *et al.* 2007).

- *Mechanical contraception* is achieved by placing foreign objects into the uterus, which was common practice in ancient times. More recently, application of non-medicated copper intrauterine devices (IUDs) has been tested in some domestic species, but was only reported to be successful in feral horses (Daels & Hughes 1995)

- *Hormonal methods* (Figure 2) include the use of steroid hormones such as progestins given orally, in slow-release implants or in the form of depot-injections. Progestins are not recommended for use in pregnant animals because of the potential for inducing prolonged gestation, stillbirth and abortion in some species (Patton *et al.* 2007). Oestrogens and androgens have been associated with serious side effects and are not recommended for wildlife (Asa 2005). GnRH-agonists administered either as implants or depot-injections have been used successfully as contraceptives in a variety of carnivores for periods of 30 months or longer (Bertschinger *et al.* 2008b). They downregulate LH and FSH release and thus reproductive function in either sex; although in some species it is not effective in males. Females treated with a GnRH-agonist should be considered fertile for the first three weeks after treatment, as an initial surge of oestradiol and transient rise in progesterone

metabolites that can cause oestrus behaviour has been observed (Asa 2005; Patton *et al.* 2007; Bertschinger *et al.* 2008b).

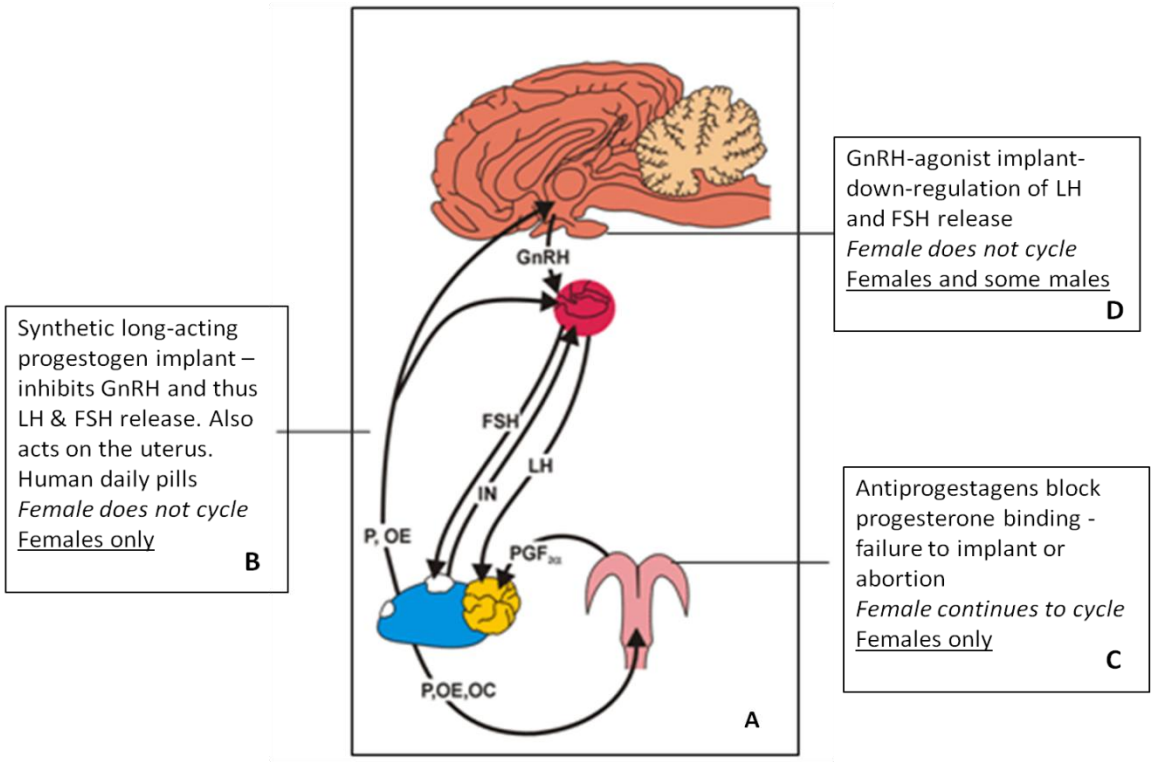

Figure 2. Endocrine control of ovarian function (A) and the mechanisms by which synthetic progestins (B), antiprogestagens (C) and GnRH super agonists (D) exert their contraceptive effects (modified from Bertschinger 2010).

- *Immunocontraception* involves stimulating immune responses that interfere in key aspects of reproduction, without the side-effects of hormonal contraceptives (Fayrer-Hosken *et al.* 2000; Cooper & Larsen 2006). The contraceptive vaccines target reproductive hormones or gametes. Vaccines against reproductive hormones such as gonadotrophin-releasing hormone (GnRH) and follicle-stimulating hormone (FSH) affect gamete production, block ovulation and induce anoestrus. Vaccines against gametes target sperm antigens and oocyte *zona pellucida* (ZP) affecting gamete function (Naz *et al.* 2005). Immunocontraception is a developing field for control of domestic and wildlife populations, as well as sex-related behaviours (Pimm & Aarde 2001; Naz *et al.* 2005; Cooper & Larsen 2006; Delsink *et al.* 2006).

2.5.3.2 Methods used in elephants

Surgical sterilization requires full immobilization of the animal (Patton *et al.* 2007) which is impractical for elephants, particularly in large populations. Furthermore it is invasive, expensive, and reversal requires highly skilled microsurgical techniques which are again impractical in elephants (Bertschinger *et al.* 2008a).

Hormonal methods in the form of synthetic steroids are not a consideration in elephants, as steroids are excreted in the faeces and may enter the food chain and could possibly affect reproductive performance of other species exposed to the elephant faecal material (Bertschinger *et al.* 2008a). Slow-release oestradiol silicone implants have been tested in elephant cows resulting in major undesirable side effects related to a permanent state of oestrus, which leads to aberrant behaviour by separation of individuals within the family unit (Fayrer-Hosken *et al.* 2000). Moreover the practicality and expenses related to administration of oestradiol implants in free-ranging elephants makes it a non-viable option (Fayrer-Hosken *et al.* 2000; Bertschinger *et al.* 2008a). Currently, GnRH super-agonist implants are being tested in several species and a remote delivery system that avoids immobilization is also being developed (Bertschinger *et al.* 2008a).

Immunocontraception seems currently to be the most acceptable method amongst the available options for controlling free-roaming African elephants. Of the contraceptive vaccines tested in African elephants the porcine *zona pellucida* (pZP) vaccine has proven to be effective, safe, reversible, and remotely deliverable (Fayrer-Hosken *et al.* 2000; Delsink *et al.* 2006). Trials on seven reserves in South Africa (n=108) achieved a contraceptive efficacy of 100%, births were reported to have ceased three years after inception of the pZP program in all treated cows (Delsink *et al.* 2007; Bertschinger 2010). Social disruption as a result of vaccine delivery (ground or aerial vaccinations) was minimal and no effects on social behaviour could be determined. The pZP-vaccine is an effective contraceptive that can be used as part of a long-term management strategy in small to medium-sized free-ranging populations of African elephants (Delsink *et al.* 2002; Delsink *et al.* 2007). The disadvantages of using pZP-immunocontraception is that cows continue to cycle normally, the production of pZP vaccine is labour intensive and the amounts that can be produced are limited by the availability of slaughter house material, plus the vaccine has to be stored at -20 °C and must be mixed with an adjuvant before use which is impractical on field conditions (Bertschinger *et al.* 2008a). For the treatment of large populations a slow or sequential release formulation is needed (Bertschinger 2010). In long-term studies with other species, pZP contraception has been associated with permanent ovarian damage causing irreversibility of the contraceptive (Kirkpatrick *et al.* 1997; Perdok *et al*. 2007).

2.5.3.3 GnRH vaccine

An additional option that shows promise is immunization against gonadotrophin-releasing hormone (GnRH). This decapeptide is a hormone in mammalian reproduction, secreted by the hypothalamus and that promotes the release of both luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary (Figure 3), thereby controlling the endocrine functions of the gonads and the production of male and female gametes (Delves 2005). The GnRH has remained practically unchanged through evolution and has been conserved across many animal species, being identical in all mammals (Miller *et al.* 2004; Delves & Roitt 2005*)*. A number of vaccines based on mammalian GnRH (or a modified form of the hormone) coupled to a carrier and then combined with an adjuvant to create the vaccine, have proved successful on previous trials as contraceptive and controlling sex-related behaviour in rats, pigs, cattle, deer, horses, sheep, squirrels, dogs, and cats (Becker & Katz, 1993; Ladd *et al.* 1994; Miller *et al.* 1997; Oonk *et al.* 1998; Miller *et al.* 2000; Dunshea *et al.* 2001; Ferro *et al.* 2001; Dalin *et al.* 2002; Zeng *et al.* 2002; Janett *et al.* 2003; Miller *et al.* 2004; Delves & Roitt 2005; Burger *et al.* 2006; Imboden *et al.* 2006; Killian *et al.* 2006; Stout 2007; Janett *et al.* 2009).

The vaccine stimulates the production of anti-GnRH antibodies and these bind to endogenous GnRH within the hypothalamic-pituitary portal vessels and prevent the molecules from binding to receptors on the pituitary gonadotrophs (Miller *et al.* 2004; Stout *et al.* 2007). The down-stream effects are to inactivate endogenous GnRH and consequently reduce the release of gonadotrophic hormones (LH and FSH) which results on reduced spermatogenesis in the male, or failure of ovulation and/or follicle development in the female. Subsequently gonadal regression and suppression of sexual behaviour and secondary sex characteristics occurs, returning treated animals to a pharmacological prepuberty (Turkstra *et al.* 2003; Stout *et al.* 2004; Perdock *et al.* 2007; Janett *et al.* 2009). It is used as a means of preventing sexual maturation, reducing androgen associated odours, improving carcass characteristics and feed efficiency, as a contraceptive, and reduces mating and aggressive behaviour, which can also prevent the transmission of sexually transmitted diseases (Miller *et al.* 2000; Dunshea *et al.* 2001; Miller *et al.* 2004; Burger *et al.* 2006; Stout *et al.* 2007; Hennesy 2008).

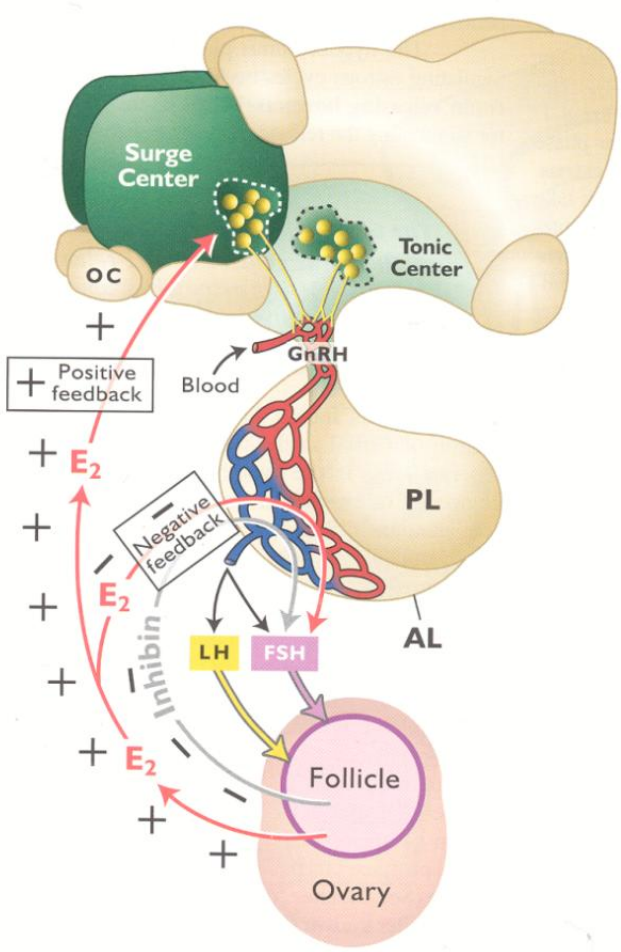

Figure 3. The relationship between the hypothalamus, the pituitary and the ovary during the follicular phase, modified from Senger (2005). (AL, anterior lobe; E_2, oestradiol; OC, optic chiasm; PL, posterior lobe)

Immunization against GnRH seems to be highly specific, and does not appear to affect other hypothalamic-releasing hormones. GnRH-antibody complexes produced in the portal blood are subsequently destroyed by the immune system as they pass through the liver, but the contraceptive effect will continue for as long as there is a continual source of available GnRH antibody (Miller *et al.* 2004). Natural reversal, in the majority of immunized animals, occurs once antibody titres drop below a threshold after an interval of about one to four years, depending on the dose given, the species involved and the age of the target animal (Miller *et al.* 2000; Miller *et al.* 2004; Delves & Roitt 2005; Turkstra *et al.* 2005; Burger *et al.* 2006; Imboden *et al.* 2006; Killian *et al.* 2006; Stout *et al.* 2007).

Because of some species differences, an immunization protocol must be determined for each species (Ladd *et al.* 1994; Ferro *et al.* 2004; Imboden *et al.* 2006; Janett *et al.* 2009). It has to be decided the adjuvant, carrier protein and GnRH peptide used, as well as the administration site,

number of boosters, booster interval, duration of treatment and specifically the dosage (Miller *et al.* 2004; Turkstra *et al.* 2005).

A previous study in male African elephants with a GnRH-vaccine (modified GnRH-tandem-dimer-ovalbumin conjugate, Pepscan Systems, Netherlands) suggested that it can inhibit testosterone secretion and improve behaviour in aggressive bulls, with no evidence of adverse side effects due to the immunization. Thus the vaccine offers a safe option to suppress aggressive behaviour and musth, period in which bulls can be dangerous and difficult to handle (De Nys 2005; De Nys *et al.* 2010).

A potential, but significant advantage of GnRH-immunocontraception over the pZP-vaccine in African elephant cows will be the induction of anoestrus (Bertschinger *et al.* 2008a). With pZP-immunocontraception, cows continue to cycle approximately every 15 weeks, as demonstrated by Bates (2010), and continuously attract bulls which could potentially disrupt the herd (Bertschinger *et al.* 2008a). In addition, research is lacking on the effects of continual infertile mating behaviour, which could possibly cause stress and ultimately health problems (Patton *et al.* 2007). If the GnRH vaccine proves to be successful in female elephants, this problem may be avoided, as it reduces hormone levels to the point that they do not ovulate or come into oestrus (Miller *et al.* 2004). Another advantage is that the GnRH vaccine is synthesized *in vitro,* thus the vaccine production is unlimited and freely available, is made ready for use (already contains the adjuvant) and can be stored at 4 °C.

It has been suggested that immunocontraception may be selecting animals with poor immune systems as the intensity of the biological response is related to the antibodies titres produced (Schanbacher 1984; Malmgren *et al*. 2001; Perdok *et al.* 2007), consequently only immunizing animals that will generate a vigorous immune response, and encouraging the reproduction of individuals with a weak or compromised immune system (Muller *et al.* 1997; Nettles 1997). Other authors explain that the individual response to the immunization is closely related to the dose, adjuvant and route of administration of the vaccine and not to immune competence of the individual itself (Kirkpatrick *et al.* 1997), this can be supported by studies where levels of antibodies against GnRH were measured after the vaccination with GnRH, finding different levels per individual but the response on reducing reproduction and secondary sex characteristics was achieved (Miller *et al.* 2000; Dunshea *et al.* 2001; Killian *et al.* 2006; Janett *et al.* 2009). In female deer this is explained by the level in which the antibodies act, and when does have a high antibody titre they do not ovulate or produce a functioning CL, evidenced by the low progesterone level during the period when ovulation should be occurring having an immunocontraceptive effect. Otherwise, if the

antibody titre is low, the doe will ovulate and conceive but the progesterone produced by the CL will not be enough to maintain pregnancy and thus having an immunocongestive effect (Miller *et al.* 2000).

2.5.3.3.1 IMPROVAC® vaccine

The vaccine IMPROVAC® is commercially manufactured for the swine industry by Pfizer Laboratories. It was initially used in Australia in 1998 and since then has been approved for use in 50 other countries. The antigen in IMPROVAC® is comprised of a synthetic, incomplete analogue of natural gonadotrophin-releasing factor (GnRF) which is covalently linked to a carrier protein, formulated with an aqueous non-oil based adjuvant into a ready-to use-injection (Dunshea *et al.* 2001; Hennesy 2008). The full immunization course consists of an initial priming dose followed by a single booster dose administered four or more weeks later (Hennesy 2008).

The qualitative composition of the vaccine is GnRF analogue covalently conjugated to Diphtheria toxoid molecule. DEAE-dextran is used as the adjuvant and thiomersal (0.1 mg/ml) has been added as a preservative and urea used as solubilizing agent (EMA 2010).

The synthetic GnRF peptide analogue is too small to be an effective immunogen and needs to be coupled to a larger immunogenic molecule in order to provoke an antibody response. Diphtheria toxoid is a stable and safe, highly immunogenic substance that delivers this effect, and is extensively used for vaccination of humans and as carrier protein in vaccines for paediatric use. The conjugation chemistry provides a highly stable linkage between the peptide and the carrier protein, thus contributing to the stability of the product (EMA 2010). The GnRF analogue used on the vaccine contains nine amino acids, differing form the natural GnRF which has 10 amino acids, this modification to the smaller end of the analogue followed by the conjugation to the carrier protein prevents binding to the GnRF receptor in the pituitary and thus devoid of any hormonal activity (Hennesy 2009) (Figure 4A, 4B).

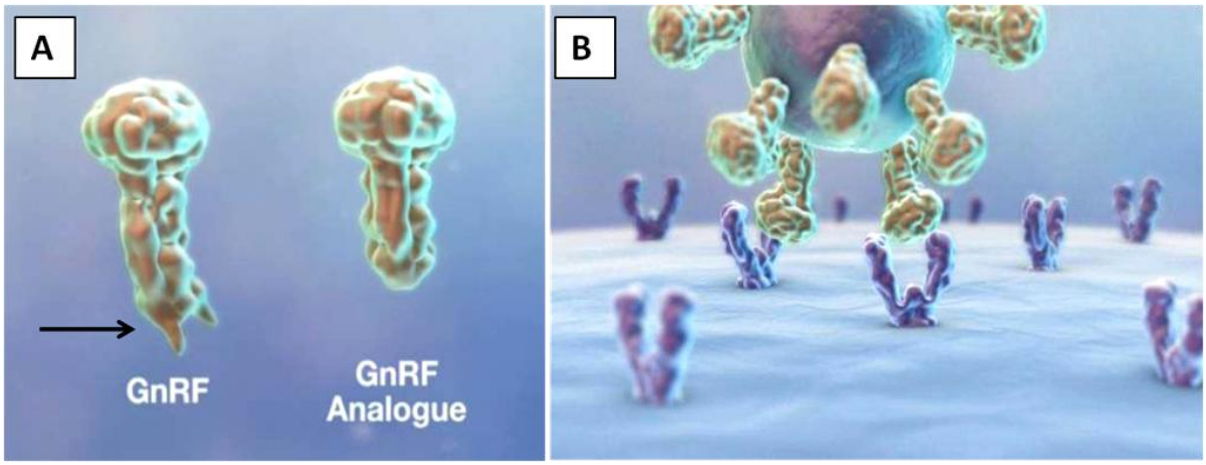

Figure 4. A: Synthetic GnRF analogue on IMPROVAC® vaccine is incomplete compared to the natural GnRF, arrow indicates the area that binds to pituitary receptor.
B: The Improvac® antigen lacks the area to bind to the pituitary receptor and thus has no hormonal activity, modified from Hennesy (2009).

2.6 Non-invasive monitoring of oestrous cycle and pregnancy in elephants

2.6.1 Non-invasive monitoring of the oestrous cycle and pregnancy

Urinary and faecal steroid profiles are the two methods that can be used to non-invasively monitor reproduction in wildlife and domestic species. They offer a practical alternative to invasive methods such as repeated collection of blood samples for hormonal evaluation (Schwarzenberger *et al.* 1996, Schwarzenberger 2007a, 2007b). Invasive monitoring methods are impractical, costly and mostly impossible for application in wildlife, especially where stress is being monitored.

Difficulties associated with urine collection limit its use in free-ranging animals. Urine collection is better suited to zoo conditions. Faecal samples are a more practical choice for evaluating reproductive status provided that the sample can be positively identified with the targeted individual and the interval between voiding and collection is short. Faecal steroid metabolites that can be assayed are oestrogens, androgens, progestagens, and glucocorticoids (Schwarzenberger *et al.* 1996, Schwarzenberger 2007a). Faecal steroid monitoring is currently commonly used and accepted as a diagnostic tool for evaluating reproductive endocrinology in farm, wild and zoo animals. It is used to provide information regarding oestrous cycle length, time of ovulation, pregnancy, abortion, puberty, reproductive behaviour, seasonality, success of contraceptive treatments, oestrus synchronization, and endocrine responses to ovulation-induction protocols (Schwarzenberger *et al.* 1996, Schwarzenberger 2007a).

The excretion of steroids into the gastrointestinal tract is mainly *via* the bile duct. Steroid concentrations in faeces exhibit a similar pattern to that in plasma, but with a species-dependant lag times. The metabolites formed from each group of steroids are species specific. These need to be identified and the specific enzyme immunoassay validated before they can be used for an individual species in order to demonstrate that excreted hormonal measures in faeces accurately reflect physiological events (Schwarzenberger *et al.* 1996, Wasser *et al.* 1996).

Faecal oestrogens determination using specific oestrogen (specific metabolites or total oestrogens) antibodies are reliable indicators for pregnancy diagnosis in those species where the foeto-placental unit is the source of large quantities of oestrogens. They can also be used to monitor the preovulatory oestrogen surge in carnivores (Schwarzenberger *et al.* 1996).

Faecal progesterone-metabolite (FPM) analyses have been successfully used for monitoring luteal function, pregnancy and abortion, puberty and seasonality in several species, as well as to monitor the success of reproductive treatment therapies (Schwarzenberger *et al.* 1996, Wasser *et al.* 1996).

2.6.2 Non-invasive monitoring of oestrous cycle and pregnancy in elephants

Non-invasive reproductive monitoring has been used to benefit the conservation and management of African elephants. This provides information on reproductive endocrine status, such as ovarian function and pregnancy, which are useful in complementing behavioural and demographic observations for evaluating the effectiveness of fertility control measures, such as remotely deliverable contraceptive preparations (Wasser *et al.* 1996; Fieβ *et al.* 1999; Schwarzenberger 2007a; Wittemyer *et al.* 2007a).

The quantitative analysis used for analyzing excreted steroids and gonadotrophins vary with the metabolites of interest (Peter *et al.* 1996; Hodges *et al.* 2010). Some studies on elephants have used a nonspecific radioimmunoassay (RIA), although the specific requirements and risk of using radioisotopes have led to alternatives such as enzyme immunoassay (EIA) and high-performance liquid chromatography (HPLC) (Peter *et al.* 1996).

EIA which is the specific analysis used on this study is based on the antibody-antigen reaction which uses an enzyme- or biotin-labelled preparation, and the amount of bound labelled hormone is determined by the amount of colour changes measured by a spectrophotometer (Mutinda 1996; Peter *et al.* 1996). EIA avoids the problems associated with use and disposal of radioactivity of RIA and is also less costly (Hodges *et al.* 2010). As with all immunoassays, is highly sensitive, therefore

assay performance has to be carefully assessed both during the initial setup phase and during routine use. There are four main criteria of validation for the EIA: sensitivity (minimum amount of hormone that can be detected), precision (within- and between- assay repeatability), accuracy (ability to detect the correct amount of hormone in the sample), and specificity (ability to detect only the specific hormone and not detect closely related substances) (Hodges *et al.* 2010).

The lag-time of faecal steroids is about 48 to 50 h in elephants (Schwarzenberger *et al.* 1996; Wasser *et al.* 1996). The faecal progestin metabolites in the form of 5α-reduced progestins: 5α-pregnane-3,20-dione (5α-DHP) and 5α-pregnane-3 α -ol-20-one (5α-P-3-OH) (Heistermann *et al.* 1997; Hodges *et al.* 1997; Fieβ *et al.* 1999) represent 55% of the total excreted progesterone (Wasser *et al.* 1996). Some other hormones that can be monitored in elephant's faeces are: androgens, oestrogens and adrenal hormones (glucocorticoids) (Schwarzenberger 2007b)

Non-invasive monitoring of progestins on faeces of elephants offers the advantage of easy and safe collection of samples, avoids restraining or capturing, and turns out to be a reliable method for evaluating physiological differences in long-term reproductive function as it does not introduce variables that may alter the results (Wasser *et al.* 1996; Schwarzenberger 2007a). Some of the applications have been the confirmation of the elephant oestrous cycle length and pregnancy (Wasser *et al.* 1996; Fieβ *et al.* 1999); endocrinology of cycling and non-cycling elephants in captivity (Brown *et al.* 2004a); adrenocortical function and welfare in captive and wild populations (Stead *et al.* 2001; Laws *et al.* 2007*;* Viljoen *et al.* 2008); endocrinology in males and musth in captive and wild populations (Ganswindt *et al*. 2003; Ganswindt *et al*. 2005a; Ganswindt *et al*. 2005b); stress, social and ecological pressures and reproduction (Foley *et al.* 2001); and ecological conditions and reproduction in wild populations (Wittemeyer *et al.* 2007a, 2007b), amongst others. As non-invasive monitoring for evaluating the effectiveness of contraceptive vaccines in African elephants, the studies include pZP vaccine in free-ranging cows (Bates 2010) and GnRH vaccine for suppressing aggressive behaviour and musth in bulls (De Nys 2005; De Nys *et al.* 2010).

Faecal progestin analysis represents the only feasible approach to longitudinal assessment of reproductive status in free-ranging elephants, providing a more reliable basis for the development of improved strategies for reproductive management and conservation of African elephants in the wild (Wasser *et al.* 1996; Fieβ *et al.* 1999).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site

Entabeni Safari Conservancy is a private game reserve situated in the Waterberg region of the Limpopo Province, located between latitude 28° 39' S and 28° 44' and longitude 24° 11' and 24° 15' E. The reserve is 11 200 hectares in size and is sub-divided into two separate sections (Figure 5):

Upper Escarpment (3 900 ha): this section of land lies above the plateau located at an average altitude of 1500 m above sea level, separated from all land below by the steep cliffs of the escarpment, and additionally by a fence across the narrow valley which otherwise forms a link between Upper and Lower Entabeni (Centre for Wildlife Management 1998).

Lower Escarpment (7 300 ha): this section of land with an average altitude of 1150 m above sea level is located at the foot of the escarpment, is further to the south and was formerly known as Lower Entabeni or Mmadikiri (Centre for Wildlife Management 1998).

A road connects the upper and lower portions of Entabeni, although wildlife movement is restricted to either side of the reserve separated physically by fences, cliffs and an electrified livestock grid.

3.1.1 Topography

Entabeni is characterized by rather diverse topography. This ranges from the high-lying mountainous areas of Upper Entabeni with many moderate to steep slopes, a number of peaks, plains and plateaus that extend to the steep cliffs of the Waterberg escarpment; down to the expansive plains of Lower Entabeni with only a single, rather large, but low hill in the central-east portion (Centre for Wildlife Management 1998).

Upper Entabeni has a large dam constructed near the north-western boundary of the reserve in the upper reaches of the Klein-Sterkrivier which originates on Entabeni and cuts through the western portions of the reserve (Centre for Wildlife Management 1998). In the Lower Escarpment a series of dams were constructed in the main tributary of the Mmadikiri stream which cuts from west to east, and from north to south there is also a series of dams. All these dams plus the natural pans make water freely available all year round throughout the Lower and Upper Escarpment.

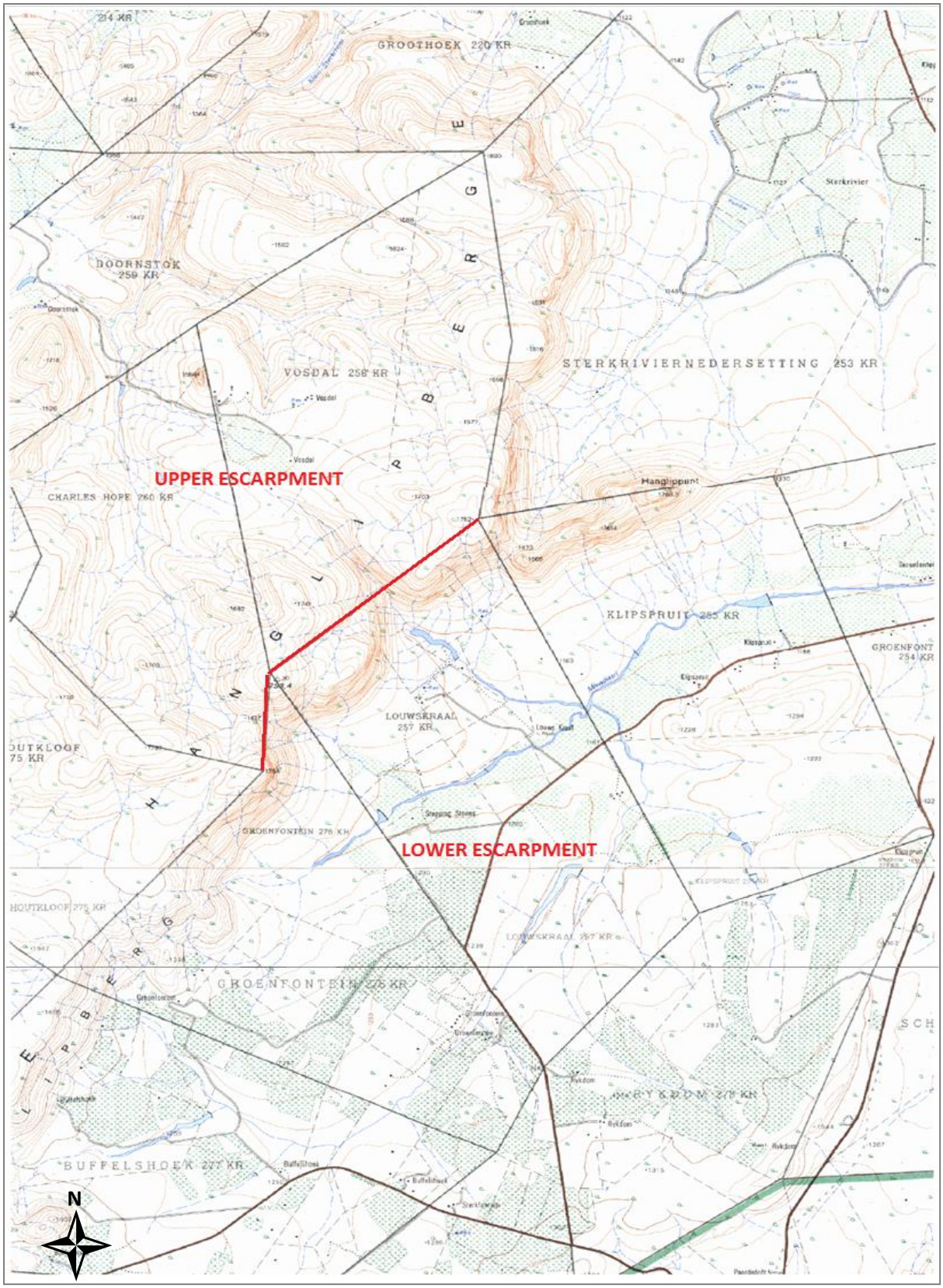

Figure 5. Map of Entabeni Safari Conservancy (11 200 ha)

3.1.2 Climate and rainfall

The reserve lies in the summer rainfall zone with wet summers and cool dry winters. The climate can be described as moderately low summer rainfall with a dry, mildly frosty, winter period. In general, the climate is semi-arid, with three rather distinct seasons; a hot wet season from November to April, a cool dry season from April to August, and a hot dry season from August to October (Centre for Wildlife Management 1998). Occasional ground frost may occur from May until September (with the highest occurrence throughout July), during which mean monthly minimum temperatures range from 2.8 to 10.4 °C.

For the *Lower Escarpment* mean annual temperature is 18.2 °C. Maximum daily temperatures range from a mean of 29.2 °C in December/January to 21.0 °C in June/July. Minimum daily temperatures vary between 16.8 °C in December/January and 4.0 °C in June/July (Centre for Wildlife Management 1998). The long-term mean annual rainfall is 589 mm, of which about 85-90% falls from October to March. During January the rainfall is the highest with an average of 115 mm (Centre for Wildlife Management 1998) (Table 1).

The *Upper Escarpment* is cooler and moister, with a higher average rainfall. The maximum temperature is 34 °C in December /January and the minimum temperature is -2 °C in June/July, with 35-45 frost nights per year. The long-term annual rainfall is on average 650 mm (R. Wilmot, personal communication 2009) (Table 2).

Table 1. Monthly rainfall (mm) for the Lower Escarpment from October 2008 to September 2010.

YEAR	SEASON	MONTH	RAINFALL (mm)	TOTAL RAINFALL (mm)
2008	Wet 2008/2009	October	14	860
		November	236.5	
		December	98	
2009		January	280.5	
		February	107	
		March	124	
	Dry 2009	April	1	70
		May	18	
		June	24	
		July	2	
		August	0	
		September	25	
	Wet 2009/2010	October	69	590
		November	131	
		December	188	
2010		January	125	
		February	10	
		March	67	
	Dry 2010	April	113	143
		May	30	
		June	0	
		July	0	
		August	0	
		September	0	

Table 2. Monthly rainfall (mm) for the Upper Escarpment from May 2009 to September 2010.

YEAR	SEASON	MONTH	RAINFALL (mm)	TOTAL RAINFALL (mm)
2009	Dry 2009	April		30
		May	0	
		June	0	
		July	0	
		August	0	
		September	30	
	Wet 2009/2010	October	72	635
		November	141	
		December	216	
2010		January	116	
		February	15	
		March	75	
	Dry 2010	April	149.5	202.5
		May	32	
		June	0	
		July	0	
		August	0	
		September	21	

3.1.3 Vegetation

The vegetation of the area is described as Sour Bushveld (Veld type A20) as described by Acocks (1975) or Waterberg Moist Mountain Bushveld (Veld type 12) according to Low & Rebelo (1998), and is characterized by a distinct upper layer of woody plants (trees and shrubs) and a grassy ground layer.

The dry winter period results in a cessation of growth of grass and trees. Trees rapidly shed their leaves after drying westerly winds are followed by cold winds from the south. The perennial grasses retract their nutrients from the aboveground parts and store it as reserves in the tuft base's and roots. Both of these result in a severe reduction in available browse and quality of grazing during the winter months (Centre for Wildlife Management 1998).

For the *Lower Escarpment* the tree layer is characterized by Transvaal Beech (*Faurea saligna)*, common hook thorn (*Acacia caffra)*, buffalo thorn (*Ziziphus mucronata)*, wild seringa (*Burkea africana)*, silver cluster leaf (*Terminalia sericea)*, marula (*Sclerocayra birrea caffra),* and African

wattle (*Peltophorum africanum)* on the deep sandy soils. The velvet bush willow (*Combretum molle)*, common sugar bush (*Protea caffra)*, fever berry (*Croton gratissimus)*, red bush willow (*Combretum appiculatum)*, horn pod (*Diplorhynchus condylocarpon)*, sickle bush (*Dichrostachys cinerea),* kudu berry (*Pseudolachnostylis maprouneifolia)*, and the Transvaal milk plum (*Englerophytum magalismontanum)* are characteristic of the rocky slopes. The shrub-tree layer may vary from 1 to 20 m in height and is represented by sandpaper raisin (*Grewia flavescens)*, peeling plane (*Ochna pulchra)* and blue guarrie (*Euclea crispa)*. Common russet grass (*Loudetia simplex)*, giant spear grass (*Trachypogon spicatus)*, guinea grass (*Panicum maximum),* couch grass *(Cynodon dactylon),* yellow thatch grass (*Hypothelia dissoluta),* broom love grass *(Eragrostis pallens)*, and red autumn grass *(Schizachyrium sanguineum)* are the conspicuous species of the grassy layer (Centre for Wildlife Management 1998).

The veld condition assessment for the Lower Escarpment reports an ecological status of medium tending towards poor score. The species density is composed of a total of 46 grass species (80.75%), various forbs (15.52%), rocks (0.62%), sedges (1.54%) and bare ground (1.54%). The overall palatability score for the Lower Escarpment is 1.64 which indicates a poor to medium score. Palatable species have a 19.84%, medium palatable have a 23.84% and unpalatable species have a 56.32% representation (Spaan 2008).

The *Upper Escarpment* vegetation is nutritionally poorer compared to the Lower Escarpment, due to the mountainous terrain and old farmlands, leaving large areas being taken over by the invasive and unpalatable specie of *Stoebe vulgaris.* There are fewer palatable tree species suitable for elephant consumption. This area has also over-stocked game in general thus leaving fewer resources for elephants. The tree layer is characterized by wild seringa (*Burkea africana),* velvet bush willow (*Combretum molle)*, large-fruited bush-willow (*Cobretum zeyheri)*, Transvaal Beech (*Faurea saligna)*, bushveld gardenia *(Gardenia volkensii),* lavender fever tree *(Croton gratissimus)*, Transval milkplum *(Englorophytum magalismontanum),* Transvaal red milkwood *(Mimusaps zeyheri)*, lavender tree (*Heteropyxis natalensis), Lannea discolor,* round-leaved teak *(Pterocarpus rotundifolius),* buffalo thorn (*Ziziphus mucronata)*, nana-berry (*Rhus dentate),* tree wisteria *(Bolusanthus speciosus), and* common sugar bush (*Protea caffra)*. The shrub-tree layer is represented by sandpaper raisin (*Grewia flavescens)*, blue guarrie (*Euclea crispa)*, fever tea *(Lippia javanica),* wild asparagus (*Asparagus sp.)*, bracken fern *(Pretidium aquilinum)* and healing leaf *(Solanum giganteum).*The more abundant grass species are: common russet grass (*Loudetia simplex)*, giant spear grass (*Trachypogon spicatus),* stab grass (*Andropogon schirensis), Aristida scabrivalvis,* Natal red top *(Melinis repens),* gum grass

(Eragrostis gummiflua), Tassel three-awn *(Aristida congesta* subsp. *congesta),* and hairy trident grass *(Tristachya leucothrix)*.

The veld condition assessment for the Upper Escarpment reports an ecological status of medium tending towards poor score. The species density is composed of a total of 30 grass species (82.99%), various forbs (10.75%), rocks (5.73%), *Stoebe vulgaris* (0.26%), and bare ground (0.27%). The overall palatability score for the Upper Escarpment is 1.374 which indicates a poor score. Palatable species have a 7.01%, medium palatable have a 23.20% and unpalatable species have a 69.79% representation (Spaan 2008).

3.2 Experimental design

This was a twelve-month study to determine the efficacy of a GnRH-contraceptive vaccine in inducing anoestrus in eight African elephant cows in the Entabeni Private Game Reserve, Limpopo Province, South Africa. The reproductive status of the cows was monitored through non-invasive faecal progesterone metabolite evaluation and analyzed by means of EIA. Observations of oestrous behaviour during the twelve-month period were also recorded.

3.2.1 Elephant sampling and study procedures timeline

1) Study subjects
   - 8 adult African elephant cows were used as the treatment group
   - 4 adult African elephant cows were used as the control group

2) Treatment of study subjects
   - The 8 cows of the treatment group received the GnRH vaccine (primary injection and a booster)

3) Study procedures and timeline (study cows: *n=12*)
   - See figure 6

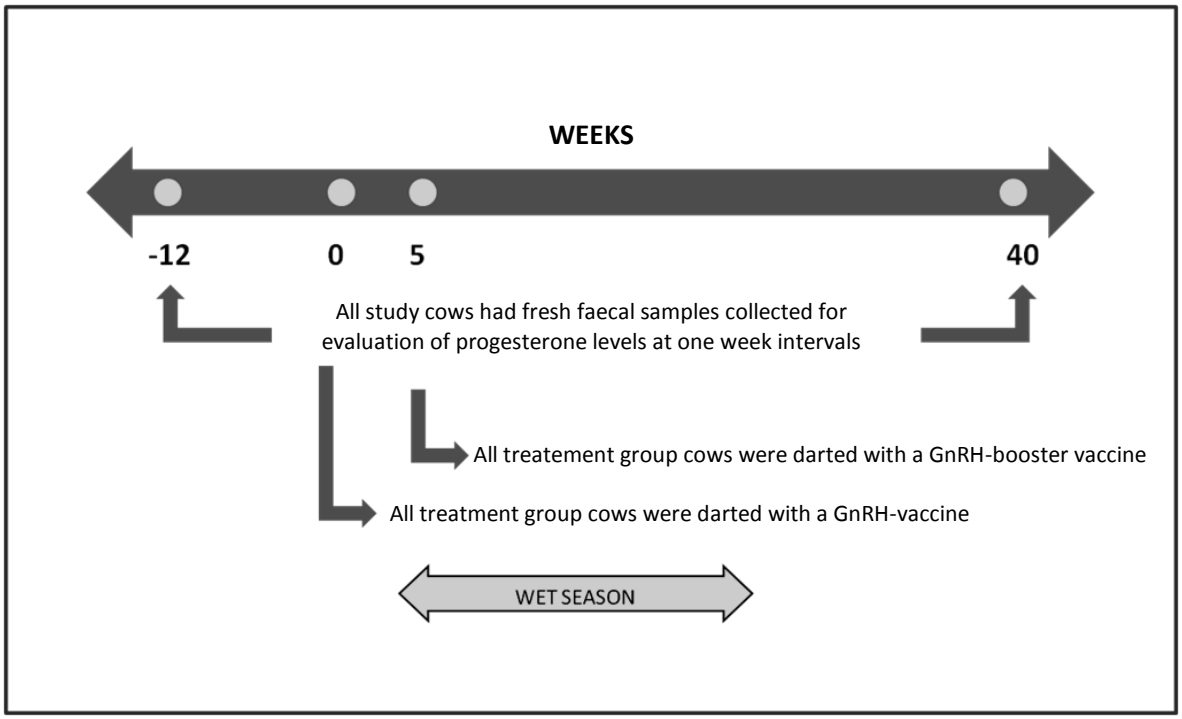

Figure 6. Scheme of timeline for non-invasive faecal steroid monitoring and experimental procedures.

3.2.2 Elephant population

The twelve African elephant cows were distributed in two herds, one consisting of 10 individuals (9 females + 1 male calf) on the Lower Escarpment (see Table 3) and the second one of six individuals (4 females + 2 male calves) on the Upper Escarpment (see Table 4).

The Lower Escarpment herd was originally captured in Sabi Sands, Kruger National Park in 2004 and translocated to Shambala Game Reserve in Limpopo where they remained until 2008. During this period cows 1, 2, 8, and 9 were treated with pZP vaccine as follows: June 2005 (primary vaccination), July 2005, July 2006, and August 2007 (booster vaccinations). In July 2008 they were moved to Entabeni. The Upper Escarpment herd was translocated from Kruger National Park around 1998 with two adult bulls that were later culled (2007); the females had not been on any contraceptive. There is currently no adult bull living on either Upper or Lower Escarpment Entabeni.

The elephants were identified according to individual characteristics including size, age, unique ear patterns (consisting of notches, nicks, tears and holes), tusks (size, shape and position), and other distinguishing features such as lumps, scars and tail hairs (Moss 1996; Delsink *et al.* 2007). Prior to starting the collection of samples, all the elephants in both herds had a complete identification kit comprising sketches of the ear patterns with corresponding photographs and marked characteristics.

The estimated age was determined from a combination of the known ages of some individuals and the visual estimates on shoulder height and size, compared with that of an average adult cow (Du Toit 2001, based on Laws 1966; Moss 1996), as well as known dates of birth. Age class groups for the experimental population were defined as an adult from ≥ 15 years of age or a sub-adult, 15 years and younger, according to the age criteria for African elephants (Du Toit 2001, based on Laws 1966).

The elephants were located on a daily basis on the Lower Escarpment by means of radio telemetry using the VHF radio-collar of Cow No. 9. The herd was then enticed out of the bush with supplementary feed (Appendix A). The procedure was different for the Upper Escarpment herd, which was found with traditional tracking methods or during winter months located on a feeding spot which had been used to provide supplementary feed for years, observations of this herd was far less frequent than with the Lower Escarpment herd and averaged 2 to 3 times per week during the dry season and once per month during the wet season. Both herds were given supplementary feed when found and observed during these periods, which were used to record behaviours and faecal sample collection from the focal cows.

Dominance/ranking was assigned on the basis of individuals showing aggressive interactions such as charges, chases, pushes, and pokes, as well as milder forms of aggression, including displacements and supplants which led to a dominant individual, and the sub-dominant individual was defined as the receiver of the aggression which showed a submissive posture and avoidance of aggressor (i.e., turning away, backing up, flight) (Estes 1999; Archie *et al.* 2006).

The Upper and Lower Escarpment herds age structure differ from the reported on the literature (Estes 1999), in that there was no new offspring and most of the females on reproductive age were nulliparous. The Lower Escarpment herd seemed to be formed by two family units, Cow No. 1, 2, 3, 6, and 7 conform one and Cow No. 4, 8, 9, and 10 the second one; Cow No. 5 didn´t seem to belong to any of these family units. Both herds had been translocated and in the case of the Lower Escarpment herd it was moved twice, which makes both herds extremely nervous towards game drive vehicles and would flee with no apparent reason. The only way to achieve valuable observation time with both herds was providing supplementary feed.

Table 3. Lower Escarpment herd composition.

ID	SEX	ESTIMATED AGE	AGE CLASS*	REMARKS	TREATMENT (T) CONTROL (C)	RANKING*
1	Female	30 years plus	Adult	Matriarch Calf no. 7	T	Dominant
9	Female	30 years plus	Adult	Calf no. 10 VHF Collar	T	Dominant
2	Female	25 years	Adult	Calf no. 6	T	Dominant
8	Female	20 years	Adult	None	T	Sub-dominant
3	Female	18 years	Adult	None	T	Sub-dominant
5	Female	15 years	Sub-adult	None	T	Sub-dominant
4	Female	12 years	Sub-adult	None	C	Sub-dominant
7	Female	10 years	Sub-adult	None	C	Dominant
6	Female	6 years	None	Born in 2004	None	None
10	Male	4 years	None	Born in 2005	None	None

*Groups assigned for statistical comparison

Table 4. Upper Escarpment herd composition.

ID	SEX	ESTIMATED AGE	AGE CLASS*	REMARKS	TREATMENT (T) CONTROL (C)	RANKING*
11	Female	30 year plus	Adult	Matriarch Calf no. 15 & 16	T	Dominant
12	Female	30 years plus	Adult	Calf born on 2008[a]	T	Sub-dominant
13	Female	15 years	Sub-adult	None	C	Sub-dominant
14	Female	15 years	Sub-adult	None	C	Sub-dominant
15	Male	8 years	None	None	None	None
16	Male	4 years	None	None	None	None

*Groups assigned for statistical comparison

[a] Euthanised shortly-after due to knee fracture

3.3 Vaccination of elephants

3.3.1 Vaccine formulation and dose

The commercially-available GnRH-vaccine Improvac® (Pfizer Animal Health, Sandton, South Africa) was used as treatment (Fig. 7A). The vaccine was stored at 4 °C and shortly before each day´s vaccination the darts were loaded with the vaccine and stored in cooler boxes until used.

The targeted animals received a primary vaccination containing 600 µg RnRF-protein conjugate (3 ml) followed by a single booster (3 ml) five to seven weeks after the primary vaccination, administered via deep intramuscular injection into the S*emi-membranosus-Semi-tendinosus* groups of muscles of the hind leg, *triceps* muscle above the elbow of the forelimb, or *gluteal* muscles.

3.3.2 Vaccination procedure

The primary vaccination was administered by remote delivery from the ground with a Dan-Inject® darting system (Dan-Inject® International, Denmark) which operates with a CO_2 -cartridge. The booster vaccination was administered from either ground delivery with a Dan-Inject® darting system or from a helicopter above the herd using a Pneu-Dart® rifle.

The darts used were reusable 3 ml Dan-Inject® with 60 mm 13 gauge plain needles with side-ports and disposable Pneu-Dart® with 60 mm 13 gauge with gel collar and marker chamber filled with cattle colour mark paint which sprays onto the elephant at the dart site.

The elephants were located with VHF radio telemetry and/or traditional tracking methods. Once located, target cows were identified and vaccinated (Fig. 7B).

The primary vaccinations were performed from the ground on September 3 *(n* = 4*)*, September 8 *(n* = 2*)*, September 9 *(n* = 1*)*, and September 18 *(n* = 1*)*, 2009. The booster vaccinations for the Lower Escarpment elephants were performed from a helicopter on October 19, 2009 *(n* = 6*)* and for the Upper Escarpment elephants from the ground on October 22, 2009 *(n* = 2*)*; the latest two elephants (cows No. 11 and No. 12) received partial doses (2 ml and 1.5 ml, respectively) only due to incomplete delivery from the darts which were retrieved after falling out.

Figure 7. A: Improvac® vaccine (Pfizer Animal Health, Sandton, South Africa)
B: Elephant cow showing vaccine dart (Pneu-Dart®) in the hind quarter (circle) and a close-up (inner square)

Following each vaccination, number of cow, injection site, darting system used, and darting source (vehicle or helicopter) were recorded (Table 5).

Table 5. GnRH immnunocontraceptive vaccinations administered to the Entabeni Safari Conservancy elephant population in 2009.

COW ID.	PRIMARY VACCINATION					BOOSTER					COMMENTS
	DATE	TIME OF DARTING	INJECTION SITE	DART USED	SOURCE	DATE	TIME OF DARTING	INJECTION SITE	DART USED	SOURCE	
1	18/9/2009	11.00	Left hind leg	Dan-inject	Ground	19/10/2009	10.00	Left hind leg	Pneu-dart	Helicopter	
2	9/9/2009	16.18	Right shoulder	Dan-inject	Ground	19/10/2009	10.02	Left hind leg	Pneu-dart	Helicopter	
3	3/9/2009	11.30	Left hind leg	Pneu-dart	Ground	19/10/2009	10.05	Left hind leg	Pneu-dart	Helicopter	
5	3/9/2009	11.41	Right hind leg	Pneu-dart	Ground	19/10/2009	10.09	Left hind leg	Pneu-dart	Helicopter	
8	8/9/2009	11.36	Left hind leg	Dan-inject	Ground	19/10/2009	10.04	Left hind leg	Pneu-dart	Helicopter	
9	8/9/2009	11.31	Right hind leg	Dan-inject	Ground	19/10/2009	10.00	Right hind leg	Pneu-dart	Helicopter	
11	3/9/2009	15.55	Left hind leg	Pneu-dart	Ground	22/10/2009	17.50	Right hind leg	Dan-inject	Ground	Delivered only 2 ml of booster
12	3/9/2009	15.50	Right shoulder	Pneu-dart	Ground	22/10/2009	17.30	Right gluteum	Dan-inject	Ground	Delivered only 1.5 ml of booster

3.4 Observations

3.4.1 Side-effects of vaccination

Post-vaccination, daily observations of the target animals were conducted with binoculars to determine side-effects such as swellings, abscesses, suppuration or ulceration at the vaccination site, as well as signs of lameness or illness (Mikota 2006; Mikota & Fowler 2006).

3.4.2 Behavioural observations

Dominance/ranking of individuals was assigned through observations of the herds, mainly while they gathered to consume supplementary feed (see Appendix A). In addition, ad lib notes were recorded of all sexual behaviours, including signs of oestrus as described in the literature (Moss 1983; Estes 1999; Ortolani *et al.* 2005). Because there were no bulls of reproductive age on either part of the reserve, sexual behaviours were limited to interactions between females such as placing trunk and sniffing the genital opening or urine of another female and presence of a vaginal discharge. These observations were correlated to FPM concentrations.

3.4.3 Faecal samples

The monitoring was conducted through non-invasive faecal steroid analysis. Starting with a three-month control period prior to vaccination during which faeces were collected from each study animal, directly following defecation to ensure positive identification of the individual. The three-month period was followed by vaccination and continued monitoring until the end of the twelve-month observation period.

3.4.3.1 Sample collection and storage

After the elephants moved off to a safe distance from the feeding spot, fresh faeces samples were collected from each study elephant cow once or twice a week, according to availability of samples. Five to 10 g of the central portion of the faecal bolus was removed using rubber gloves and placed in individual collection glass vials (Fig. 8A, 8B). These were labelled with the date, number of cow and sequence number; the samples were frozen immediately upon collection in a mobile freezer and later that day stored in a chest freezer at -20 °C until extraction and analysis (Wasser *et al.* 1996;

Fieβ *et al.* 1999; Schwarzenberger 2007b; Wittemyer *et al.* 2007a). No faecal samples were collected if they were contaminated with urine to avoid cross-contamination. For each sample, a unique sequence number was assigned and the date, elephant number, time of dropping, time of collection, environmental temperature, time of storage in chest freezer, and other notable variables were recorded on a data sheet.

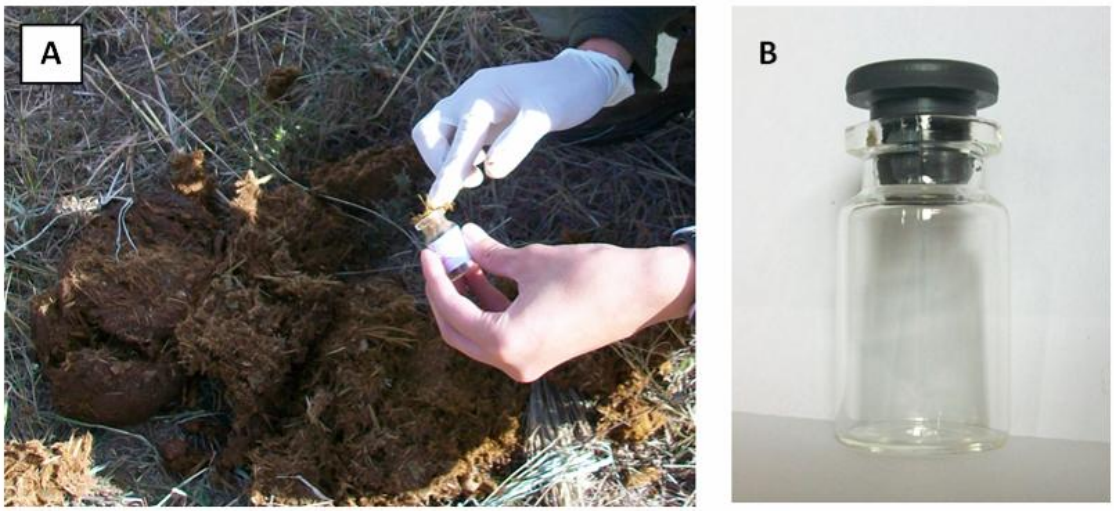

Figure 8. A: Collection of elephant faeces sample
B: Glass vial with stopper for faecal sample collection, storage and freeze-drying

3.4.3.2 Extraction of faecal samples

Faecal samples were analyzed by the Hormone Laboratory of the Section of Reproduction, Department of Production Animal Studies, at the University of Pretoria.

The samples were freeze-dried in the collection vials before extraction for 48 h at -54 °C and approximately 672 Torr vacuum in an Instruvac freeze-drier (Air and Vacuum Technologies; Model No. VFDT02.50). Dried samples were crushed using a pestle and sieved through a metallic mesh. After removing the fibrous material, 0.05 g of the powder was mixed with 3 ml of 80% aqueous ethanol (Wasser *et al.* 1996; Fieβ *et al.* 1999; Ganswindt *et al.* 2002; Ganswindt *et al.* 2003; Ganswindt *et al.* 2005a). Steroids were extracted by shaking for 15 min on a multi-tube vortex according to the procedure described by Ziegler *et al.* (2000). Following centrifugation at 3000 rpm for 10 min, 1.5 to 2 ml of clear supernatant was transferred to Eppendorf tubes and stored upright at -20 °C until hormone analysis.

3.4.3.3 Progesterone enzyme immunoassay

FPM concentrations were measured by means of EIA on microtitre plates using biotinylated steroids as labels, as it has previously been shown to provide reliable information on luteal function and pregnancy in the African elephant (Fieβ *et al.* 1999). The method makes use of a polyclonal antibody raised in rabbits against 5α-pregnan-3β-ol-20-one-3-HS-BSA and 5α-pregnan-3β-ol-20-one-3-HS-peroxidase as label (Szdzuy *et al.* 2006).

Cross-reactivities of the antibody to progestins were as follows: 5α-pregnan-3α-ol-20-one, 650%; 5α-pregnan-3β-ol-20-one, 100%; 4-pregnen-3,20-dione (progestagene) 72%; 5α-pregnan-3,20-dione, 22%; <0.1% for 5β-pregnan-3α,20α-diol, 4-pregnen-20α-ol-3-one, 5β-pregnan-3α-ol-20-one, 5α-pregnan-20α-ol-3-one, 5α-pregnan-3β,20α-diol and 5α-pregnan-3α,20α-diol (Hodges *et al.* 1997).

The sensitivity of the assay at 90% binding was 3 pg per well, and intra- and inter-assay coefficients of variation, determined by repeated progestagen measurements of high and low concentration controls ranged between 3.64% and 12.19%.

3.5 Data analysis

Data generated by the project was recorded on Microsoft Excel spreadsheets and grouped into two categories: before treatment and after treatment. Definition of the oestrous cycle was based on FPM profiles expressed as µg/g dried weight (DW) and plotted against time (weeks).

Individual averages were tested for normality using Shapiro-Wilk W where a probability value greater than 0.05 signified a normal distribution. Data that were normally distributed were analyzed using Student´s *t*-tests while data not meeting the normal distribution test were analyzed using the Wilcoxon Rank Sum Test.

3.5.1 Defining the oestrous cycles, lengths and its component phases

For each female, a baseline of FPM was calculated using an iterative process in which values that exceeded the mean plus 2 standard deviations (SD) were considered luteal elevations and temporarily removed from the data set. The mean was continuously recalculated and the elimination process was repeated until no values exceeded the mean plus 2 SD. The mean of

remaining values was reported as baseline concentration per individual (Brown *et al.* 1994a; Brown *et al.* 1999; Moreira *et al.* 2001; Powell & Monfort 2001; Brown *et al.* 2004a; Ortolani *et al.* 2005; De Haas van Dorsser *et al.* 2007) as variability in FPM concentrations between individual females has been well-documented (Wasser *et al.* 1996; Fieβ *et al.* 1999).

The phases of the ovarian cycle were determined on the basis of a defined rise in FPM concentrations. An increase above threshold values and persistence above baseline for at least two consecutive weeks was taken to indicate the beginning of the postovulatory luteal phase (LP) of the ovarian cycle (Brown *et al.* 2001; De Haas van Dorsser *et al.* 2007). Correspondingly, a fall in hormone concentrations below this baseline with at least two consecutive values indicated the commencement of the following inter-luteal or follicular phase (ILP) of the cycle (Hodges *et al.* 1997; Hodges 1998; Fieβ *et al.* 1999; Brown *et al.* 2001). The cycle length was defined as the combined length of LP and subsequent ILP (Heistermann *et al.* 1997, Lueders *et al.* 2010). The increase in FPM following the end of the ILP indicates the ovulatory period which has been reported to coincide with maximum male interest and mating (Hodges 1998; Ortolani *et al.* 2005). Phase lengths estimates are given as mean ± SD. The behavioural oestrus and oestrus signs were compared with the time of FPM increase, as it has been reported to last from 2 to 8 days (Moss 1983; Western & Lindsay 1984).

Females were considered to have irregular cycles when overall cycle length exceeded or fell short of the reported length of 13 to 17 weeks (Brannian *et al.* 1988; Plotka *et al.* 1988; Wasser *et al.* 1996; Heistermann *et al.* 1997; Hodges *et al.* 1997; Schwarzenberger *et al.* 1997; Hodges 1998; Fieβ *et al.* 1999; Brown 2000; Ortolani *et al.* 2005; Brown 2006; Bertschinger *et al.* 2008a; Hildebrandt *et al.* 2010). Individuals were designated on irregular cycles based on extended ILP (>9 weeks), shortened LP (<6 weeks), and/or the presence of marked, random fluctuations in FPM concentrations during ILP or LP (Brown 2000). Periods of anoestrus in some females were considered if they had an ILP lasting longer than twice the duration of an average normal ILP (range 4-6 weeks in the African elephant) (Plotka *et al.* 1988; Heistermann *et al.* 1997; Schwarzenberger *et al.* 1997; Hodges 1998; Fieβ *et al.* 1999; Brown 2000; Ortolani *et al.* 2005; Brown 2006; Bertschinger *et al.* 2008a; Hildebrandt *et al.* 2010).

For the analysis of the effects of age, season and ranking on FPM concentrations, only the luteal (above baseline) values of the six treated individuals of the Lower Escarpment herd were used. Too few samples were collected from the two treated individuals on the Upper Escarpment (less than 30 samples per individual). The α-level of significance was set at $P < 0.05$ for all tests. Statistical analyses and graphs were performed using NCSS Statistical Software ©2007 (version 07.1.19) and Microsoft Office Excel 2007. Data are presented as mean ± SD, unless otherwise stated.

3.5.2 Effect of treatment on oestrous cycle

To test the effect of treatment (GnRH vaccine) on FPM concentrations a two-tailed T-test was performed and compared to oestrous cycle means of untreated cows.

3.5.3 Effect of age class on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

A chi-square test was used to assess the effects of age (adult or sub-adult) on FPM concentrations. Age class groups for treated individuals were defined as adult (>15 years) (*n=177* for 5 individuals) or sub-adult (≤15 years) (*n=36* for 1 individual) (Du Toit 2001, based on Laws 1966).

3.5.4 Effect of seasonality on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

The potential confounder season (wet/summer, characterized by rain, warm temperatures and long photoperiods – October through March or dry/winter, characterized by arid conditions, cool temperatures and short photoperiods – April through September), was assessed with respect to FPM concentrations using a one-way ANOVA. Elephant cows were the subjects and season was the between subject source of variation. The season of the year was defined as dry season 2009 (May through September 2009, *n=41*), wet season (October 2009 through March 2010, *n=124*), and dry season 2010 (April through June 2010, *n=48*).

3.5.5 Effect of dominance/ranking on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

A chi-square test was used to assess the effects of dominance/ranking on FPM concentrations. Dominance/ranking groups were defined as dominant individuals (*n=111* for 3 individuals) and sub-dominant individuals (*n=102* for 3 individuals) (Estes 1999; Archie *et al.* 2006).

CHAPTER 4

RESULTS

4.1 GnRH vaccine treatment

4.1.1 Administration of the vaccine

The Dan-Inject® with 60 mm 13 gauge with plain needles with side-ports and the disposable Pneu-Dart® with 60 mm 13 gauge with gel collars and marker chambers were both found to be practical systems to deliver the vaccine. The incomplete delivery of two Dan-Inject® booster vaccinations were attributed to the inexperience of the shooter and not to the system itself.

For the first vaccination performed from the ground, it required 15 days to dart all the treatment cows as they became increasingly wary of the vehicle. The booster vaccination on the Lower Escarpment was delivered from a helicopter and required approximately 9 minutes to dart all 6 cows.

The post-vaccination effects of the immunization were limited to local swellings and all resolved after a maximum of two weeks. The affected cows never showed signs of irritation or discomfort as a result of these reactions and they were not associated with lameness or other signs of illness.

4.1.2 Non-invasive faecal steroid monitoring

From May 2009 to June 2010 a total of 566 samples were collected from the 12 study individuals (see Appendix B), of which 474 were from the Lower Escarpment herd (*n=8*) with 352 from the treatment group (*n=6*) and 122 from the control group (*n=2*). From the Upper Escarpment herd 92 samples were collected (*n=4*) with 52 from the treatment group (*n=2*) and 40 from the control group (*n=2*). These four cows were removed from the data to test the effect of age, dominance/ranking and seasonal rainfall due to the low sample sizes which also led to large gaps in the weekly data which limit any inferences regarding oestrous cycle phases and effects of treatment.

Behaviour and other signs that could be related to oestrus were recorded on 44 occasions for the study animals. There were 37 for the Lower Escarpment herd and 7 for the Upper Escarpment herd (see Appendix C). The observations included placing the trunk and sniffing at another female's

genital orifice or urine, as well as presence of vaginal discharges. The majority (86.48%, *n=32*) of these observations for the Lower Escarpment herd showed a close temporal relationship with increases in FPM concentrations.

4.1.3 Effect of treatment on oestrous cycle

The 12 females of the study group showed evidence of luteal activity as evidenced by FPM concentrations exceeding the baseline more than once during the study period.

4.1.3.1 Effect of GnRH vaccine treatment on oestrous cycle of individuals

The summaries of the FPM concentrations of each cow are as follows:

a) Lower Escarpment herd

Cow No. 1 (Figure 9):

- Matriarch, 30+ years of age, lactating cow No. 7
- Received pZP contraception from 2005 to 2007
- Received primary GnRH vaccine treatment and 4.5 weeks later a booster
- Showed one abnormal cycle before treatment, two normal cycles and one abnormal cycle after treatment
- Had lower FPM concentrations during the dry season after treatment
- The average FPM concentration for the luteal phase was 4.83 ± 3.44 µg/g DW and 1.27 ± 0.34 µg/g DW for the inter-luteal phase.

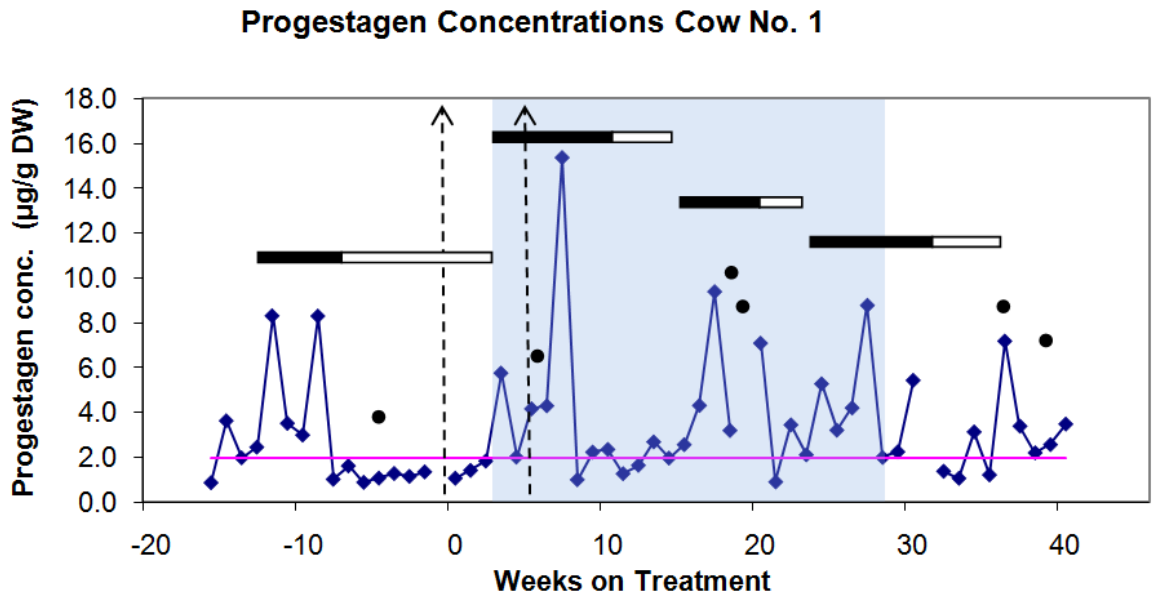

Figure 9. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 1) treated with the GnRH vaccine. Red solid line represents baseline concentration, horizontal solid bars represent luteal phase, horizontal open bars represent inter-luteal phase, and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening or urine observations. Wet season is illustrated in blue background.

Cow No. 2 (Figure 10):

- Cow 25 years of age, lactating cow No. 6
- Received pZP contraception from 2005 to 2007
- Received primary GnRH vaccine treatment and 6 weeks later a booster
- Two cycles with long inter-luteal phases and one normal cycle post-treatment
- After the onset of the 2010 dry season levels appeared to be lower again
- The booster was probably administered too late to affect the second luteal phase
- The average FPM concentration for the luteal phase was 6.18 ± 3.35 µg/g DW and 2.03 ± 0.76 µg/g DW for the inter-luteal phase.

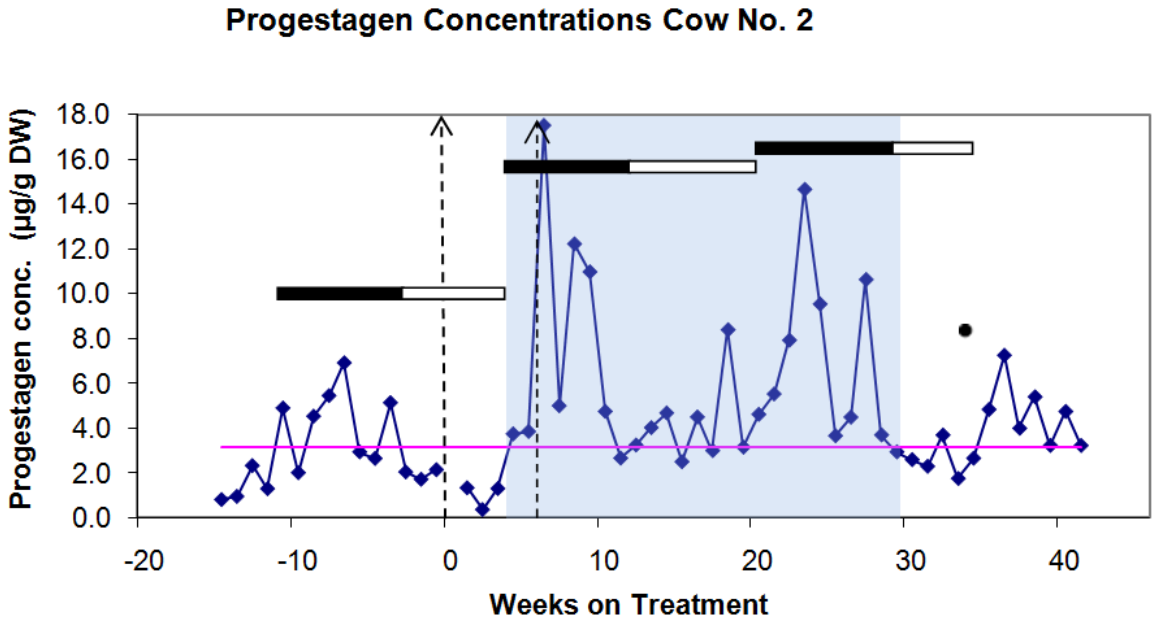

Figure 10. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 2) treated with the GnRH vaccine. Red solid line represents baseline concentration, horizontal solid bars represent luteal phase, horizontal open bars represent inter-luteal phase, and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening or urine observations. Wet season is illustrated in blue background.

Cow No. 3 (Figure 11):

- Cow 18 years of age, no calves
- Received primary GnRH vaccine treatment and 6.5 weeks later a booster
- Presented one abnormal cycle, and after treatments showed above baseline luteal levels and only decreased by the beginning of the next dry season.
- Tendency to lower FPM levels during the dry seasons
- The average FPM concentration for the luteal phase was 6.13 ± 3.50 µg/g DW and 1.93 ± 0.69 µg/g DW for the inter-luteal phase.

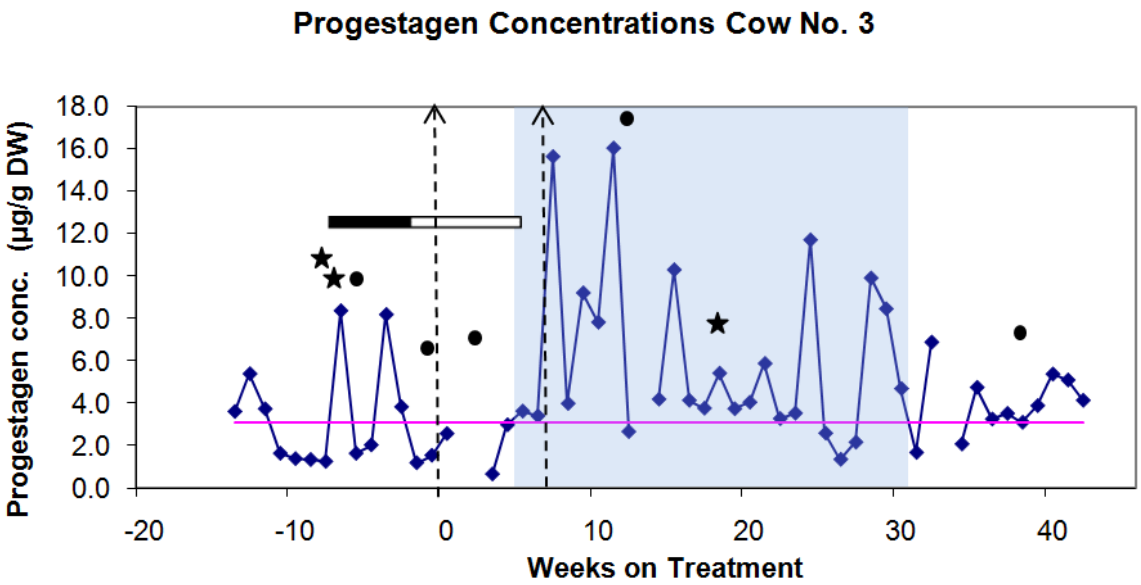

Figure 11. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 3) treated with the GnRH vaccine. Red solid line represents baseline concentration, horizontal solid bars represent luteal phase, horizontal open bars represent inter-luteal phase, and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening or urine and stars for vaginal discharge observations. Wet season is illustrated in blue background.

Cow No. 4 (Figure 12):

- Cow 12 years of age, no calves
- Control individual, received no GnRH treatment
- Three abnormal luteal cycles with short inter-luteal phases
- Period of anoestrus during the dry season of 2009 and lower levels during the start of the 2010 dry season
- The average FPM concentration for the luteal phase was 5.84 ± 3.57 µg/g DW and 1.33 ± 0.54 µg/g DW for the inter-luteal phase.

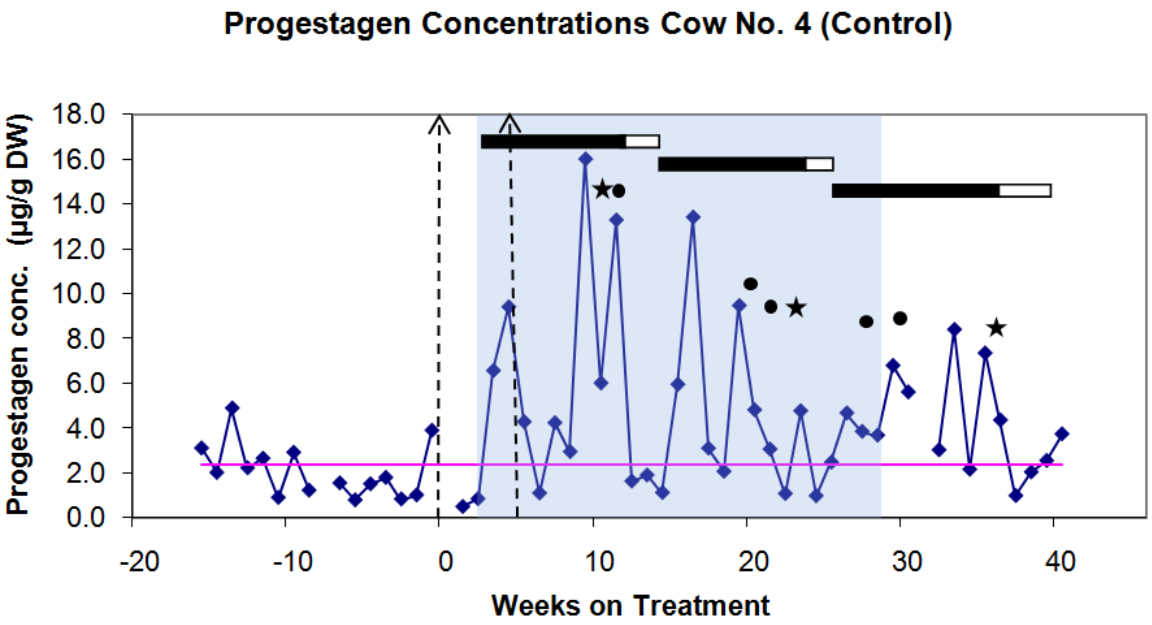

Figure 12. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 4; control group). Red solid line represents baseline concentration, horizontal solid bars represent luteal phase, horizontal open bars represent inter-luteal phase, and dotted line arrows serve as reference to darting dates of primary vaccine and subsequently booster vaccine on treated individuals. Circles stand for sniffing into genital opening or urine and stars for vaginal discharge observations. Wet season is illustrated in blue background.

Cow No. 5 (Figure 13):

- Cow 15 years of age, no calves
- Received primary GnRH vaccine treatment and 6.5 weeks later the booster
- Period of anoestrus during dry season 2009 followed by possibly a long luteal phase
- Booster too late to interfere with luteal phase
- The average FPM concentration for the luteal phase was 4.28 ± 2.04 µg/g DW and 1.30 ± 0.35 µg/g DW for the inter-luteal phase.

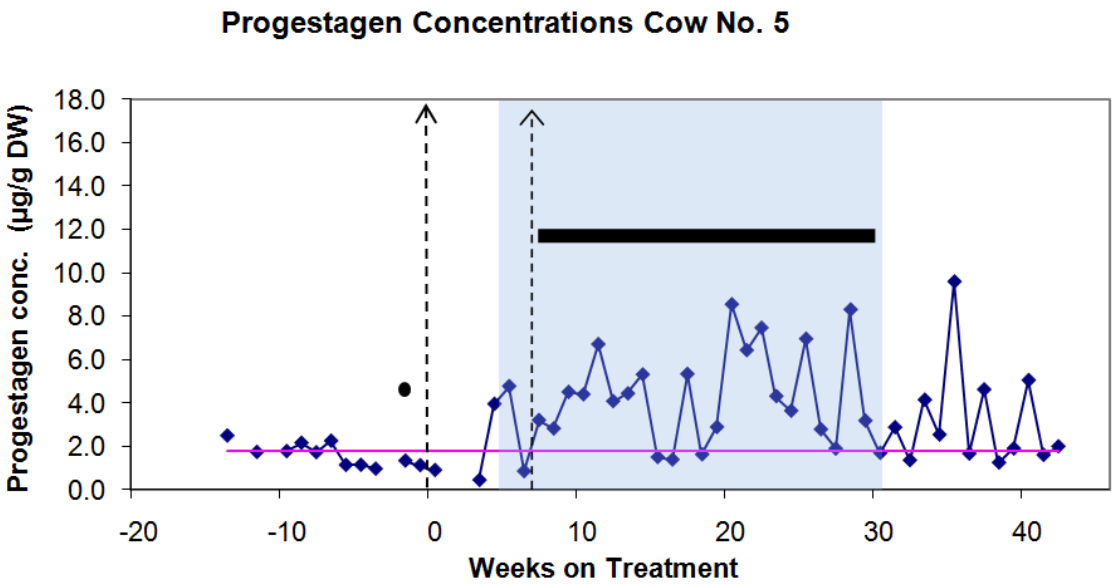

Figure 13. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 5) treated with the GnRH vaccine. Red solid line represents baseline concentration, horizontal solid bar represents luteal phase and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening or urine observations. Wet season is illustrated in blue background.

Cow No. 7 (Figure 14):

- Cow 10 years of age, no calves, and still suckling from mother (Cow No. 1)
- Control individual, received no GnRH treatment
- Period of anoestrus during dry season 2009 followed by a long luteal phase
- The average FPM concentration for the luteal phase was 3.80 ± 2.73 µg/g DW and 1.10 ± 0.27 µg/g DW for the inter-luteal phase.

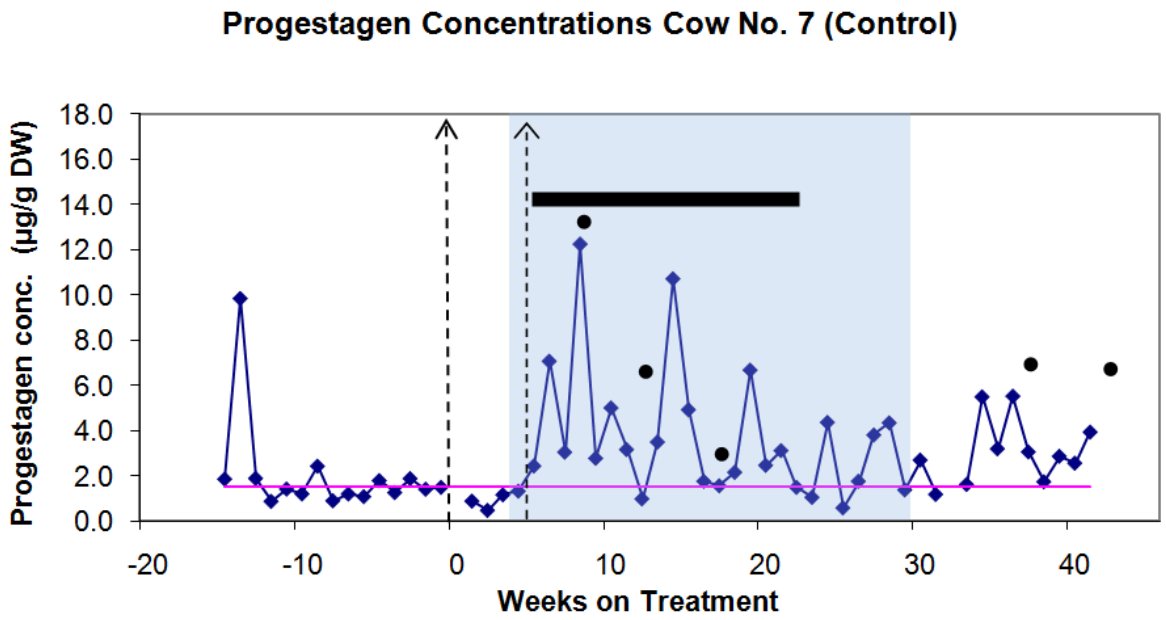

Figure 14. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 7; control group). Red solid line represents baseline concentration, horizontal solid bar represents luteal phase and dotted line arrows serve as reference to darting dates of primary vaccine and subsequently booster vaccine on treated individuals. Circles stand for sniffing into genital opening or urine observations. Wet season is illustrated in blue background.

Cow No. 8 (Figure 15):

- Cow 20 years of age, no calves
- Received pZP contraception from 2005 to 2007
- Received primary GnRH vaccine treatment and 6 weeks later a booster
- Period of anoestrus during dry season 2009 and showed one normal cycle post-immunization and a second luteal cycle with a short follicular phase.
- Effects of first and second dry season obvious, showing lower FPM levels
- The average FPM concentration for the luteal phase was 5.47 ± 2.76 µg/g DW and 1.64 ± 0.42 µg/g DW for the inter-luteal phase.

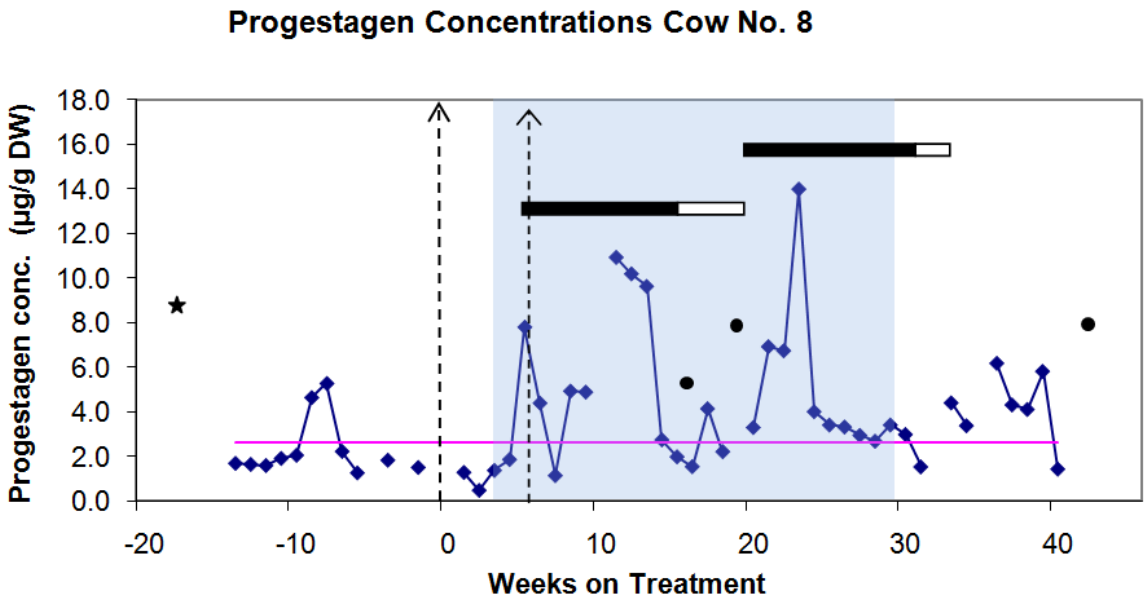

Figure 15. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 8) treated with the GnRH vaccine. Red solid line represents baseline concentration, horizontal solid bars represent luteal phase, horizontal open bars represent inter-luteal phase, and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening or urine and stars for vaginal discharge observations. Wet season is illustrated in blue background.

Cow No. 9 (Figure 16):

- Cow 30+ years of age, lactating male calf No. 10
- Received pZP contraception from 2005 to 2007
- Received primary GnRH vaccine treatment and 6 weeks later a booster
- Presented one long luteal phase after being treated
- Tendency to lower progesterone levels during the dry seasons
- The average FPM concentration for the luteal phase was 6.32 ± 3.55 µg/g DW and 2.09 ± 0.64 µg/g DW for the inter-luteal phase.

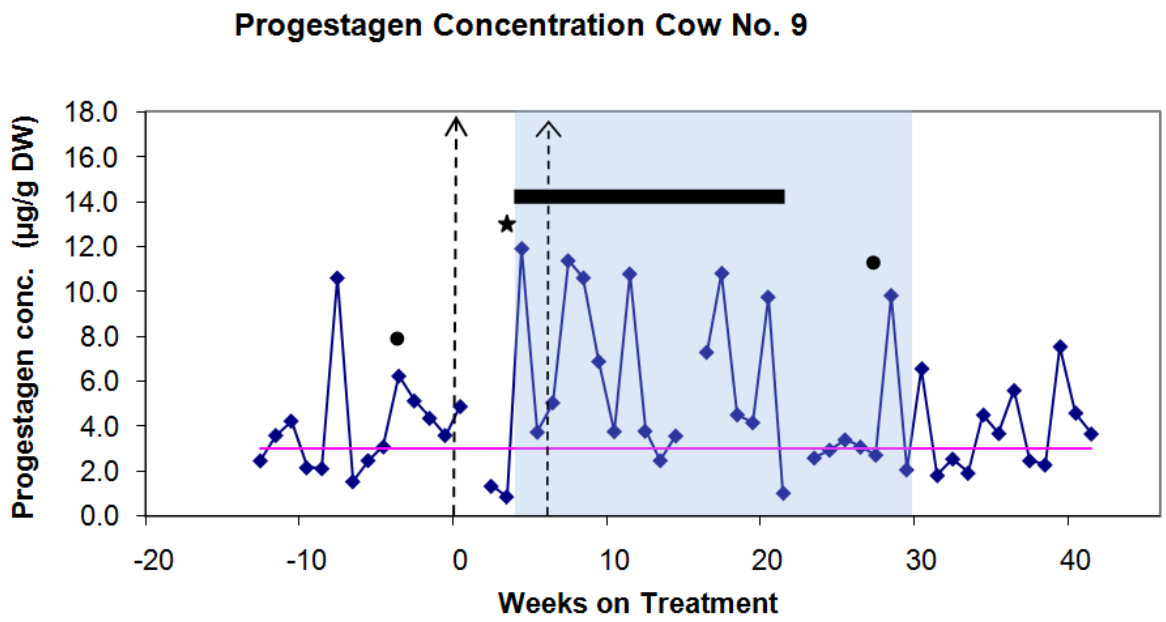

Figure 16. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 9) treated with the GnRH vaccine. Red solid line represents baseline concentration, horizontal solid bar represents luteal phase and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening or urine and stars for vaginal discharge observations. Wet season is illustrated in blue background.

b) Upper Escarpment herd

Cow No. 11 (Figure 17):

- Matriarch, 30+ years of age, lactating male calf No. 16
- Received primary GnRH vaccine treatment and 7 weeks later received booster. Due to incomplete delivery, she only received 2 ml out of the 3 ml dose.
- Long anoestrus period during 2009 dry season, and resumed cycling with one short luteal phase during the wet season.
- Booster probably too late to interfere with luteal phase
- The average FPM concentration for the luteal phase was 11.12 ± 15.94 µg/g DW and 1.96 ± 0.58 µg/g DW for the inter-luteal phase.

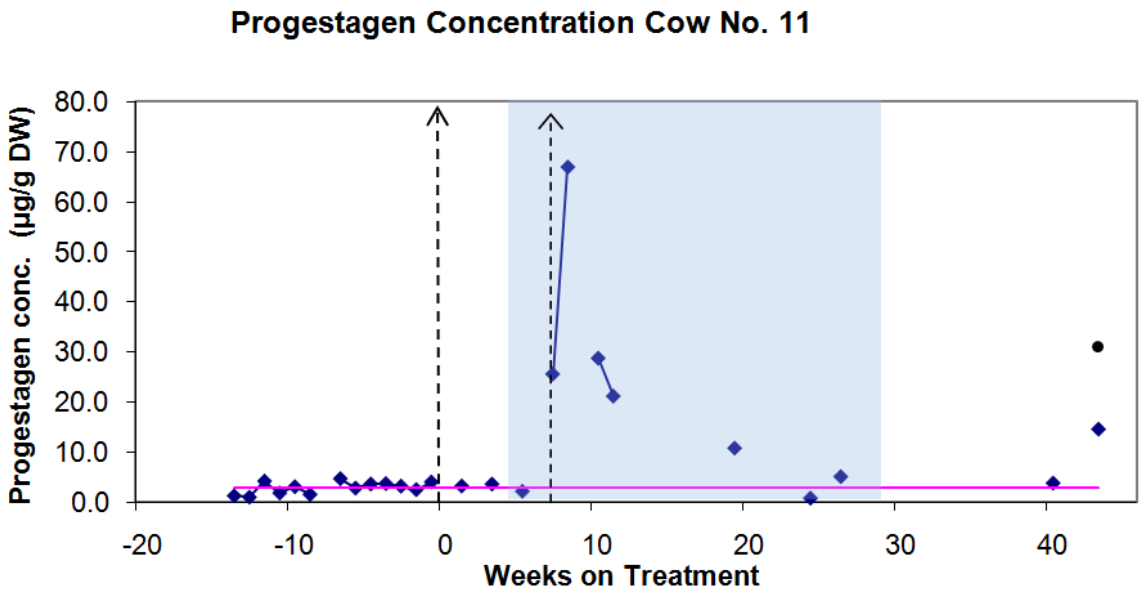

Figure 17. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 11) treated with the GnRH vaccine. Red solid line represents baseline concentration and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening observations. Wet season is illustrated in blue background.

Cow No. 12 (Figure 18):

- Cow 30+ years of age, her last calf was euthanised in November 2007 at the age of 2.5 years because of a knee fracture
- Received primary GnRH vaccine treatment and 7 weeks later a booster. Due to incomplete delivery, she only received 1.5 ml out of the 3 ml dose.
- Long anoestrus period during 2009 dry season, and resumed cycling during the wet season
- Booster probably too late to affect first luteal phase
- The average FPM concentration for the luteal phase was 11.33 ± 17.75 µg/g DW and 1.70 ± 0.43 µg/g DW for the inter-luteal phase.

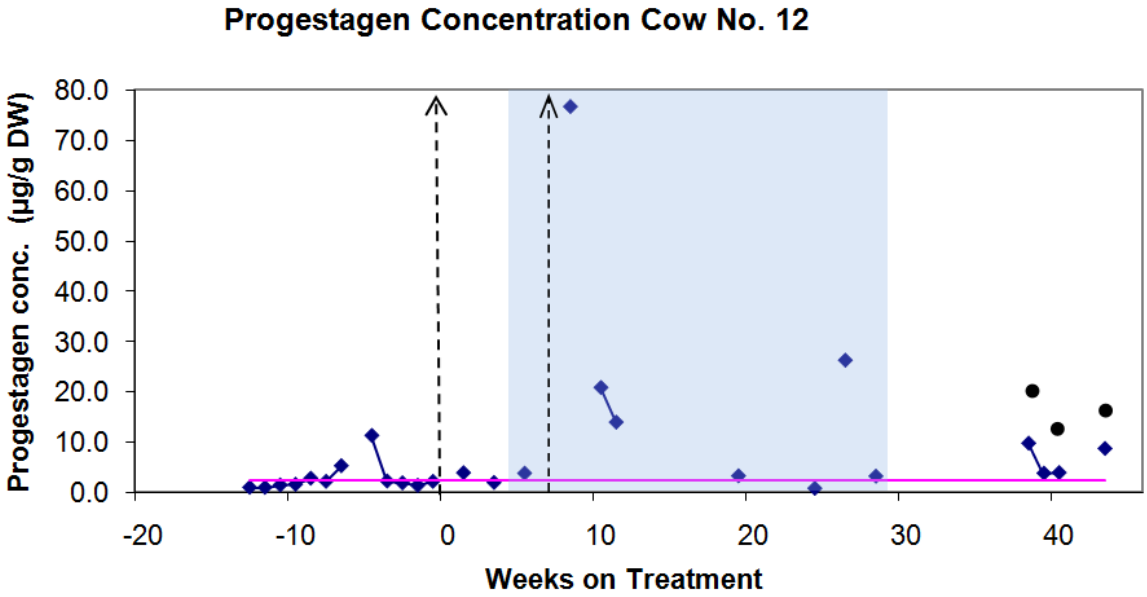

Figure 18. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 12) treated with the GnRH vaccine. Red solid line represents baseline concentration and dotted line arrows correspond to darting dates of primary vaccine and subsequently booster vaccine. Circles stand for sniffing into genital opening observations. Wet season is illustrated in blue background.

Cow No. 13 (Figure 19):

- Cow 15 years of age, no calves
- Control individual, received no GnRH treatment
- Started with an anoestrus period during dry season 2009, resumed one cycle before the start of the wet season.
- The average FPM concentration for the luteal phase was 5.89 ± 2.56 µg/g DW and 2.54 ± 0.75 µg/g DW for the inter-luteal phase.

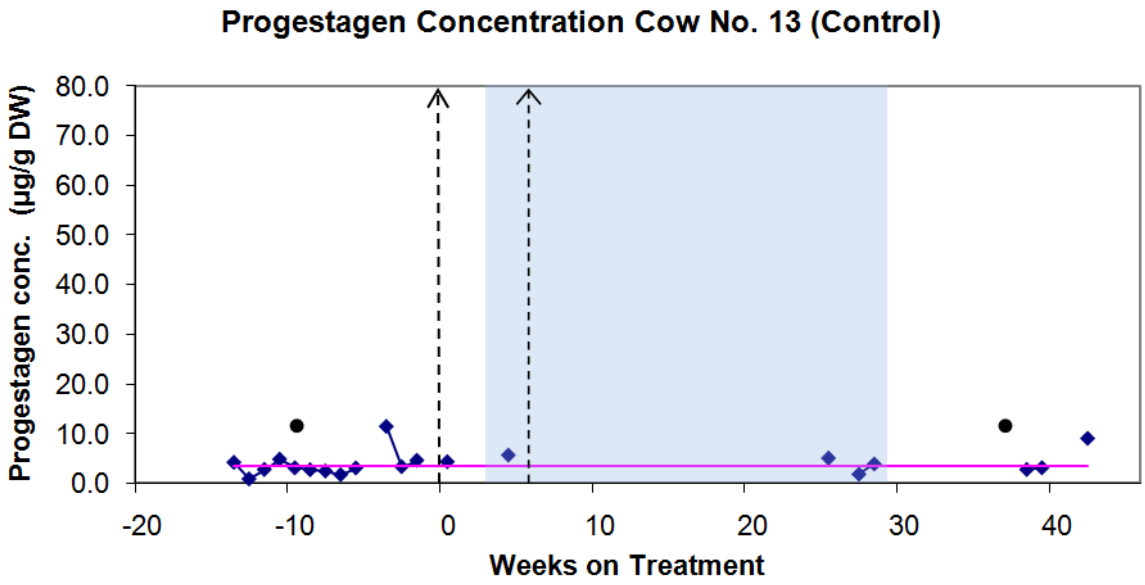

Figure 19. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 13; control group). Red solid line represents baseline concentration and dotted line arrows serve as reference to darting dates of primary vaccine and subsequently booster vaccine on treated individuals. Circles stand for sniffing into genital opening or urine observations. Wet season is illustrated in blue background.

Cow No. 14 (Figure 20):

- Cow 15 years of age, no calves
- Control individual, received no GnRH treatment
- Presented one luteal phase during the wet season
- The average FPM concentration for the luteal phase was 8.28 ± 6.37 µg/g DW and 1.83 ± 0.59 µg/g DW for the inter-luteal phase.

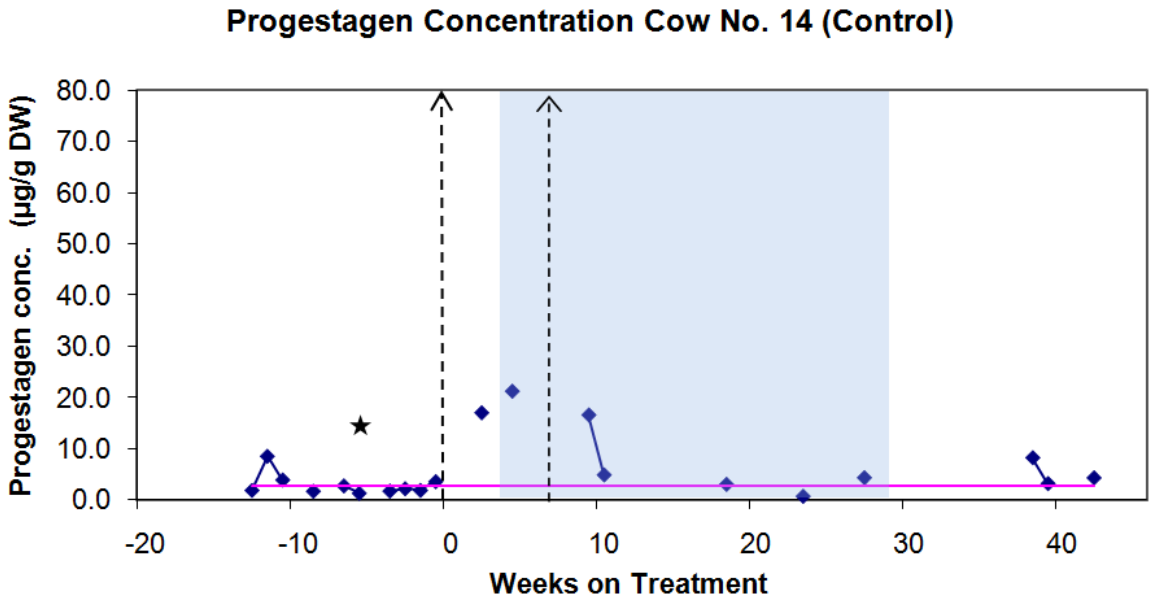

Figure 20. Faecal progestagen concentrations for an individual adult African elephant female (Cow No. 14; control group). Red solid line represents baseline concentration and dotted line arrows serve as reference to darting dates of primary vaccine and subsequently booster vaccine on treated individuals. Stars stand for vaginal discharge observations. Wet season is illustrated in blue background.

4.1.3.2 Effect of GnRH vaccine treatment on oestrous cycle of groups

The baseline values, average luteal and inter-luteal phase FPM concentrations per individual of the Lower and Upper Escarpment herds are summarized in Table 6.

Table 6. Baseline, luteal phase, inter-luteal phase means ± SD of faecal progestagen metabolite concentrations (µg/g DW) of African elephant female individuals at Entabeni Safari Conservancy, South Africa.

COW No.	n	BASELINE	LUTEAL PHASE MEAN	INTER-LUTEAL PHASE MEAN
1	64	1.96 ± 0.86	4.83 ± 3.44	1.27 ± 0.34
2	64	3.13 ± 1.39	6.18 ± 3.35	2.03 ± 0.76
3	59	3.10 ± 1.30	6.13 ± 3.50	1.93 ± 0.69
4	59	2.34 ± 1.30	5.84 ± 3.57	1.33 ± 0.54
5	59	1.81 ± 0.75	4.28 ± 2.04	1.30 ± 0.35
7	63	1.53 ± 0.55	3.80 ± 2.73	1.10 ± 0.27
8	50	2.63 ± 1.23	5.47 ± 2.76	1.64 ± 0.42
9	56	3.03 ± 1.16	6.32 ± 3.55	2.09 ± 0.64
11	25	2.90 ± 1.25	11.12 ± 15.94	1.96 ± 0.58
12	27	2.39 ± 1.13	11.33 ± 17.75	1.70 ± 0.43
13	20	3.36 ± 1.25	5.89 ± 2.56	2.54 ± 0.75
14	20	2.82 ± 1.25	8.28 ± 6.37	1.83 ± 0.59

n= number of samples collected from each individual

The average luteal FPM concentration of the Lower Escarpment herd treatment group was 5.53 ± 3.23 µg/g and 4.72 ± 3.28 µg/g for the control group. Average baseline concentration was 2.63 ± 1.26 µg/g for treatment group and 1.95 ± 1.09 µg/g for the control group. There were no significant differences in mean luteal phase ($P > 0.45$) or baseline ($P > 0.24$) FPM concentrations between

treatment (*n=6*) and control (*n=2*) groups. Peak luteal phase concentrations per individual ranged from 9.58 to 19.88 µg/g. Average inter-luteal concentration for the treatment group was 1.71 ± 0.65 µg/g and 1.22 ± 0.44 µg/g for the control group (Table 7).

The average luteal FPM concentration of the treatment group on the Upper Escarpment herd was 11.22 ± 16.61 µg/g and 7.25 ± 5.14 µg/g for the control group. Average baseline concentration was 2.64 ± 1.20 µg/g for the treatment group and 3.12 ± 1.27 µg/g for the control group. There was no significant difference in mean luteal phase ($P > 0.70$) or baseline ($P > 0.69$) FPM concentrations between treatment (*n=2*) and control (*n=2*) groups. Peak luteal phase concentrations per individual ranged from 11.39 to 76.87 µg/g. Average inter-luteal concentration for the treatment group was 1.80 ± 0.50 µg/g and 2.24 ± 0.76 µg/g for the control group (Table 7).

Table 7. Baseline, luteal phase, inter-luteal phase means ± SD and peak luteal range of faecal progestagen metabolite concentrations (µg/g DW) of African elephant female groups.

GROUP	n	BASELINE MEAN	LUTEAL PHASE MEAN	INTER-LUTEAL PHASE MEAN	PEAK LUTEAL RANGE
Lower Escarpment Treatment	6 (352 samples)	2.63 ± 1.26	5.53 ± 3.23	1.71 ± 0.65	9.58 - 19.88
Lower Escarpment Control	2 (122 samples)	1.95 ± 1.09	4.72 ± 3.28	1.22 ± 0.44	12.28 - 15.98
Upper Escarpment Treatment	2 (52 samples)	2.64 ± 1.20	11.22 ± 16.61	1.80 ± 0.50	67.06 - 76.87
Upper Escarpment Control	2 (40 samples)	3.12 ± 1.27	7.25 ± 5.14	2.24 ± 0.76	11.39 - 21.21

n= number of samples collected from each individual

4.1.3.3 Oestrous cycle length and component phases

Sixteen cycles could be identified in 8 females of the Lower Escarpment herd during the study period (Table 8), but only 4 (25%)of these fell within the normal 13-17 weeks oestrous cycle range described for African elephants (Brannian *et al.* 1988; Plotka *et al.* 1988; Wasser *et al.* 1996; Heistermann *et al.* 1997; Hodges *et al.* 1997; Schwarzenberger *et al.* 1997; Hodges 1998; Fieβ *et al.*

1999; Brown 2000; Ortolani *et al.* 2005; Brown 2006; Bertschinger *et al.* 2008a; Hildebrandt *et al.* 2010). These were the cycles of 3 treatment cows (Cow No. 1, 2 normal cycles; Cows No. 2 and No. 8, 1 normal cycle each). The mean length of these 4 cycles was 14.11 ± 1.35 weeks with luteal and inter-luteal phases of 9.39 ± 0.75 and 4.72 ± 1.11 weeks, respectively. The 12 remaining cycles had shorter (*n=3,* 25%), longer (*n=3,* 25%) or normal (*n=6,* 50%) luteal phases combined with shorter (*n=5,* 41%) or longer than expected (*n=4,* 33%) inter-luteal lengths.

Table 8. Summary of oestrous cycle lengths (weeks) of the Lower Escarpment herd.

		COW No.							
		1	**2**	**3**	**4**	**5**	**7**	**8**	**9**
PRE-TREATMENT	**LP**	4.43	8.43	5.71					
	ILP	9.57	7.86	7.14					
	TC	**14.00**	**16.29**	**12.86**					
POST-TREATMENT	**LP**	9.00	6.00		10.00	25.86	15.86	10.43	16.86
	ILP	3.86	8.86		1.71			5.29	
	TC	**12.86**	**14.86**		**11.71**	**25.86**	**15.86**	**15.71**	**16.86**
	LP	5.57	8.71		10.14			10.57	
	ILP	2.43	6.00		0.71			2.14	
	TC	**8.00**	**14.71**		**10.86**			**12.71**	
	LP	9.43			12.14				
	ILP	3.71			2.43				
	TC	**13.14**			**14.57**				

LP= Luteal phase; ILP= Inter-luteal phase; TC= Total cycle length
Yellow= short phase; red= long phase; green= normal cycle

4.2 Effect of age class on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

The mean FPM concentration for adult cows (5.78 ± 3.36 µg/g; *n= 177*) was significantly higher (DF=1, *P*=0.006) than the mean FPM concentrations for sub-adult cows (4.27 ± 2.03 µg/g; *n=36*) (Figure 21).

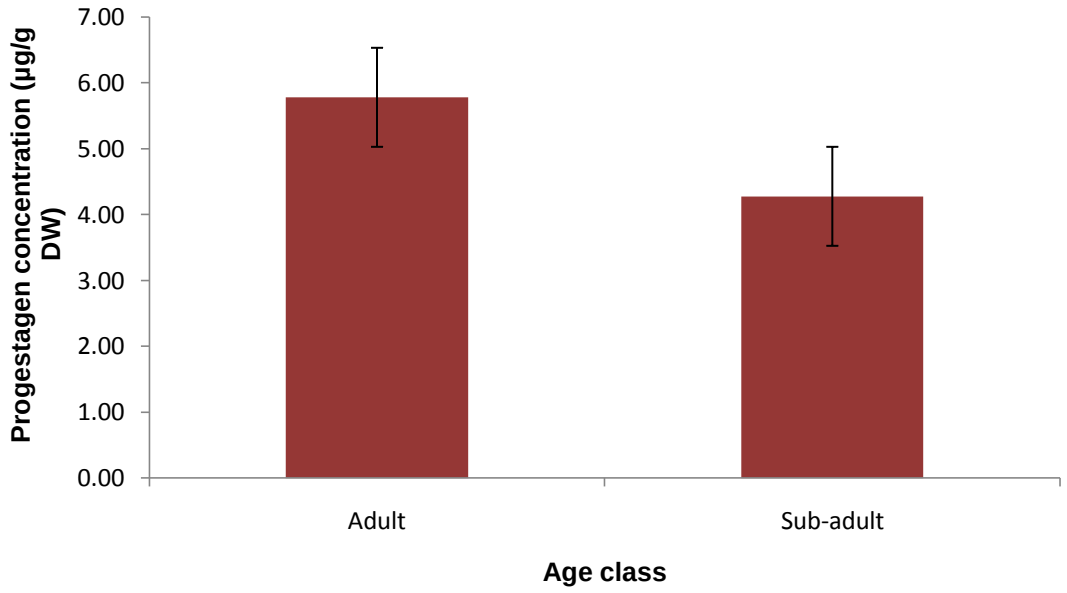

Figure 21. Average progestagen luteal concentrations (µg/g DW) for adult (*n=5*) and sub-adult (*n=1*) GnRH vaccine treated African elephant females from the Lower Escarpment herd from May 2009 to June 2010 at Entabeni Safari Conservancy, South Africa. Error bars are mean ± SEM.

4.3 Effect of seasonality on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

There was a significant effect of season (dry or wet) on mean FPM concentration (DF=2, F=5.67, *P*=0.004). The mean FPM concentration ± SEM for the wet season (2009-2010) was 6.12 ± 0.28 µg/g (*n= 124*) and significantly higher than both the 2009 (4.95 ± 0.49 µg/g; *n=41*) and 2010 (4.45 ± 0.45 µg/g; *n=48*) dry seasons. There was no statistical difference between the two dry seasons (DF=1, *P*=0.92) (Figure 22).

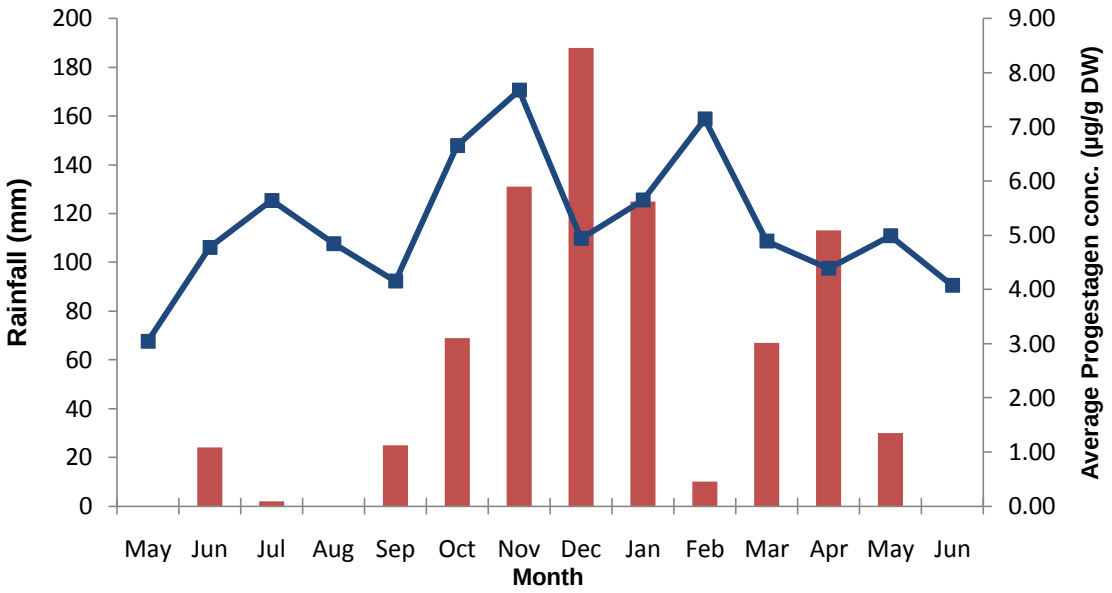

Figure 22. Mean monthly rainfall (horizontal bars) and average progestagen luteal concentrations (blue line) for GnRH vaccine treated African elephant females from the Lower Escarpment herd from May 2009 to June 2010.

For the Upper Escarpment herd there was insufficient data to run a statistical analysis, although the drastic changes from a period of anoestrus on the dry season 2009 to high concentration in FPM during the rainy season were noteworthy (Figure 23).

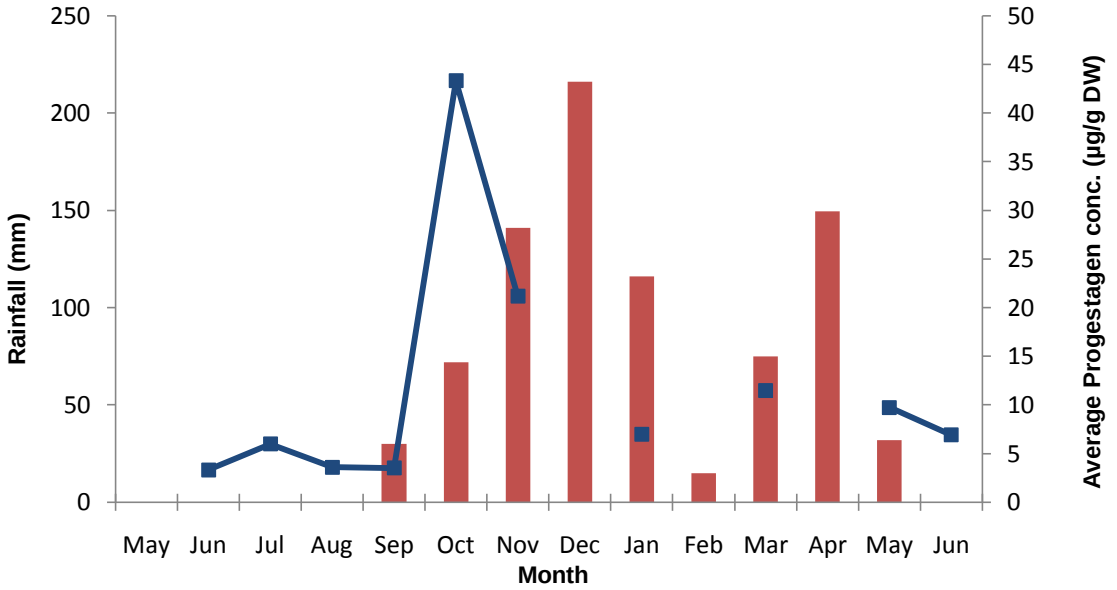

Figure 23. Mean monthly rainfall (horizontal bars) and average progestagen luteal concentrations (blue line) for GnRH vaccine treated and control African elephant females from the Upper Escarpment herd from May 2009 to June 2010.

4.4 Effect of dominance/ranking on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

There was no effect of ranking on the mean FPM concentrations (DF=1, *P*=0.4853). The mean concentration of the dominant cows was 5.74 ± 3.48 µg/g (*n= 111*) compared to 5.28 ± 2.92 µg/g (*n=102*) for sub-dominant individuals (Figure 24).

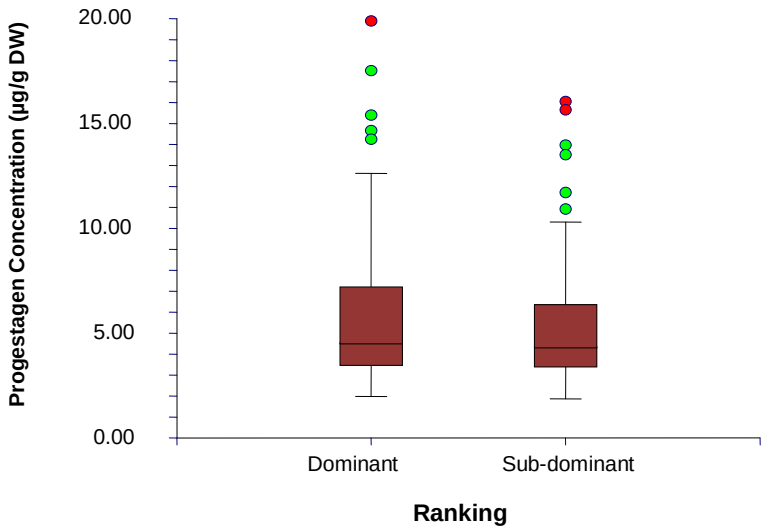

Figure 24. Median, interquartil range, range and outliers of progestagen luteal concentrations (µg/g DW) for dominant and sub-dominant GnRH vaccine treated African elephant females from the Lower Escarpment herd.

CHAPTER 5

DISCUSSION

5.1 GnRH vaccine treatment

5.1.1 Administration of the vaccine

The delivery system (ground vs. aerial) appeared to cause a similar effect in all treated animals. The behavioural changes observed during ground-darting, which took 15 days, were avoidance of the research vehicle, increased wariness, reduced time on supplementary feeding spot, and animals fled easily. The elephants resumed normal behaviour (i.e., more relaxed and longer observation time during supplementary feed) a few days after the last dart was fired. Darting from the helicopter caused far less disturbance and was more effective than ground-darting. This was probably because it only lasted 9 minutes and it was not associated with the research vehicle. The animals returned to pre-darting behaviour 2 days later.

The approximate cost of the ground-darting, which was used with the first vaccination of the Lower Escarpment herd plus the first and booster vaccination of the Upper Escarpment elephants cost about R 800.00 in total which includes fuel and salary of the driver for the 15 days. There was no veterinary fee included as the darting was performed by HJ Bertschinger and G Benavides. The cost for the aerial-vaccination was R 9 000.00 in total, inclusive of 30 min of Entabeni´s helicopter time (R 7 000.00; the rest of the time flown was used to try to locate the Upper Escarpment herd), plus the visit and consultation fee of the veterinarian who performed the aerial-darting (R 2 000.00). If a veterinarian had been hired to perform the ground-vaccination as well, the veterinary fee would had been around R 24 000.00 extra. The expense of R 9000.00 of the helicopter in delivering the vaccinations compared to R 24 800.00 from the ground-vaccination justified the disadvantages of the ground-vaccinations by saving time and resulting in only one stressful event for the herd.

The side effects post-vaccination were minimal and similar to any dart wound, which were not of veterinary concern.

5.1.2 Non-invasive faecal steroid monitoring

The monitoring of the progesterone metabolites through faeces was shown to be a reliable method in this study to monitor the oestrous cycles of free-ranging elephants, as described previously by Foley 2001, Wittemeyer *et al.* 2007a, and Bates 2010. Samples could be collected on a regular basis from the Lower Escarpment cows without causing disturbances to the herd. The habituation of the herd to supplementary feeding provided an ideal opportunity for behavioural observations as well as faecal sample collection.

The small number of samples collected from the Upper Escarpment was due to the lack of a radio collar within the herd, which together with the difficult mountainous terrain made them difficult to locate, particularly during the wet season when supplementary feeding was not possible.

The long oestrous cycle of 13 to 17 weeks of the African elephants (First reports, Plotka et al. 1988) and the effect of the variability of the ecological conditions on FPM concentrations (Foley *et al.* 2001; Wittemeyer *et al.* 2007a) made it difficult to elucidate the effect of the GnRH vaccine treatment on the oestrous cycle on only 12 months of non-invasive monitoring performed in this study. We would recommend extending the observation period to 2 to 3 years in places with seasonal variation.

The behavioural and other signs of oestrus recorded in this study showed a close temporal relationship with subsequent increases in FPM concentrations. This confirms the observations that behavioural oestrus precedes a rise in FPM concentrations and can be used as a supplementary indicator of oestrus as reported previously (Hodges 1998; Estes 1999; Ortolani *et al.* 2005). No other oestrus or courtship behaviours were observed as reported by Moss (1983) and Estes (1999) which may be attributable to the lack of a mature bull on the reserve.

5.1.3 Effect of GnRH vaccine treatment on oestrous cycle

The dose of 3 ml (600 µg RnRF-protein conjugate) of Improvac® used in this research was based on the successful down-regulation of aggression and androgen secretion from a previous report in an elephant bull treated with Improvac® (De Nys *et al.* 2010; Bertschinger, personal communication 2011) and another 30 bulls in which the same dose has been used and repeated every 6 months (Bertschinger, personal communication 2011). Trials on other species show that the effectiveness of the GnRH contraceptive response is dose-related and in large-sized animals the administration of

1,500 to 2,000 µg of conjugate is recommended to achieve the contraceptive effect (Miller *et al.* 2004). During the trials in elephant bulls, 2,000 µg GnRH (modified GnRH-tandem-dimer-ovalbumin conjugate, Pepscan Systems, Netherlands) was injected three or four times at approximately three weeks intervals. The bulls (three out of six) with raised faecal androgen concentrations at the start of the study could all be down-regulated. This status was maintained until the end of four month observation period (De Nys *et al.* 2010).

In this study the GnRH vaccine failed to not induce anoestrus in female elephants as was expected using two doses of 600 µg. This leaves the possibility that the ineffective or incomplete action of the vaccine was a consequence of under-dosing as it is below the dose recommended by Miller *et al.* (2004). When compared to the protocol used by De Nys (2005) in male elephants, this protocol´s dose was also low, although there is proof of gender differences in the secretion pattern of GnRH and gonadotrophic hormones, which suggests that females may need a lower dose compared to males (Killian *et al.* 2006). As the results of De Nys were inconclusive in all the treated animals, it may suggest that the dose in the protocol used by De Nys could also have been increased.

There is a possibility that the booster was administered too late to downregulate the second cycle in some cows, as can be seen in the profiles of cows No. 1, 2, 8, 9, 11, and 12 (Figures 9, 10, 15, 16, 17, 18; respectively) where they had already entered the luteal phase before this vaccination. Another study in mares found that there were differences in the amount of time from vaccination to cessation of ovarian activity depending on the stage of the cycle in which the vaccine was applied, being more effective if applied during the luteal phase (dioestrus) than in mares with large follicles (oestrus) (Imboden *et al.* 2006). In this study the booster was applied five to seven weeks after the primary vaccination, as recommended by Pfizer, and suggested by the 100% success of downregulation in mares by Botha *et al.* 2008. The stage of the oestrous cycle was unknown at the time of vaccination. Other incomplete vaccinations may have occurred due to the viscous nature of the solution.

All the females showed some evidence of ovarian cyclicity during the one-year study, although most (75%) of the cycles did not fall within the normal 13-17 weeks oestrous cycle range reported. For both herds there was no significant difference in mean or baseline FPM concentrations between treatment and control groups. The Upper Escarpment herd had higher mean luteal, inter-luteal and peak FPM concentrations compared to the Lower Escarpment herd in both treatment and control groups which can be attributable to the different habitats and the resource availability, which is more pronounced in the Upper Escarpment through the seasons.

It is evident that three cows from the Lower Escarpment (No. 5, 7 & 9) showed a period of anoestrus followed by a long luteal phase (25.86, 15.86 and 16.86 weeks, respectively) after the vaccination. In domestic animals this can possibly be attributed to a persistent *corpus luteum* or a luteal cyst, although it was highly unlikely that these cows presented any of these abnormalities and it cannot be considered as an effect of the vaccine because one of these cows (No. 7) received no GnRH treatment.

The abnormal cycles in elephant cows are not well understood (Proctor *et al.* 2010) and there are several studies trying to find the pathological, environmental and/or social factors that cause the irregularities. In the wild, we presume that sexually receptive periods in cows result in mating and pregnancy. This would mean that, once a cow has conceived she will not cycle again for at least another two to three years. Repeated oestrous cycles are not a normal feature of reproduction in free-ranging elephants (Rasmussen and Schulte 1998; Moss 2001). With the exception of a study carried out by Bates (2010) there is thus a lack of information to which our data can be compared.

In the study conducted over a period of one year Bates (2010) observed that 42.9% of the females in a wild herd in South Africa treated with pZP contraceptive were not cycling and 14.3% had irregular cycles. In captive populations in North America, 43% of African elephants also present abnormal cycles, some of the causes proposed are reproductive tract pathologies, alteration in the secretion of pituitary gonadotrophins, thyroid hormones and hyperprolactinemia (Brown *et al.* 2004a; Proctor *et al.* 2010).

Other studies observed how social and environmental variables affect ovarian cyclicity. Proctor *et al.* (2010) suggest that suboptimal social and environmental conditions could contribute to the reduction of reproductive performance. A social factor studied by Freeman *et al.* (2009) found that females in captivity that had resided longer with the same herd-mates were more likely to be acyclic.

A possible cause of irregular cycles in some of Entabeni´s cows could be the effect of previous pZP treatment. Kirkpatrick *et al.* (1995) found that after 7 years of treatment with pZP vaccination in mares there was a decline in the production of ovarian oestrogen, which caused the mares to cycle intermittently and a reduction in ovulation rates. Powell and Monfort (2001) also demonstrated that the anovoulatory condition in these mares was episodic and the duration of these episodes was variable, and suggest that pZP immunocontraception may not be reversible in all mares after only one year. Some studies have also found significant ovarian pathologies associated with pZP treatment in several other species, such as multi-oestrus in female deer (Kirkpatrick *et al.* 1997; Miller & Killian 2000); altered oestrous cycles in canids (Mahi-Brown *et al*. 1985; AZA 2010), rabbits

(Wood *et al.* 1981; AZA 2010) and cynomolgus monkeys (Gulyas *et al*. 1983); changes in steroid profiles in canids (Mahi-Brown *et al*. 1985) and some primates (Sacco *et al.* 1987); and temporary cessation of oestrous cycles in some primates (Sacco *et al.* 1987; AZA 2010). The degree, range and duration of these abnormalities were variable across individuals and species (Wood *et al.* 1981; Sacco *et al.* 1987) and could be related to individual and/or species-specific differences in the immune response generated by the pZP vaccine (Powell & Monfort 2001). In our study irregular cycles were found in all the cows, but only 4 had previously been treated for 3 years with pZP contraceptive.

Elephants are social animals and live in herds that are dominated by a matriarch. The breeding herd has a pyramidal structure and consists of the matriarch and her mature daughters and their respective offspring. Inhibiting individual fertility and herd growth may have long-term consequences on behaviour and social structure (Perdok *et al.* 2007). The Lower and Upper Escarpment herds on this study last had a born calf in 2005 and 2008, respectively; in addition they had been translocated and left without mature males, and both herds are extremely nervous.

The lack of changes in group dynamics, absence of mature males, living in a fenced environment where seasonal movements are not possible, the continuous nervousness, and the potential effect of previous pZP treatment on some of these cows might have contributed to the high incidence of cyclic abnormalities observed in Entabeni´s elephant herds.

In addition to the oestrous cycle abnormalities, we observed changes to the social behaviour as a consequence of inhibiting reproduction. The last born calf of Cow No. 1 was still being nursed at 10 years of age. Normally they would be weaned at approximately two years of age (Sukumar 2003; Garaï 2005). This was the most extreme case, but all of the 4 younger (4, 4, 6, and 10 years old) elephants on the property were still suckling. Contraception also alters the normal age structure of the breeding herd by preventing the addition of newborn calves.

5.2 Effect of age class on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

Reproductive rates in free-ranging African elephants decline as they get older, as shown by physiological and demographic analyses (Laws *et al.* 1970; Smuts, 1975; Freeman *et al.* 2008).

Furthermore, Wittemayer *et al.* (2007a) found that faecal progestagen concentrations decreased linearly with age in free-ranging female elephants.

In species such as humans, a decline in anti-Müllerian hormone (AMH) circulating concentrations has been correlated with the number of morphologically-healthy oocytes within the follicular reserve and is clinically used to determine the onset of menopause in women (Visser *et al.* 2006; Dow *et al.* 2011). In elephants AMH concentration has also been found to decline with age which most probably would be an indication of reproductive cessation in aged elephants (Dow *et al.* 2011).

Our study showed that adult animals had higher FPM concentrations than sub-adults. The herds at Entabeni are still relatively young with no cows having reached 50 or more years of age, which is suggested as the age when reproductive rates start to decline in female elephants (Freeman *et al.* 2010).

Some of the variation in the response to the vaccine in other studies have included the age and state of sexual maturity of the individuals. These showed the best response in young or pre-pubertal animals in contrast with mature animals which produce greater individual variations with less marked and shorter responses (neonatal lambs: Clarke *et al.* 1998; Brown *et al.* 1994b; mature stallions: Malmgren *et al.* 2001; Janett *et al.* 2009; mature mares: Dalin *et al.* 2002; colts and fillies: Dowsett *et al.* 1993; adult antelopes: Turkstra *et al.* 2001; yearling and adult female deer: Curtis *et al.* 2002). Stout & Colenbrander (2004) and Stout *et al.* (2007), state that it is more difficult to achieve suppression of follicle development and ovulation in older mares. De Nys (2010) achieved a better response in younger elephant bulls (18-22 years of age) than in the older bulls (27-40 years of age) using GnRH immunization with significant decreases in faecal epiandrosterone concentrations and consequently reduction of aggressive behaviour.

In this study Cow No. 5 (sub-adult individual; 15 years) had the lowest average progestagen concentration levels attributed to her younger age. As a result her chances of responding to the contraceptive may possibly have been better. However contrary to the expectations this was not the case. It may be that additional boosters or increasing the dose of the vaccine are indicated to induce a better response in older cows.

5.3 Effect of seasonality on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

There is a positive linear relationship between the rainfall and production of herbaceous matter (Tainton 1981). The total availability of plant feed and quality in terms of digestibility, nitrogen and phosphorus content, differs between the dry and wet season (Centre for Wildlife Management 1998). The effect that these seasonal changes have on reproductive hormonal activity in non-pregnant free-ranging African elephants has been previously documented (Foley *et al.* 2001; Wittemeyer *et al.* 2007a). With the onset of the rainy season, the quality and availability of resources significantly increases resulting in a better body condition, increased faecal progestin levels and a peak in reproductive activity (Wittemeyer *et al.* 2007a).

In our study it was evident the effect of the dry season (starting April through September) on FPM levels in nearly all study animals. They either had lower FPM levels or a period of hormonal flat-lining (anoestrus). In the latter case cyclic activity resumed during the wet season (October through March).

This study commenced during the dry season of 2009 which would have been influenced by the previous 2008/2009 wet season. For the Lower Escarpment the wet season of 2008/2009 had higher rainfall (860 mm) than the 590 mm registered for the 2009/2010 season during the study. For the Upper Escarpment there is no information available for the wet season of 2008/2009 but considering that it receives higher rainfall compared to the Lower Escarpment it can be assumed that it also received above average rainfall.

The four Upper Escarpment cows (Cows No. 11, 12, 13, and 14), as well as four females (Cows No. 4, 5, 7, and 8) from the Lower Escarpment, appeared to experience a period of anoestrus during the dry season 2009. The fact that the effect is more marked in the Upper Escarpment is probably a consequence of the poor condition of the veld that provides low nutrition for the elephants.

The approximate start of the wet season in the Waterberg is 1st of October, although grass and trees often “green-up” before the rains, which corresponds to an increase in FPM levels in 6 of 12 cows (No. 1, 2, 3, 5, 7, and 14) commencing in September 2009.

Following the onset of the next dry season (post-treatment) in April 2010, the FPM levels decrease, and in almost all treated animals (5 of 6; Cows No. 1, 2, 3, 8, and 9) from the Lower Escarpment herd, the levels are lower than the previous dry season. This might reflect either an incomplete

effect of the GnRH vaccine or the effect of the amount of rain received during the previous rainy season.

In general, the average monthly FPM concentrations closely followed rainfall patterns and confirmed the relationship reported in the literature between ecological variation and ovarian activity (Foley *et al.* 2001; Wittemeyer *et al.* 2007a). Thus African elephants appear to optimize the timing of oestrus in order to maximize use of seasonal availability of resources to coincide with the energetic investment needed for reproduction (Wittemyer *et al.* 2007b).

5.4 Effect of dominance/ranking on faecal progestagen metabolite concentration in response to GnRH vaccine treatment

Dublin (1983) suggested that in wild herds of African elephants the dominant females use social aggression to suppress reproduction in subordinate individuals when resources are limited. Higher rank females will thus have greater access to resources such as food, shelter and reproductive mates to improve their chances of reproductive success. Archie (B. Archie, Amboseli, data unpublished, cited by Freeman *et al.* 2009) found that reproductive rates decline as females reach matriarchal status in free-ranging elephants. Contradictory findings with captive (Archie 2006) and with free-ranging African elephants (Freeman 2010) where dominance rank was not found to be a predictor of female fitness and the presence of female relatives *per se* had little effect on female reproductive performance. Bates (2010), on the other hand, found a statistical difference in FPM concentrations between dominant and less dominant herds, but showed no link between cyclicity status and dominance rank on individuals.

In this study we concur with Archie (2006), Freeman (2010) and Bates (2010) that ranking had no effect on FPM concentration or on ovarian cyclicity.

CHAPTER 6

CONCLUSIONS

Research on contraceptive techniques continues to be an important field in elephant conservation, not only for its efficacy and safety, but for providing acceptable alternatives to culling for management of overpopulation in certain areas.

This study concluded that a primary dose of 600 µg RnRF-protein conjugate (Improvac®; Pfizer Animal Health, Sandton, South Africa) followed by a single booster with the same dose 5 to 7 weeks later failed to induce anoestrus in 8 female African elephants. A review of the vaccination protocol is recommended. The doses should be increased and a second booster could be considered. As mentioned earlier, 5 to 7 weeks is regarded as being optimal for the booster.

The ecological factors that affect reproduction can be employed to enhance the efficacy of the administration of the contraceptive by optimizing the timing to achieve the highest population response, which in this case, should be most effective if conducted at the onset of the dry season. The physiological influence of age in the response to the vaccine should also be considered, as older cows might need a higher dose than younger cows.

The results of this study offer a novel insight into the ovarian activity of free-ranging non-pregnant female African elephants and confirm that abnormal oestrous cycles are a common feature amongst wild elephants (Bates 2010) and is not restricted or attributable to captivity as anticipated by several authors (Brown 2000; Schulte *et al.* 2000; Brown *et al.* 2004a; Brown *et al.* 2004b; Ortolani *et al.* 2005; Freeman *et al.* 2009; Proctor *et al.* 2010). This affirmation provides important information on the endocrine functioning and reproductive activity that can be used for improving elephant population management and control.

This study found no evidence of adverse side effects due to GnRH immunization during the observation period. Further research to optimize the protocol for using the GnRH vaccine is indicated in order to obtain consistent responses that will provide efficient and safe contraceptive management of female African elephants.

An important conclusion of this study is that an observation period of 12 months is far too short as interval. The long oestrous cycle (13-17 weeks) of the African elephant and the effects of season

serve to confound observations related to methods such as the induction of anoestrus. An interval of two (preferably three) years would be a more appropriate study period.

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Appendix A: Supplementary feed composition.

Commercial name: Wille Pille – 25 kg bags

Product Registration No. V21516 Act. 36/1947

Sussex Str. 10 Potgietersrus Mokopane

Tel/Fax: (015) 4915688

Specifications for game formulas

(Complete pellet)

NUTRIENT (%)	MIN	MAX
Moisture	-	12
Protein	12	-
Fiber	-	16
Fat	2.5	-
Urea	-	-
Calcium	0	0.8
Phosphorus	0.3	-

Basic ingredients (may vary with season availability): soya husks, lucerne, corn bran, maize, molasses meal, citrus, aloe powder*, modified soya, sunflower seed, binding agents (mycocurb and toxfin).

Perplex protein, vitamins and minerals added to enhance maximum horn-growth, fertility and immunity.

*Includes aloe powder for parasite control.

Appendix B: Summary of number of faecal samples collected from May 2009 to June 2010 at Entabeni Safari Conservancy, South Africa.

HERD	COW NO.	NO. SAMPLES PRE-TREATMENT	NO. SAMPLES POST-TREATMENT	TOTAL SAMPLES TX GROUP	NO. SAMPLES CONTROL GROUP
LOWER ESCARPMENT	1	22	42	64	
	2	21	43	64	
	3	20	39	59	
	4 (Control)				59
	5	18	41	59	
	7 (Control)				63
	8	13	37	50	
	9	16	40	56	
	TOTAL	110	242	352	122
UPPER ESCARPMENT	11	13	12	25	
	12	13	14	27	
	13 (Control)				20
	14 (Control)				20
	TOTAL	26	26	52	40
TOTAL	TX	136	268	404	
	CONTROL				162
	SAMPLES	566			

Appendix C: Summary of behaviour and signs that could be related to oestrus, recorded from May 2009 to June 2010 at Entabeni Safari Conservancy, South Africa.

DATE	COW NO.	OBSERVATION
15/08/2009	1	Genital inspection by No. 7
27/10/2009	1	Genital inspection by No. 7
21/01/2010	1	Genital inspection by No. 7
26/01/2010	1	Genital inspection by No. 7
25/05/2010	1	Genital inspection by No. 7
18/06/2010	1	Genital inspection by No. 7
09/05/2010	2	Genital inspection by No. 6
06/07/2009	3	Vaginal discharge - brownish
10/07/2009	3	Vaginal discharge
20/07/2009	3	Genital inspection by No. 1
07/09/2009	3	Genital inspection by No. 2 & 6
21/09/2009	3	Genital inspection by No. 1
25/11/2009	3	Genital inspection by No. 9
05/01/2010	3	Vaginal discharge - transparent
25/05/2010	3	Genital inspection by No. 1
01/12/2009	4	Vaginal discharge
02/12/2009	4	Genital inspection by No. 5
29/01/2010	4	Genital inspection by No. 5
08/02/2010	4	Genital inspection by No. 5
26/02/2010	4	Vaginal discharge - transparent
26/03/2010	4	Genital inspection by No. 8
13/04/2010	4	Genital inspection by No. 7
27/05/2010	4	Vaginal discharge - thick, brown
21/08/2009	5	Genital inspection by No. 1
17/08/2009	6	Genital inspection by No. 5
03/12/2009	6	Genital inspection by No. 5

DATE	COW NO.	OBSERVATION
09/03/2010	6	Genital inspection by No. 2
15/04/2010	6	Genital inspection by No. 1
03/11/2009	7	Genital inspection by No. 3
05/12/2009	7	Genital inspection by No. 4
08/01/2010	7	Genital inspection by No. 3
26/01/2010	7	Genital inspection by No. 1
25/05/2010	7	Genital inspection by No. 1
26/06/2010	7	Genital inspection by No. 2
30/05/2009	8	Vaginal discharge - transparent
29/12/2009	8	Genital inspection by No. 6
26/01/2010	8	Genital inspection by No. 10
25/06/2010	8	Genital inspection by No. 2
15/08/2009	9	Genital inspection by No. 1
03/10/2009	9	Vaginal discharge
18/03/2010	9	Genital inspection by No. 10 after urinating
28/06/2010	11	Genital inspection by No. 14
25/05/2010	12	Genital inspection by No. 11 after urinating
09/06/2010	12	Genital inspection by No. 11 after reuniting with herd
28/06/2010	12	Genital inspection by No. 11
02/07/2009	13	Genital inspection by No. 12
25/05/2010	13	Genital inspection by No. 14
28/07/2009	14	Vaginal discharge - transparent