Control of testosterone secretion, musth and aggressive behaviour in African elephant (*Loxodonta africana*) bulls using a GnRH vaccine

By

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Submitted in partial fulfilment of the requirements for the degree

Magister Scientiae

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July 2005

Acknowledgements

I would like to thank:

My parents for giving me the chance to study for a Master degree at the University of Pretoria and for their love and support throughout these years of study.

Professor Henk Bertschinger, my supervisor, for giving me the opportunity to work on this project and for his help and guidance.

Dominic Moss and Rynette Coetzee from Johannesburg zoo, the people from Imire Game Park and Tshukudu Game Reserve, Natascha Prinsloo from Shambala and Rory Hensman for giving me the opportunity to work on their elephant bulls and helping me with the sampling and for their hospitality.

Anne-Marie Human, the laboratory technician of the Veterinary Wildlife Unit, and Rupert Palme from the Institute of Biochemistry in Vienna, for the analysis of my samples.

Dr C. Foggin for helping me with the execution of this project in Zimbabwe.

Dr. H. Zulch, for her advice with the behavioural observations.
Prof. Nothling for his advice with the statistical analysis.

Andrew Taylor, SP Sasidharan, Susane Schmidt and Sven Parsons for their precious help, advice and support.

Pepscan Systems (Lelystad, The Netherlands) for providing the vaccine.

The University of Pretoria and the Veterinary Wildlife Unit for providing funding for the project. The South African Veterinary Foundation is acknowledged for funding the laboratory costs.

My friends for their support throughout this project.

Declaration

I, H.M. De Nys, 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 fulfilment of the requirements for the degree MSc in Production Animal Studies.

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Signed ……

HM De Nys

Date…….

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African elephant (*Loxodonta africana*)
Bulls
Behaviour
Musth
GnRH vaccine
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Remote delivery
Faecal epiandrosterone
Testosterone
Faecal 3α,11oxo-cortisol
GnRH antibody titres

ABSTRACT

Aggressive behaviour with or without musth can constitute a serious management problem for captive and free-ranging African elephant bulls, and can endanger the lives of both animals and man where such elephants are kept. Generally, bulls in musth have to be restrained to such an extent that it becomes an animal welfare issue. Often euthanasia constitutes the only resort since there are as yet no practical ways to control musth. Musth and aggressive behaviour seem to be related to high concentrations of testosterone. In this study, the effects of a GnRH vaccine on faecal epiandrosterone and behaviour were tested in six African elephant bulls. Anti-GnRH antibody titres could be measured in three bulls after immunization. Faecal cortisol metabolites were also monitored in order to explore a possible link with androgen secretion.

The vaccine (GnRH-tandem-dimer conjugated to ovalbumin, Pepscan Systems, the Netherlands) was used with either Montanide ISA 51 or Covaccine adjuvants. Five elephant bulls were vaccinated with 2 mg GnRH 3 or 4 times at approximately 3-week intervals by means of darting or hand-injection. Faecal samples were collected during each week prior to vaccination and 4 months after the last vaccination. Behaviour was monitored concurrently.

Three of the bulls responded to the vaccine with significant decreases in faecal epiandrosterone concentrations. The first bull had been exhibiting aggressive behaviour with temporal gland secretion for some time. Three vaccinations were carried out using ISA 51 adjuvant and the last one using Covaccine. A marked effect was observed on faecal epiandrosterone after the 4th vaccination. He also displayed calmer episodes and a reduction in temporal gland secretion. Because the emulsion formed with ISA 51 proved too viscous for darting purposes, Covaccine adjuvant was used for the remaining bulls. The second bull was extremely irritable and had been damaging properties on a regular basis for months. After the 3rd vaccination, antagonistic behaviour and temporal gland secretion ceased and epiandrosterone concentrations were significantly lower. Increased antibody titres were observed. The third bull was a trained animal that had not exhibited aggressive behaviour before vaccination. Faecal epiandrosterone was reduced after the second vaccination. No changes in faecal epiandrosterone concentrations were observed in the three remaining bulls. One of these bulls, however, was in musth before vaccination and went out of musth 10 days

after the first vaccination. Androgen levels were low just before the primary vaccination, which indicates that the primary vaccination may have coincided with the natural end of musth. Cortisol levels increased when musth signs disappeared. The two other bulls had not exhibited aggressive behaviour before vaccination and no behavioural changes were observed after immunization. Positive antibody titres, however, were observed. A positive correlation between androgen and cortisol secretion was observed in these two bulls. The vaccine produced no side effects whatsoever in any of the bulls.

The observed improvement in behaviour of bulls that were aggressive prior to vaccination and the absence of adverse side effects, suggest that this method could constitute a way of controlling musth and aggressive behaviour in African elephants. There was, however, marked individual variation in response to GnRH immunization. Possible factors influencing the response of each bull are discussed. Age seems to be important, as the youngest bulls showed a better response in terms of reduced androgen secretion. Moreover, species-specific differences could influence the efficacy of GnRH immunization as well as the detection of hormonal changes after vaccination. The cortisol and androgen metabolite results suggest that a substantial amount of androgens may be secreted by the adrenal glands in African elephants. Observed patterns of hormonal secretion in the musth bull support the hypothesis that high androgen levels associated with musth suppress cortisol secretion.

Further work to determine the optimal vaccination protocol is needed in order to obtain marked and consistent responses to the vaccine.

CHAPTER 1

INTRODUCTION

Musth is a behavioural, physical (Poole 1987c) and physiological (Hall-Martin and Van Der Walt 1984; Jainudeen *et al.* 1972a) condition exhibited periodically by adult male elephants (Poole 1987c). It is associated with increased aggressive behaviour, creating a serious management problem of captive and free-ranging elephant bulls, which endangers the lives of both animals and man where these elephants are kept. Musth is well described in Asian elephant (Elephas maximus) bulls (Eisenberg *et al.* 1971; Jainudeen *et al.* 1972a; Jainudeen *et al.* 1972b), where, for centuries, it has been known to occur. More recently it has also been described in African elephant (Loxodonta Africana) bulls (Hall-Martin and Van Der Walt 1984; Poole and Moss 1981). Characteristics of musth in Asian and African bulls are remarkably similar (Poole 1987c). The main signs of musth are heavy temporal gland secretion, continuous urine dribbling and increased aggressive behaviour (Hall-Martin and Van Der Walt 1984; Jainudeen *et al.* 1972b; Poole and Moss 1981). Adult males enter musth annually or twice-yearly, at any time of the year. Musth can last a few days to several months. In the wild, males in musth move over long distances searching for females in oestrus (Hall-Martin and Van Der Walt 1984; Poole 1987c). They are dominant towards other males, except larger males in musth (Poole and Moss 1981). Meetings with other males sometimes lead to violent fights and injuries (Hall-Martin 1987; Poole 1987a). Musth is generally inhibited by the presence of older, larger males (Poole 1987c; Poole 1989a; Slotow *et al.* 2000).

Young free-ranging elephant bulls that become problematic are usually situated in smaller reserves where, mostly, there is no natural hierarchical social structure with no or too few adult bulls to control them (Mabula Game Reserve, Tshukudu Game Lodge, personal observation) (Slotow *et al.* 2000). They generally enter musth at an earlier age and for long periods at a time. Such young bulls are a hazard to humans and have been known to attack and kill species like rhinoceros (Tshukudu Game Lodge, personal observation) (Slotow *et al.* 2000). This can lead to concerns for the safety of tourists and loss of other animals, especially in reserves too small to allow the introduction of other older males. The removal of the culprit sometimes constitutes the only resort. Captive or domesticated bulls in musth become less responsive and difficult to control (Eisenberg *et al.* 1971; Jainudeen *et al.*

1972b; Poole 1987b). Aggressive behaviour is directed towards people and other animals (Ganswindt *et al.* 2004a; Jainudeen *et al.* 1972b; Poole 1987b). Generally the animals have to be restrained (Kock and Kock 1984; Lincoln and Ratnasooriya 1996; Poole 1987b; Thakuria and Barthakur 1996) to such an extent that it becomes an animal welfare issue. Often food and water supply are reduced (Lincoln and Ratnasooriya 1996; Thakuria and Barthakur 1996), and tranquillizers need to be used to allow basic management (Imire Game Park, personal communication) (Thakuria and Barthakur 1996). In some instances, bulls have to be removed from working programs or even euthanased.

Consequently, there is an urgent need to develop methods to control musth and aggressive behaviour that could improve the safety of people and the well-being of the bulls. As yet, no practical method exists. Musth is related to high testosterone levels (Cooper *et al.* 1990; Hall-Martin and Van Der Walt 1984; Jainudeen *et al.* 1972a; Lincoln and Ratnasooriya 1996; Niemuller and Liptrap 1991; Rasmussen *et al.* 1984; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996; Rasmussen and Perrin 1999). Controlling testosterone secretion could thus be a way to control musth. The use of anti-androgens (Niemuller *et al.* 1998), GnRH agonists (Brown *et al.* 1993; de Oliveira *et al.* 2004) and GnRH antagonists (Brown *et al.* 1993) has been studied in elephant bulls but further work is still needed. Another possibility is the use of a GnRH vaccine, which has been studied and used successfully in many domestic species (see reviews by D'Occhio (1993) and Thompson (2000)) as well as in a few non-domestic species (Curtis *et al.* 2002; Miller *et al.* 2000; Turkstra *et al.* 2001) to control reproduction and androgen associated effects. Immunization generally results in suppression of testosterone secretion (Hage-van Noort *et al.* 1992; Schanbacher 1984), libido and aggressive behaviour (Enright 2003; Price *et al.* 2003; Stout *et al.* 2003) and reduction in testis size (Hage-van Noort *et al.* 1992; Schanbacher 1984) and spermatogenesis (Schanbacher 1984; Turkstra 2003). It is reversible (Ladd *et al.* 1994; Lincoln *et al.* 1982; Miller *et al.* 2000; Stout *et al.* 2003) and no toxic or adverse effects have been observed (Kumar *et al.* 2000; Ladd 1993). This vaccine has been commercialised for cattle (Hoskinson *et al.* 1990) and pigs (Dunshea *et al.* 2001; Oonk *et al.* 1998) as an alternative to surgical castration.

This is the first study in which a GnRH vaccine has been employed in African elephant bulls. The long-term objective is to develop a method to control musth and aggressive behaviour.

The aims of this study were:

- To vaccinate six bulls with the GnRH vaccine.
- To determine the effects of vaccination on testosterone secretion by monitoring epiandrosterone levels in the faecal samples of these bulls.
- To determine the effects of vaccination on the behaviour of bulls.
- To determine a possible correlation between testosterone and cortisol secretion by analysing faecal cortisol metabolites.
- To measure the antibody response to GnRH vaccination in bulls where blood sampling was feasible.

CHAPTER 2

LITERATURE REVIEW

2.1 Musth in elephants

2.1.1 Behavioural characteristics of musth

One of the main characteristics of musth is aggressive and unpredictable behaviour (Eisenberg *et al.* 1971; Poole 1987c; Poole and Moss 1981). In the wild, musth bulls display very aggressive behaviour towards other bulls (Poole 1987a; Poole 1987c). They leave their normal range and walk long distances looking for female herds with receptive females (Hall-Martin 1987; Hall-Martin and Van Der Walt 1984; Poole 1987b). They can be extremely agitated and irritable, showing aggressive behaviour towards non-elephant objects, destroying trees, tusking the ground, throwing branches, charging vehicles (personal observations) (Poole 1987b; Poole 1987c) and sometimes even charging other species like rhinos (personal observation) (Slotow *et al.* 2000). Domesticated bulls in musth also show aggression towards inanimate objects and human beings, throwing objects at them (Jainudeen *et al.* 1972b). Attacks on other elephants and keepers occur occasionally (Jainudeen *et al.* 1972b). Musth bulls also show reduced responsiveness to commands, become difficult to control and generally need to be completely restrained because they represent a real danger (Jainudeen *et al.* 1972b). In zoos, aggressive behaviour is similar, with attacks on older females and keepers (Johannesburg Zoo, personal communication) (Ganswindt *et al.* 2004a; Poole 1987b; Rasmussen *et al.* 1984). Observations on captive African elephants (Ganswindt *et al.* 2004a) showed that bulls with temporal gland secretion combined with urine dribbling were most likely to be aggressive. A musth male becomes dominant over other males, except towards larger males in musth (Poole and Moss 1981). Usually non-musth males try to avoid males in musth (Hall-Martin 1987; Poole 1987a). Encounters between males in musth can end in serious fights with injuries and even mortalities (Hall-Martin 1987; Poole 1987a). Elephants in musth lose condition because it is energetically expensive and associated with a reduced intake of food (Poole 1989a).

A series of behavioural displays, postures and vocalisations associated with musth has been described in the African elephant (Kahl and Armstrong 2002; Poole 1987c). These are as follows:

- Musth-walk

  Males in musth have a very characteristic way of walking, which can be identified by an experienced observer from a distance (Poole 1987c). They walk with the head held higher than the shoulders, the ears tensed and carried high, and with a controlled swinging motion of the head and tusks.

- Ear Wave

  At irregular intervals, the inner and upper portion of one ear is waved forcefully forward allowing the lower and outer portion to follow behind (Poole 1987c). This creates a wave diagonally across the ear. Some males seem to prefer one or the other ear for the ear-wave, while a few individuals sometimes show double ear-waves (Kahl and Armstrong 2002). This ear-wave seems to occur more often during aggressive interactions between males or during musth rumbling and the purpose may be to waft the scent from the temporal glands towards a rival (Poole 1987c).

- Trunk to head (Poole 1987c) / Trunk curl (Kahl and Armstrong 2002)

  The trunk to head behaviour described by Poole (1987c) is performed by lifting the head high and reaching up with the trunk to rub the temporal gland area. In trunk-curl behaviour described by Kahl and Armstrong (2002), males curl and uncurl their trunk in a sinuous way, often hanging it partially curled over one tusk. The trunk and adjacent areas of the face and temporal glands appear to be irritated and swollen. In some animals a deep fold is observed on the surface of the swollen trunk when bent. This is termed musth-wrinkle.

- Head oscillation (Poole 1987c)/ Head toss (Kahl and Armstrong 2002)

  The male swings his head and trunk repeatedly in a figure of eight motion. This movement is generally combined with trunk to head or trunk curl.

- Musth rumble

  This vocalization is a low pulsating sound of up to 108 decibels with frequencies as low as 14 Hz (Poole 1987c). Musth rumbles may be performed in association with ear-waves or ear-folds. Males in musth rumble in specific situations such as during agonistic encounters with other males, marking behaviour or prior to copulation. They rumble more frequently when alone or searching for females than when associated with females. Females respond with a specific loud low frequency vocalization.

- Marking

  The temporal glands are rubbed against objects more frequently during musth (Poole 1987c). When marking in mud, the bull lies down on his side while rubbing his face and entire body in the mud (Kahl and Armstrong 2002).

- Trunk bounce (Kahl and Armstrong 2002)

  A bull in musth may bounce or drag the distal portion of his trunk on the ground while walking.

- Tusking (Poole 1987c)

  Bulls get down on their knees and tusk the ground. Sometimes they tusk vegetation and throw bushes and other objects at vehicles or other elephants. This behaviour is always observed during fights between males in musth.

- Urine dribbling

  Males in full musth exhibit continuous urine dribbling (Poole and Moss 1981). The amount of urine discharged can vary from slow discrete drops to a wide stream and changes frequently, depending on the male's activity (Poole 1987c). Urine dribbling can sometimes cease for a while. The dribbling makes the insides of the back legs wet. It is associated with a strong odor, which can be detected in the urine trail left by the male. With heavy urine discharge for an extended period, the distal part of the sheath develops a greenish discoloration termed the "green penis syndrome" (Poole and Moss 1981). A male in musth with low urine dribbling urinates with the penis retained in the sheath and urine consequently sprays on the insides of the legs (Poole 1987c). Normal urination is generally not seen in males with heavy urine dribbling.

Asian elephants in musth have several of these behavioural patterns in common with African elephants (Poole 1987c): rubbing their temporal glands with their trunk (Eisenberg *et al.* 1971; Jainudeen *et al.* 1972b), head oscillations with trunk raised high, urine dribbling and frequent marking of the environment with the temporal glands (Eisenberg *et al.* 1971).

2.1.2 Temporal gland activity and musth

The temporal gland is a modified apocrine tubular-alveolar sweat gland located in the temporal area behind the eyes, and is unique to elephants (Rasmussen *et al.* 1984). Temporal glands of the African elephant appear to be active in all individuals of both sexes, including young calves (Perry 1953). Peak glandular activity seems to be associated with periods of

more frequent mating. Two types of secretion are observed in African elephants (Poole 1987c). One is observed in males and females of all ages. It is serous, evaporates quickly and often constitutes a response to stress or excitement. The other type is characteristic of males in musth. It is sticky and stains the skin for a longer period of time. Males in musth have enlarged temporal glands with continuous and abundant secretion (Eisenberg *et al.* 1971; Jainudeen *et al.* 1972b; Poole and Moss 1981). In contrast, the temporal gland secretion in the Asian elephant is confined to males over 15 years of age and is characteristic of musth. Jainudeen *et al.* (1972b) propose two arbitrary stages of musth in the Asian elephant. The first stage is characterized by gradual swelling of the temporal glands, hyperirritability and frequent erection of the penis. As the elephant progresses into the second stage of musth, the quantity of temporal gland secretion and intensity of aggressive behaviour increase. Initially a highly viscous yellowish fluid is released which flows to a distance of 5 to 10 cm. At the height of musth, the secretion is profuse and watery and reaches the angles of the mouth. As the secretion dries up, the face turns black. At this stage urine dribbling is also observed. A similar pattern is observed in African elephant bulls during musth, with temporal secretion starting earlier than urine dribbling (Ganswindt *et al.* 2004a). In Asian elephants, temporal gland secretion occurs very rarely in females (Eisenberg *et al.* 1971; Jainudeen *et al.* 1972b). Rasmussen *et al.* (1984), during three years of observations, only recorded slight temporal gland secretion in three of eight Asian cows at the Washington Park Zoo. It only occurred during periods of stress such as calving or cow group changes.

Temporal gland secretions during musth have a specific chemical composition (see 2.1.8) (Rasmussen 1988; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996) and probably play an important role in chemical communication (Rasmussen 1988). Temporal glands are used for marking by dispersing the secretions into the environment (Eisenberg *et al.* 1971; Jainudeen *et al.* 1972b). Males as well as females can distinguish between musth and non-musth states (Rasmussen and Krishnamurthy 2000; Rasmussen and Schulte 1998). Chemical signals seem to change in association with the maturity of the bull (Rasmussen *et al.* 2002). Young socially immature males in musth release honey-like odours that elicit little response from mature individuals, whereas older musth males release a malodorous, chemically distinct secretion that is generally avoided by younger males. Adult non-musth males also avoid males in musth (Hall-Martin 1987; Poole 1987a; Rasmussen and Schulte 1998). It is possible that temporal gland secretions transmit information relating to dominance (Rasmussen *et al.* 1996). Cows can be attracted by musth secretions during oestrus and avoid them at other

times (Rasmussen and Krishnamurthy 2000). The same has been observed with musth urine (Rasmussen 1988; Rasmussen and Schulte 1998; Schulte and Rasmussen 1999), and cows are more responsive during the follicular phase of the cycle (Schulte and Rasmussen 1999). Bulls also seem to show more interest in musth urine than non-musth urine of other bulls (Rasmussen 1988). In summary, temporal gland secretions, urine, and musth rumbles inform other elephants on the stages of musth and non-musth of a bull and hence play an important role in social structure and reproductive interactions (Rasmussen and Krishnamurthy 2000; Rasmussen and Schulte 1998).

2.1.3 Seasonal occurrence and individual periodicity

Musth can be observed throughout the year but in regions where rainfall is seasonal, the occurrence seems to be higher during wet seasons (Hall-Martin 1987; Jainudeen *et al.* 1972b; Kahl and Armstrong 2002; Poole 1987c). Peaks in the incidence of musth have been observed during the rainy seasons in Ceylon (Jainudeen *et al.* 1972b), Kenya (Poole 1987c), Kruger National Park (Hall-Martin 1987) and other reserves in southern Africa (Kahl and Armstrong 2002). The availability of oestrus cows also increases during the rainy season as does reproductive activity (Hall-Martin 1987; Laws 1969; Poole 1987c). In contrast, in Addo Elephant National Park, there is little seasonal change in the quality of the vegetation (Hall-Martin 1987). It is not surprising therefore that oestrus, reproductive activity and musth show no seasonal pattern. The effect of environmental conditions on musth activity was clearly demonstrated in Kenya. Poor or good rainfall years were associated with low or high numbers of receptive females and low or high numbers of males in musth (Poole 1987c). In contrast, in captive African elephants, no seasonality of musth is observed (Ganswindt *et al.* 2004a).

Musth in individual bulls has a cyclic pattern and occurs annually or twice-yearly (Eisenberg *et al.* 1971; Jainudeen *et al.* 1972b; Poole and Moss 1981). Musth periods are not synchronised between bulls (Eisenberg *et al.* 1971; Poole 1987b; Poole and Moss 1981), but generally one individual enters musth around the same time every year (Poole 1987b; Poole 1987c; Poole and Moss 1981). The individual cyclic pattern is less regular in young animals (Jainudeen *et al.* 1972b; Poole 1987c) or bulls in poor condition (Jainudeen *et al.* 1972b). Musth has also been shown to become more sporadic when animals decline in rank (Poole 1987c).

2.1.4 Influence of condition, cows, other bulls and age on the occurrence of musth

Many factors can influence musth. Poor physical condition, parasitism, overwork, wounds and poor nutrition have been observed to reduce the incidence of and sometimes suppress musth (Cooper *et al.* 1990; Jainudeen *et al.* 1972b). In a study by Cooper *et al.* (1990), where the grain and hay ration of an Asian bull was restricted, there was a two to four week delay in the onset of temporal gland flow as well as a qualitatively less intense behavioural musth than usual. According to Jainudeen *et al.* (1972ab), sexual activity may induce the onset of musth, with musth occurring earlier than normally in animals that experience musth annually. On the other hand, other studies show no evidence of a correlation between the exposure to oestrus cows and the onset of musth (Cooper *et al.* 1990; Rasmussen *et al.* 1984). One should remember, however, that observations are often made under unnatural conditions where, for instance, there is no competition from other bulls (Cooper *et al.* 1990). Moreover, Poole's (1987c) observations on free-ranging elephants in Kenya indicate an influence of the presence of oestrus cows on the onset of musth for certain age groups. The oldest males (over 50 years old) came into musth before associating with female groups, while younger males (35-50 years old) showed signs of musth only after having been associated with females for several weeks. During this period they actively tested females and competed with other bulls for access to oestrous females. Twenty-five - 35 year old males came into musth only after having been with female groups for up to a month and would come into musth for just a few days, several times during one sexually active period.

The presence of older dominant bulls can suppress musth patterns in younger animals (Poole 1987c; Poole 1989a; Slotow *et al.* 2000). Smaller, younger males seem to be out of musth more often when there is a dominant musth male present (Poole 1989a). An aggressive interaction with a higher–ranking male in musth can also force a bull out of musth (Poole 1987c; Poole 1989a) and sometimes suppresses musth for several years (Poole 1987a). The onset of musth in young animals can also be delayed by larger, older musth bulls (Milius 2000; Poole 1987c; Slotow *et al.* 2000). In wild populations, African elephants enter their first musth at around 25-30 years of age (Poole 1987c; Poole and Moss 1981). Younger bulls are generally not large enough to compete with older, stronger males in musth and even males that are around 30 years old and enter musth generally go out of musth when threatened by older, larger musth males (Poole 1987b). In Pilanesberg, before the introduction of older bulls, the male population consisted of young bulls aged 15 to 25 years. Musth was regularly observed in these bulls (Slotow *et al.* 2000). The same seems to happen

with elephants in captivity where the intensity of dominant relationships is reduced (Poole 1987b; Poole 1987c). Some domesticated Asian bulls (Cooper *et al.* 1990; Eisenberg *et al.* 1971; Jainudeen *et al.* 1972b) and captive African bulls (Cooper *et al.* 1990; Ganswindt *et al.* 2004a) show their first musth episode as early as 10-20 years of age. Other factors that may contribute to the early appearance of musth in captive animals are good nutrition and a reduction in environmental stresses (Cooper *et al.* 1990; Poole 1987b; Poole 1987c).

2.1.5 Duration of musth

The duration of musth is extremely variable and ranges from a few days to several months (Jainudeen *et al.* 1972b; Poole 1987c). It seems to be age-related; in young bulls, musth generally only lasts a few days to a few weeks, while in older bulls musth is more predictable and lasts 2 to 5 months (Jainudeen *et al.* 1972b; Poole 1987a; Poole 1987b; Poole 1987c). Individual animals show little variation in the duration of musth from one year to the next (Jainudeen *et al.* 1972b; Poole 1987c). Musth will usually be shorter in the presence of larger musth bulls (Slotow *et al.* 2000). In captivity, the duration of musth can be longer (Rasmussen *et al.* 1984) and, in contrast to free-ranging bulls, it does not seem to be age-related (Ganswindt *et al.* 2004a) .

2.1.6 Musth and breeding behaviour

Males in musth are more likely to be found associated with female herds than non-musth males (Eisenberg *et al.* 1971; Hall-Martin 1987; Hall-Martin and Van Der Walt 1984; Poole 1987c; Poole and Moss 1981). There is still a lot of uncertainty and controversy concerning the role of musth and its relationship to breeding activity. The likelihood that bulls in musth mate is greater than for non-musth bulls (Hall-Martin 1987; Poole 1987a; Poole 1989b; Poole and Moss 1981). Musth-males walk long distances compared to the more sedentary non-musth males (Hall-Martin 1987). Hence musth increases the opportunities for contact with oestrous cows. Musth also allows a male to compete with other males and to reach a higher ranking status (Eisenberg *et al.* 1971; Hall-Martin 1987; Poole 1987c; Poole and Moss 1981).

A positive relationship exists between age and mating success (Poole 1989b). Normally the larger, older males have the best access to females in oestrus, but a younger bull in musth will be able to compete with a non-musth bull and enhance his chances of mating (Hall-Martin 1987). Furthermore, cows in oestrus show more interest in large bulls in musth than in other males (Moss 1983; Poole 1987a; Poole 1999; Rasmussen and Schulte 1998) and

solicit guarding behaviour more often from musth bulls (Poole 1989b). They seem to select their mates and facilitate mating with larger musth males by standing still (Moss 1983). Musth bulls consort cows more frequently than non-musth males (Kahl and Armstrong 2002; Moss 1983) and, to prevent access of rival males to the female, the guarding male must be more aggressive (Poole 1989b).

Musth could be considered as a reproductive strategy to increase access to oestrous females. This would enhance an individual's reproductive success and his chances of producing more offspring (Hall-Martin 1987; Moss 1983; Poole 1987c; Poole and Moss 1981). Bulls, however, do not need to be in musth to be able to mate (Hall-Martin 1987). In a study by Cooper *et al.* (1990), musth had no effect on breeding behaviour in a captive Asian bull. He displayed sexual behaviour towards oestrous cows when he was not in musth as well as when he was in musth. Under natural conditions, however, musth could be critical to the success rate of a bull (Cooper *et al.* 1990; Poole 1987c). Based on observations of free-ranging African elephants, Ganswindt *et al.* (2004b) showed that there are at least two distinct states of sexual activity; one with musth-typical signs (temporal gland secretion and urine dribbling) and the other without. It has been suggested that a musth bull clearly signals his intentions of contesting access to an oestrous cow (Poole 1989a; Poole 1999), and reduces competition by other males by discouraging them (Kahl and Armstrong 2002). The choice of larger, and thus older bulls could have the long-term advantage of transmitting the trait for longevity to the offspring (Moss 1983). Moreover, musth is a reliable indicator of good condition of a male (Poole 1989b). It has also been suggested that by choosing musth bulls, cows are more likely to have a fertile mating, because spermatogenesis is increased during musth (Moss 1983). With limited data, Howard *et al.* (1984) noticed no significant difference in sperm motility, sperm concentration and overall semen quality between musth and non-musth bulls. However, a recent study (Hildebrandt 2003) showed a correlation between semen quality, genital development and social status of free-ranging African bulls. Bulls that were identified as breeding-bulls based on semen quality and development of the genital organs were dominant solitary animals, whereas non-breeding bulls, characterized by low ejaculate volumes, underdeveloped accessory sex glands and low testosterone concentrations were generally found in bachelor groups. It thus seems that social-rank can induce sexual inactivity. A similar pattern was seen in captive animals. In captive bulls, the correlation between social rank and reproductive status was reversed when one of the bulls came into musth. Vesicular glands were large and testosterone concentrations high. Semen collection

was however difficult in musth bulls and precise information on semen quality could therefore not be obtained. In a further study on free-ranging African elephants (Hildebrandt *et al.* 2004), it appeared that the semen quality of musth bulls was poor compared to non-musth bulls. The author's interpretation was that musth bulls do not contribute to breeding. A study on non-musth semi-wild Asian elephants also showed that higher androgen levels were correlated to lower sperm quality (Schwarzenberger *et al.* 2001). These findings raise questions about the role of musth in reproduction. Musth is a complex phenomenon for which good descriptive data is limited.

2.1.7 Dangers associated with bulls in musth

Because musth is associated with increased aggression and unpredictable behaviour, it can be a cause of serious concern under certain circumstances. This applies especially to captive and domesticated animals (Imire Game Park, personal communication) (Poole 1987b; Thakuria and Barthakur 1996). As discussed previously, captive animals enter musth earlier and for longer periods than in the wild. In musth they present a threat to the safety of both people and animals and therefore create a real management problem. Generally the management of such animals consists of keeping them leg-chained, isolated (Lincoln and Ratnasooriya 1996; Poole 1987b; Thakuria and Barthakur 1996) and treated with sedatives (Imire Game Park, personal communication) (Thakuria and Barthakur 1996). Sometimes food and water supply is restricted in order to shorten the duration of musth (Lincoln and Ratnasooriya 1996; Thakuria and Barthakur 1996). Musth is thus an important animal welfare issue and a well-known problem in captive Asian bulls (Rasmussen *et al.* 1984). Increasing numbers of African elephants are being housed in captivity and domesticated. As they get older, these bulls are also likely to show an increased incidence of musth (Rasmussen *et al.* 1984).

Free-ranging bulls in musth are also sometimes considered to be dangerous. The problem is usually confined to smaller reserves (Mabula Game Reserve, Tshukudu Game Lodge, personal communication) (Slotow *et al.* 2000). The problem bulls were either introduced with family units, or as orphans from previous culls in the Kruger National Park. In either case, the bulls grew up in the absence of older dominant bulls. Orphaned bulls that were moved to Pilanesberg, South Africa, entered musth at an early age and killed many white rhinoceros, until older male elephants were introduced (Slotow *et al.* 2000). Generally the removal of the offending elephant constitutes the only solution since there are as yet no practical ways to control musth. For safety and welfare reasons, there is an urgent need to

develop methods that prevent or control musth and aggressive behaviour in elephant bulls of both species.

2.1.8 Endocrinology of musth

The link between testosterone secretion and musth has been the topic of many studies. Most studies have concluded that musth is associated with and possibly caused by increased androgen levels (Cooper *et al.* 1990; Ganswindt *et al.* 2004a; Hall-Martin and Van Der Walt 1984; Jainudeen *et al.* 1972a; Lincoln and Ratnasooriya 1996; Niemuller and Liptrap 1991; Rasmussen *et al.* 1984; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996; Rasmussen and Perrin 1999). Studies in African (Hall-Martin and Van Der Walt 1984; Rasmussen *et al.* 1996) and Asian (Cooper *et al.* 1990; Jainudeen *et al.* 1972a; Lincoln and Ratnasooriya 1996; Niemuller and Liptrap 1991; Rasmussen *et al.* 1984; Rasmussen and Perrin 1999) elephant bulls showed that blood testosterone concentrations associated with heavy musth are generally greater than 20 ng/ml and sometimes higher than 100 ng/ml (Brown *et al.* 1993; Niemuller and Liptrap 1991; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996). Incipient musth, post-musth or moderate musth seem to be associated with concentrations of 10-20 ng/ml (Cooper *et al.* 1990; Hall-Martin and Van Der Walt 1984; Jainudeen *et al.* 1972a; Niemuller and Liptrap 1991; Rasmussen *et al.* 1984; Rasmussen *et al.* 1996; Rasmussen and Perrin 1999). Dihydrotestosterone concentrations also increase during musth, but to a lesser degree than testosterone (Rasmussen *et al.* 1984; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996). Blood androstenedione increases as well. The androstenedione/testosterone ratio is in favour of testosterone during musth, whereas during non-musth the principal androgen present in the plasma is androstenedione (Niemuller and Liptrap 1991). Shifts in the ratio also occur during sporadic increases in blood testosterone concentrations.

Males in non-musth usually have testosterone concentrations comparable to other species with values varying from 0.2 ng/ml to 10 ng/ml (Cooper *et al.* 1990; Hall-Martin and Van Der Walt 1984; Jainudeen *et al.* 1972a; McNeilly *et al.* 1983; Rasmussen *et al.* 1984; Rasmussen *et al.* 1996; Rasmussen and Perrin 1999). They often show inconsistent fluctuating profiles, generally with low testosterone concentrations and occasional peaks up to 10 ng/ml (Cooper *et al.* 1990; Lincoln and Ratnasooriya 1996). This can partly be explained by the pulsatile release of testosterone combined with a random sampling procedure (Lincoln and Ratnasooriya 1996). Before puberty, testosterone concentrations seem to be lower than 0.5

ng/ml (McNeilly *et al.* 1983). Around 12 years of age an increase is observed, corresponding with the beginning of puberty.

During musth, elevated androgen levels have also been reported in urine (Brannian *et al.* 1989; Ganswindt *et al.* 2002; Poole *et al.* 1984), faeces (Ganswindt *et al.* 2002; Ganswindt *et al.* 2004a; Ganswindt *et al.* 2004b) and temporal gland secretions (Rasmussen *et al.* 1984; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996). In a study on the use of faecal androgen concentrations to assess testicular endocrine function in the male elephant (Ganswindt *et al.* 2002), epiandrosterone was the dominant androgen detected in the faeces by the enzyme-immunoassays used. Testosterone in temporal gland secretions is present in greater concentrations than in blood, which indicates that there is a trapping mechanism for androgens in the temporal glands (Rasmussen *et al.* 1984; Rasmussen *et al.* 1990; Rasmussen *et al.* 1996). Levels of dihydrotestosterone in temporal gland secretions are also higher than in blood during musth.

Androgen concentrations during musth and non-musth can be extremely variable from one individual to another. Testosterone levels are also correlated with dominance rank (Hall-Martin and Van Der Walt 1984; Lincoln and Ratnasooriya 1996; Rasmussen *et al.* 1984) and aggression (Dickerman *et al.* 1997; Rasmussen *et al.* 1984). Higher-ranking animals, which are generally more aggressive, have higher blood testosterone concentrations (Dickerman *et al.* 1997; Hall-Martin and Van Der Walt 1984; Lincoln and Ratnasooriya 1996; Rasmussen *et al.* 1984) as well as higher testosterone and dihydrotestosterone concentrations in temporal gland secretions (Rasmussen *et al.* 1984). Variations in androgen levels between musth bulls are considerably higher than in other bulls, but the factors causing this are unknown (Ganswindt *et al.* 2004b). Elevated androgen levels seem not to be linked to the age of the individual during musth (Ganswindt *et al.* 2004b).

Faecal cortisol metabolites using an 11-oxo-etiocholanolone EIA measuring 3α,11-oxo-CM were monitored in captive (Ganswindt *et al.* 2003) and free-ranging (Ganswindt *et al.* 2004b) African elephants in order to determine if musth is associated with increased stress. The metabolites found during musth periods were no higher than those found during non-musth periods (Ganswindt *et al.* 2003). Periodic increases were observed but generally did not correspond with the occurrence of musth. Later on, a longitudinal study in captive elephants (Ganswindt *et al.* 2004a) showed that glucocorticoid levels are in fact reduced during periods

with elevated androgen levels, musth signs and increased aggressive behaviour. Consequently there is no indication that musth in the African elephant represents a stress. Moreover, there seems to be a direct downstream effect of elevated androgens or musth on adrenal corticoid secretion since a decrease in corticoids occurres after the elevation in androgens and terminates with the end of the androgen elevation.

2.1.9 Role of androgens in musth

The precise role of testosterone in the onset of musth is not fully understood. In contrast to Poole *et al.* (1984), Ganswindt *et al.* (2004b) showed that elevated androgen levels do not occur during sexually active periods without musth signs (temporal gland secretion and urine dribbling). Increases in androgen levels were shown to be higher during sexually active periods with both temporal gland secretion and urine dribbling than with temporal gland secretion alone (Ganswindt *et al.* 2004a; Ganswindt *et al.* 2004b). The occurrence of temporal gland secretion could be seen as a transitional condition associated with moderate elevations in androgens and possible aggressive behaviour (Ganswindt *et al.* 2004a). Normally testosterone secretion starts increasing one to several weeks before the onset of the signs of musth (Cooper *et al.* 1990; Poole *et al.* 1984; Rasmussen and Perrin 1999). In a longitudinal study performed on captive African bulls using faecal samples Ganswindt *et al.* (2004a) confirmed this. They showed that temporal gland secretion and urine dribbling are downstream effects of elevated androgens. Sporadic increases in blood testosterone have been observed in Asian elephants during non-musth periods and generally they correspond with special events like mating, fighting and the presence of oestrous cows (Niemuller and Liptrap 1991). The African bull studied by Cooper *et al.* (1990) had occasional rises in testosterone concentration as high as those observed during musth, but without any manifestations of musth. Periods of elevated androgen levels in the absence of musth signs have also been detetected using faecal samples (Ganswindt *et al.* 2004a). They were of shorter duration, and the androgen levels were lower, than during musth. All these findings support the idea that elevated androgen levels act as a stimulus for the onset of musth. A minimum duration of elevated androgens above a certain threshold seems to be required (Ganswindt *et al.* 2004a). Lincoln and Ratnasooriya (1996) showed a positive correlation between mean testosterone concentrations during the two months preceding musth and the duration of that musth. The most intense part of musth, however, occurred after the testosterone peak. It is likely that high blood testosterone concentrations constitute one of the factors initiating musth. The exact mechanism by which testosterone exerts its effect however is still unclear. As discussed

earlier, other factors also influence musth and high androgen levels possibly act in association with these factors.

Testosterone has a low frequency pulsatile secretory pattern (Lincoln and Ratnasooriya 1996). Niemuller and Liptrap (1991) showed that testosterone pulses follow LH pulses, which demonstrates that LH controls testosterone secretion. There seem to be no significant differences in testosterone pulse rates between musth and non-musth, but pulse amplitude, pulse area and mean concentration are higher during musth. These parameters are more important for inducing strong musth than mild musth. The same pulstile patterns are seen for LH, but, unlike testosterone, the increases in pulse amplitude and area appear to be similar for different intensities of musth. Maybe the duration of exposure of the testes to increased LH secretion is the factor determining the degree of testosterone response. Brannian *et al.* (1989) showed increased LH levels in urine of an African elephant during musth, suggesting that elevated androgen levels during musth result from LH stimulation. In contrast, Brown *et al.* (1993) found that LH concentrations in a musth bull with high androgen levels were similar to those observed in non-musth bulls. Testes of bulls in musth appear to be hyper-responsive to LH. LH secretion can be stimulated by exogenous GnRH and is thus most likely regulated endogenously by pulsatile release of GnRH (Lincoln and Ratnasooriya 1996).

The question of whether or not testosterone and its metabolites are the cause of the aggressive behaviour remains. Niemuller and Liptrap (1991) showed a link between the intensity of behavioural changes during musth and testosterone secretion. Rasmussen and Perrin (1999) found that changes in behaviour during musth in an Asian bull only took place when major changes in serum testosterone levels occurred. Some studies showed evidence that testosterone plays a role in hierarchical status and thus aggressive behaviour (Lincoln and Ratnasooriya 1996; Rasmussen *et al.* 1984). In a study on the relationship between aggressive behaviour and altered steroid hormone levels, Dickerman *et al.* (1997) came to the conclusion that androgens do enhance aggression and that the link between aggressive behaviour and dihydrotestosterone is even stronger than with testosterone. Finally, Ganswindt *et al.* (2004a) suggested that increased aggression during musth is most likely androgen-mediated because aggressive males have higher androgen levels.

2.1.10 Lipid metabolism and musth

Important metabolic changes related to lipid catabolism seem to occur during musth (Rasmussen and Perrin 1999). Serum triglyceride concentrations are increased and positively correlated with testosterone concentrations. Serum lipase activity increases and blood pH is more alkaline during musth. This suggests that there is a mobilization of fat reserves during musth and that there is a relationship between high androgen levels and changes in fat metabolism. Ketone concentrations increase simultaneously in blood, temporal gland secretions and urine. During musth the animal reduces his food intake, which probably results in rapid fat catabolism and, consequently, high ketone levels. This could have an effect on brain chemistry with subsequent behavioural changes. Metabolic changes alter the chemical composition of urine, temporal gland secretion and breath. Qualitative and quantitative changes of the volatile compounds of temporal gland secretions occur during musth (Rasmussen *et al.* 1990; Rasmussen *et al.* 1996; Rasmussen and Perrin 1999). The compounds involved are phenols, alcohols, aliphatic acids, carboxylic acids and ketones. Different patterns are related to different stages of musth (Rasmussen *et al.* 1990). Moreover, males experiencing their first years of musth have a different chemical composition to older males (Rasmussen 2000; Rasmussen *et al.* 2002). By collecting volatiles from the dry orifice before the start of secretion, Rasmussen and Perrin (1999) showed that chemical changes take place before overt temporal gland secretion (Rasmussen and Perrin 1999). Very high levels of ketones are present in urine during musth and at different stages of musth (Rasmussen and Perrin 1999; Rasmussen and Wittemyer 2002). It is possible that these volatile compounds play a role in chemical communication.

2.2 Control of musth

2.2.1 Methods used to control musth

Captive males in musth are usually managed through their musth cycle by isolating them and restraining them completely with leg chains (Kock and Kock 1984; Lincoln and Ratnasooriya 1996; Poole 1987b; Thakuria and Barthakur 1996). Musth can last for a long time and permanent leg chains can cause severe wounds on the legs (Kock and Kock 1984). Bulls sometimes have to be immobilized several times during musth in order to change the leg chains, treat associated wounds and clean the enclosure (Kock *et al.* 1984; Kock and Kock 1984). Some keepers use traditional remedies and methods which are sometimes cruel and

inhumane (Kock and Kock 1984; Lincoln and Ratnasooriya 1996). Food and water supply are commonly reduced in order to minimise the intensity and duration of musth (Lincoln and Ratnasooriya 1996; Schmidt 1993; Thakuria and Barthakur 1996). In some cases, chemical sedatives and tranquillizers like diazepam and azaperone are used during the entire musth period to allow basic management (Imire Game Park, personal communication) (Thakuria and Barthakur 1996).

As discussed previously, musth appears at least in part to be related to high concentrations of androgens. In general, gonadal steroids like testosterone, dihydrotestosterone and oestradiol play an important role in the physiology of male sexual behaviour in mammals, even if there are still many uncertainties concerning the importance and effect of each steroid at the level of the central nervous system (Meisel and Sachs 1994). Consequently, the manipulation of the secretion or action of gonadal steroids could be a way to control musth. Surgical castration has been tried on some captive bulls (Foerner *et al.* 1994; Fowler and Hart 1973; Olsen and Byron 1993). After castration, animals were more tractable and showed no signs of musth for up to four years (Foerner *et al.* 1994; Olsen and Byron 1993; Rasmussen *et al.* 1984). This is not a practical option, however, because the testes are intra-abdominal, making the surgery difficult and risky for the animal (Fowler and Hart 1973). It is also irreversible. Moreover, complications such as peritonitis and respiratory distress during prolonged recumbency are frequently encountered (Foerner *et al.* 1994; Fowler and Hart 1973). Musth prevention may be possible by inhibiting testicular activity with an anti-androgen such as cyproterone acetate (Jainudeen *et al.* 1972a). Administration of cyproterone to Asian bulls during musth reduced testosterone to baseline levels but musth behaviour was still present (Niemuller *et al.* 1998).

Inhibiting gonadal secretion of steroids can also be achieved by intervening at the hypothalamic-pituitary axis. GnRH is synthesised and secreted by hypothalamic neurons (Beattie 1982). It is released into the capillary plexus of the hypothalamo-pituitary portal system in the median eminence and transported to the anterior pituitary gland. GnRH stimulates the release of LH and FSH from the pituitary gland and thus regulates gonadal steroidogenesis, and spermatogenesis and oogenesis in males and females respectively. Continuous exposure to high concentrations of GnRH results in the down-regulation of gonadotrophin output as a result of pituitary desensitization (Fink 1988). The use of GnRH agonists to induce down-regulation of LH and subsequently testosterone secretion has been tried in mature male elephants (Brown *et al.* 1993; de Oliveira *et al.* 2004). Brown *et al.*

(1993) showed that the GnRH agonist Lupron Depot disrupts normal pituitary and gonadal function. It induced an initial stimulation of LH and testosterone secretion, followed by a decline to baseline values. However, while the pituitary became partially desensitised, the testes became hyper-responsive to gonadotrophic stimulation. One of the males treated with Lupron Depot was in musth and went out of musth 22 days later. de Oliveira *et al.* (2004) showed that the administration of leuprolide acetate to a 52 year-old Asian bull for several years decreased testosterone levels. Musth behaviour was prevented when the drug was administered during the first premusth manifestations. But the fact that this study included only one bull and that it was a 6-year study on an already older bull makes the interpretation of the results difficult. The effectiveness of GnRH antagonists has also been tested on male elephants; they resulted in partial inhibition of LH and testosterone secretion two days after treatment (Brown *et al.* 1993). A musth male that was treated seemed to be out of musth two days after treatment. Appropriate dosages and treatment protocols as well as long-term effects of these drugs still need to be determined. Lincoln *et al.* (2003) suggest research on the use of progestins, like depot medroxy-progesterone acetate, to inhibit gonadotrophin secretion and thereby prevent musth. The use of GnRH as an antigen in a vaccine to inhibit the hypothalamic-pituitary-gonadal axis could be a useful way to control musth, because immunization against GnRH has proven to be efficient and reversible in many species (see reviews D'Occhio (1993) and Thompson (2000)).

2.2.2 GnRH immunization

Immunological techniques have been widely used during the past decade to study reproductive physiology and to find appropriate targets for contraceptive vaccines (D'Occhio 1993). Active immunization against GnRH was developed in 1970 and has been used for two major fields of application (Schanbacher 1984; Thompson 2000). One of the fields is immunocontraception, in particular the immunocastration of males of domestic species to suppress fertility (Ladd *et al.* 1994; Schanbacher 1984; Thompson 2000), reduce sexual and aggressive behaviour (Bonneau and Enright 1995; D'Occhio 1993; Enright 2003; Meloen 1995; Stout *et al.* 2003; Stout and Colenbrander 2004; Thompson 2000), reduce androgen associated odours (Bonneau and Enright 1995; Hage-van Noort *et al.* 1992; Thompson 2000) and improve carcass characteristics and feed efficiency (Enright 2003; Hage-van Noort *et al.* 1992; Huxsoll *et al.* 1998). The vaccine has also been used in females as a contraceptive or means of behavioural control (Dalin *et al.* 2002; Miller *et al.* 2000; Zeng *et al.* 2002a). GnRH immunization is reversible and avoids the problems and complications associated with

surgical castration (D'Occhio 1993; Hage-van Noort *et al.* 1992; Schanbacher 1984). It has also been used in human medicine to treat androgen-dependent pathologies (Ladd 1993; Schanbacher 1984; Thompson 2000) and research is ongoing to develop a male contraceptive (Ladd 1993). In a wildlife context, GnRH vaccines could be used for reproductive and behavioural management of zoo animals (Meloen 1995; Turkstra *et al.* 2001) and for population control of wildlife populations (Curtis *et al.* 2002; Meloen 1995; Miller *et al.* 2000). The other major field of application of GnRH immunization is the investigation of the hypothalamic-pituitary axis and the role of GnRH in reproductive endocrinology (Schanbacher 1984; Thompson 2000).

2.2.2.1 Mode of action

Immunization with a GnRH-conjugate stimulates the formation of antibodies against GnRH molecules. The antibodies probably act mainly at the level of the hypothalamo-pituitary portal system, where GnRH travels a short distance from the hypothalamic neurons to the pituitary gonadotrophs (Schanbacher 1984; Thompson 2000). Their action is believed to be direct and immediate (Schanbacher 1984). Sufficient specific antibodies must be present in the circulating blood entering the pituitary portal system to bind all GnRH released (Thompson 2000). The antibodies neutralize GnRH by preventing it from diffusing through the capillary walls, or by masking the receptor binding-site on the GnRH molecule. GnRH production probably increases after immunization, because of feedback loops, and antibody titres must therefore stay high if all the GnRH produced is to be neutralized (Hage-van Noort *et al.* 1992). Inactivation of GnRH blocks the pituitary secretion of gonadotrophins and thereby suppresses testicular activity (D'Occhio 1993; Hage-van Noort *et al.* 1992; Turkstra 2003).

After immunization, atrophy of the pituitary gonadotrophs and testes occurs, with a marked reduction in plasma gonadotrophin and sex steroid concentrations (Hage-van Noort *et al.* 1992; Schanbacher 1984; Turkstra 2003). Efficient vaccination generally results in delayed puberty in young animals (D'Occhio 1993; Schanbacher 1984). In mature males, testosterone levels are reduced to castrate levels and the testes undergo atrophy. Atrophy of the accessory sex glands is also observed (Falvo *et al.* 1986; Schanbacher 1984). Immunocastration causes reduced and incomplete spermatogenesis (Schanbacher 1984; Turkstra 2003), which can be monitored by measuring testicular size (Schanbacher 1984). Suppression of reproductive function is expected, including fertility, sexual behaviour and secondary sex characteristics. It has been suggested and, in certain instances, observed that the intensity of the biological

response is related to the antibody titres (Lincoln *et al.* 1982; Malmgren *et al.* 2001; Schanbacher 1984). Much controversy and uncertainty surrounds this. In some cases, correlation between GnRH binding activity of the antibodies and the degree of response in parameters such as testicular regression was absent or poor (Meloen *et al.* 1994; Meloen 1995; Turkstra *et al.* 2002). Other studies suggested that the length of time that the antibodies persist in the circulation (Ferro *et al.* 2003) or the antibody avidity for free GnRH (Ferro *et al.* 2002b; Ferro *et al.* 2002a) are more important than the actual titre. Long-term suppression of reproductive function, on the other hand, could possibly be due to disruption of tissues in the hypothalamic median eminence (D'Occhio *et al.* 2001; Molenaar *et al.* 1993).

2.2.2.2 Applications

GnRH vaccines have been used successfully in numerous species. The effects described above were generally observed. Immunization of rats results in markedly reduced plasma concentrations of LH, FSH and testosterone, atrophy of the testes, epididymes and vesicular glands and suppression of spermatogenesis (Schanbacher 1984). Studies in domestic cattle produced similar findings: reduction in testis size, suppression of plasma testosterone concentrations to those typical of steers (Cook *et al.* 2000; D'Occhio *et al.* 2001; Finnerty *et al.* 1994; Huxsoll *et al.* 1998) and reduction of sexual and aggressive behaviour (Enright 2003; Finnerty *et al.* 1997; Huxsoll *et al.* 1998; Price *et al.* 2003). No undesirable effects were observed on growth and carcass characteristics (Cook *et al.* 2000; D'Occhio *et al.* 2001; Enright 2003; Finnerty *et al.* 1994; Huxsoll *et al.* 1998). Vaccination of pre-pubertal bulls seems to be more efficient than vaccination after puberty and could constitute a practical way of reducing aggressive behaviour around and after puberty while maintaining a growth rate similar to that of intact bulls (Enright 2003; Huxsoll *et al.* 1998).

Immunocastration of young pigs was demonstrated to be a viable and practical alternative to surgical castration for preventing boar taint (Bonneau *et al.* 1994; Dunshea *et al.* 2001; Falvo *et al.* 1986; Meloen *et al.* 1994; Oonk *et al.* 1995; Zeng *et al.* 2001). Testis size and androstenone levels in fat are markedly reduced after vaccination (Bonneau *et al.* 1994; Meloen *et al.* 1994; Oonk *et al.* 1998; Zeng *et al.* 2001; Zeng *et al.* 2002b). Moreover, there was a beneficial effect on growth performance compared to surgically castrated pigs (Bonneau *et al.* 1994; Dunshea *et al.* 2001; Zeng *et al.* 2001). This is probably due to the partial presence of testosterone for a longer time in immunocastrated pigs, a conclusion that is supported by the fact that feed efficiency and growth performance are superior when

immunocastration is applied later in young pigs (Turkstra *et al.* 2002). One study showed that pituitary LH, plasma LH and plasma testosterone are significantly reduced, while plasma and pituitary FSH are not affected in boars immunized at 12 weeks of age (Awoniyi *et al.* 1988). GnRH immunization also seems to impair Leydig cell function.

Immunization of stallions induces a marked decrease in testosterone, distinct changes in semen quality, a decrease in testicular size (Malmgren *et al.* 2001; Stout *et al.* 2003; Stout and Colenbrander 2004; Turkstra 2003) and histological changes in the testes (Malmgren *et al.* 2001). Oestrone sulphate concentrations are also reduced (Malmgren *et al.* 2001). Immunized animals are generally easier to handle and libido is depressed (Malmgren *et al.* 2001; Stout *et al.* 2003), but the age at which the stallion is vaccinated influences the response to the vaccine significantly (Stout *et al.* 2003; Stout and Colenbrander 2004). Vaccination of 3-year old stallions seems to be very efficient, whereas vaccination of older breeding stallions requires more booster vaccinations, there is a higher individual variability, the effects are of shorter duration and the animals often continue to display sexual behaviour.

Efficient inhibition of male reproductive function following active immunization against GnRH has also been induced in dogs (Ladd *et al.* 1994), cats (Levy *et al.* 2004), rams (Hotzel *et al.* 1997; Kiyma *et al.* 2000) and deer (Freudenberger *et al.* 1993; Lincoln *et al.* 1982; Miller *et al.* 2000), whose rutting behaviour is blocked by vaccination. Immunization of white-tailed deer seems to be a feasible way to control numbers in wild populations of this species (Curtis *et al.* 2002; Miller *et al.* 2000). Immunization against GnRH has been tested in goats and several zoo-animals, including blackbuck and springbok (Turkstra *et al.* 2001). GnRH antibodies were induced in all vaccinated animals but testosterone concentrations only dropped to undetectable levels in young animals, and were not reduced in mature adults.

There appear to be species differences in response to GnRH vaccination (Ferro *et al.* 2004; Ladd *et al.* 1994; Meloen 1995). Therefore, trials must be performed on each species to determine the effect of a particular vaccine formulation and administration protocol (Hage-van Noort *et al.* 1992; Meloen 1995). For instance, after immunization of rodents, sheep and dogs with the same GnRH vaccine, Ferro *et al.* (2004) observed different responses in each species, with the dog being the least responsive.

In many studies important individual variations in response are observed (Dalin *et al.* 2002; Lincoln *et al.* 1982; Malmgren *et al.* 2001; Meloen 1995; Miller *et al.* 2000; Schanbacher 1984; Stout *et al.* 2003; Turkstra *et al.* 2002). The reason for this is unclear. Age and state of sexual maturity seem to be important factors, as in certain studies the vaccine was most effective in young or pre-pubertal animals (Curtis *et al.* 2002; Enright 2003; Malmgren *et al.* 2001; Stout *et al.* 2003; Turkstra *et al.* 2001). Response may also be related to factors such as immune competence and genetic differences between individuals.

Examples of commercialised GnRH vaccines are Improvac® (Dunshea *et al.* 2001) and Pepscan® TDK (described by Oonk *et al.*,1998), both used in the pig industry, and Vaxtrate® (Hoskinson *et al.* 1990), designed for use in cattle.

2.3 Formulation of GnRH vaccines

GnRH is a decapeptide with low species-specificity (Schanbacher 1984). This has made its use across species successful. Because it is a hapten, it needs to be conjugated to a large molecule such as a hydrocarbon or protein to induce an immune response (Hage-van Noort *et al.* 1992; Meloen 1995; Thompson 2000; Turkstra 2003). A large number of conjugates have been employed. Examples of carrier molecules that have been used successfully are keyhole limpet haemocyanin (KLH) (Huxsoll *et al.* 1998; Kiyma *et al.* 2000; Meloen *et al.* 1994; Miller *et al.* 2000; Schanbacher 1984), human serum albumin (Finnerty *et al.* 1994; Lincoln *et al.* 1982), bovine serum albumin (Hotzel *et al.* 1997; Malmgren *et al.* 2001; Oonk *et al.* 1995), porcine thyroglobulin (Lincoln *et al.* 1982), ovalbumin (D'Occhio *et al.* 2001; Hoskinson *et al.* 1990; Oonk *et al.* 1998), leukotoxin produced by *Pasteurella haemolytica* (Cook *et al.* 2000), and bacterial toxoids like tetanus toxoid (Ladd *et al.* 1994) and diphteria toxoid (Ferro and Stimson 1998). Studies comparing different carriers showed that branched polylysine constructs, lipo-thioester (Beekman *et al.* 1999), tetanus toxoid and synthetic T-helper epitopes derived from malarial circumsporozite protein and measle virus fusion protein (Ferro and Stimson 1998) are highly immunogenic and effective. Various methods of conjugation are used. Examples are the carbodiimide reaction (Falvo *et al.* 1986; Lincoln *et al.* 1982; Oonk *et al.* 1998), glutaraldehyde reaction (Hotzel *et al.* 1997), use of bifunctionnal chemical linkers like m-maleimidobenzoyl-N-hydroxysuccinide ester (MBS) (Hage-van Noort *et al.* 1992; Meloen *et al.* 1994; Meloen 1995) and use of GnRH analogues containing

an additional amino group through which the molecule can be linked to a protein (Finnerty *et al.* 1994; Miller *et al.* 2000). A range of carriers and conjugation methods have resulted in a good GnRH antibody response.

In order to increase immunogenicity, the GnRH peptide can be modified by changing its structure or configuration (Hage-van Noort *et al.* 1992; Meloen 1995; Turkstra 2003). For instance, it can be dimerized or foreign amino acids can be introduced. In a study on immunocastration of piglets, Meloen *et al.* (1994) used a 20 amino acid tandem repeat of the GnRH peptide, conjugated to KLH. It appeared to be more efficient than the monomer, completely suppressing the development and endocrine function of the testes, and gave a less variable response in boars. In a further study (Oonk *et al.* 1998), a modified dimerized form, tandem-GnRH-dimer (G6k-TD), conjugated to ovalbumin, was shown to be effective at a lower dose rate, with only two vaccinations using Specol as adjuvant, a milder adjuvant than Freund's complete. This vaccine currently provides a practical method for chemically castrating pigs. It has also been proven effective in Chinese male (Zeng *et al.* 2001; Zeng *et al.* 2002b) and female (Zeng *et al.* 2002a) pigs. In young stallions, this vaccine combined with Covaccine™ adjuvant (Covaccine BV, Lelystad, The Netherlands) resulted in baseline testosterone concentrations and a decrease in testis size, sperm numbers and libido after two injections (Stout *et al.* 2003; Stout and Colenbrander 2004; Turkstra 2003). Finally, the vaccine has been tested in several zoo-species and the preliminary results suggest that it is a promising tool for suppressing fertility in young male goats, blackbucks and springbok antelopes (Turkstra *et al.* 2001).

To improve immune response while minimizing the dose and number of vaccinations, adjuvants are added to antigens (Hage-van Noort *et al.* 1992; Meloen 1995; Turkstra 2003). Adjuvants generally enhance the immune response and prolong the release of the antigen (Hage-van Noort *et al.* 1992; Meloen 1995). Several kinds of adjuvants can be used. Freund's complete adjuvant (FCA) is extremely effective but causes granulomas and localized tissue damage, which limits its use (Meloen 1995; Thompson 2000). Moreover, it interferes with tuberculin testing. Other examples of adjuvants are Freund's incomplete (Lincoln *et al.* 1982; Meloen *et al.* 1994), non-ionic surfactant vesicles, which are very effective and non-toxic compared to FCA (Ferro *et al.* 1996), polyl(lactide-co-glycolide)/triacetin (PLGA), a very effective slow release formulation (Ferro *et al.* 2003), aluminium hydroxide (Meloen 1995), muramyldipeptide (Falvo *et al.* 1986), DEAE dextran in oil-based vehicle (Freudenberger *et*

al. 1993), NUFA oil with Corynebacterium parvum (Freudenberger *et al.* 1993), Equimune (Malmgren *et al.* 2001), Specol , a mild oil adjuvant (Oonk *et al.* 1998; Zeng *et al.* 2002a), Covaccine (Stout and Colenbrander 2004) and oil-based Montanide ISA (Kiyma *et al.* 2000).

2.4 Vaccination protocol

The most common administration routes used are the intra-muscular (Malmgren *et al.* 2001; Oonk *et al.* 1998; Zeng *et al.* 2002b) and subcutaneous (D'Occhio *et al.* 2001; Ladd *et al.* 1994; Miller *et al.* 2000) routes. Successful delivery of vaccines has been achieved using darting systems (Curtis *et al.* 2002). Doses of GnRH peptide ranging from 5 µg (Oonk *et al.* 1998) to several milligrams (Huxsoll *et al.* 1998; Malmgren *et al.* 2001) have been used effectively. Booster immunizations are generally necessary (Meloen 1995; Turkstra 2003). They enhance existing antibody titres, and routine boosters are required for continued effectiveness (Thompson 2000). Vaccination protocols that have been successful usually consist of two (Miller *et al.* 2000; Oonk *et al.* 1998; Stout *et al.* 2003; Turkstra 2003) to five (Malmgren *et al.* 2001) vaccinations with booster intervals varying from two weeks (Ladd *et al.* 1994; Malmgren *et al.* 2001) to several months (D'Occhio *et al.* 2001). The adjuvant, carrier protein, GnRH peptide, number of boosters, booster interval, duration of treatment and dose all influence the efficacy and variability of response to the vaccine.

2.5 Reversibility and Toxicity

Natural reversal takes place in the majority of immunized animals, with the males reverting to normal sexual behaviour and fertility; this is an important advantage of this approach. Reversibility seems to be correlated to the rate of antibody decline (D'Occhio 1993; Keeling and Crighton 1984; Kumar *et al.* 2000; Ladd 1993; Ladd *et al.* 1994; Lincoln *et al.* 1982; Miller *et al.* 2000). Several studies on immunocastration in different species have showed the reversibility of the procedure (Ladd *et al.* 1994; Lincoln *et al.* 1982; Miller *et al.* 2000; Stout *et al.* 2003). The recovery to normality varies between animals but generally occurs between one and two years after the last immunization (Keeling and Crighton 1984; Miller *et al.* 2000; Stout *et al.* 2003). Results of studies on chronic toxicity and reversibility of immunization in rats (Kumar *et al.* 2000; Ladd 1993) and rabbits (Kumar *et al.* 2000) indicated that there are

no acute or chronic side-effects. Furthermore, no adverse effects were observed during clinical and toxicology trials in human patients (Ladd 1993).

2.6 Faecal steroid assays for non-invasive monitoring of hormone concnetrations in wild life

2.6.1 Introduction

Faecal steroid analysis for the non-invasive monitoring of reproductive status and cortio-adrenal activity has been studied extensively in domestic and non-domestic species (see reviews by Schwarzenberger *et al.* (1996a) and Whitten *et al.* (1998)). It constitutes an important and useful tool for the investigation of reproductive endocrinology (Schwarzenberger *et al.* 1996a), the detection of reproductive events (Schwarzenberger *et al.* 1996a; Whitten *et al.* 1998) and the assessment of stress or well-being in wildlife (Möstl and Palme 2002). Consequently it can be used to improve breeding programmes, to facilitate assisted reproductive technology (Brown *et al.* 2001b; Morrow and Monfort 1998; Schwarzenberger *et al.* 1996a), to assess the efficacy of reproductive treatment therapies (Brown *et al.* 2001b) and to develop better management systems for free-ranging and captive wildlife (Brown *et al.* 2001b; Ganswindt *et al.* 2002; Lasley and Kirkpatrick 1991; Velloso *et al.* 1998).

Faecal analysis is a practical alternative to blood and urine sampling. Collection of blood samples at regular intervals is impractical, especially in wildlife species (Ganswindt *et al.* 2002). The stress of handling and immobilization, which is sometimes necessary for blood sampling, can influence hormone secretion (Möstl and Palme 2002; Whitten *et al.* 1998). Urine samples are usually difficult to obtain (Brown 2000; Ganswindt *et al.* 2002) and supplementary time-consuming procedures are sometimes necessary to separate it from the soil (Kirkpatrick *et al.* 1992; Lasley and Kirkpatrick 1991). Steroid metabolites are generally present in high concentrations in the faeces (Lasley and Kirkpatrick 1991; Whitten *et al.* 1998) and faecal profiles generally reflect plasma steroid profiles (Möstl and Dehnhard 2002). Faecal samples offer the advantages of easy identification from a distance, easy location (Kirkpatrick *et al.* 1992; Whitten *et al.* 1998), reduced risk to the investigator (Gual-Sill *et al.* 1999; Lasley and Kirkpatrick 1991) and absence of handling stress during collection (Lasley and Kirkpatrick 1991; Möstl and Palme 2002; Whitten *et al.* 1998).

Moreover, faecal concentrations represent secretions over a number of hours as opposed to blood concentrations, providing a better average endocrine status (Brown *et al.* 1996a; Whitten *et al.* 1998). The use of faecal steroid analysis allows frequent sampling and provides long-term longitudinal studies on hormone secretion (Brown *et al.* 1996a; Fieβ *et al.* 1999; Lasley and Kirkpatrick 1991; Möstl and Palme 2002).

2.6.2 Steroid metabolites, excretion route and lag time of excretion

Using the administration of radioactively labelled steroids the excretion of steroid hormones and the metabolites present in faeces have been investigated in numerous species (see review by Schwarzenberger *et al.* (1996a)). Species as well as steroids differ as far as metabolites and routes of excretion are concerned (Palme *et al.* 1996) (see reviews by Lasley and Kirkpatrick (1991); Möstl and Palme (2002); Schwarzenberger *et al.* (1996a); Wasser *et al.* (2000) and Whitten *et al.* (1998)).

In sheep, ponies, pigs (Palme *et al.* 1996), hares (Teskey-Gerstl *et al.* 2000), primates (Foley *et al.* 2001; Wasser *et al.* 2000) and African elephants (Ganswindt *et al.* 2003) cortisol metabolites were shown to be mainly excreted in urine, while in rats (Bamberg *et al.* 2001) and cats (Brown *et al.* 2001b) they mainly appear in the faeces. Testosterone metabolites are predominantly excreted in the urine in ponies and pigs, almost equally distributed in urine and faeces in sheep, and excreted mainly in the faeces in wolves (Velloso *et al.* 1998), felids (Brown *et al.* 1996a; Brown *et al.* 2001b), African (Ganswindt *et al.* 2003) and Asian elephants (see review Brown (2000)).

In most species, faeces contain a higher amount of unconjugated than conjugated steroids (Foley *et al.* 2001; Möstl *et al.* 1999; Möstl *et al.* 2002; Palme *et al.* 1996; Palme and Möstl 1997; Velloso *et al.* 1998) (see review by Schwarzenberger *et al.* (1996a)). This also applies to elephants (Ganswindt *et al.* 2003; Wasser *et al.* 1996). Faeces usually contain multiple cortisol metabolites and nearly no parent hormone (Huber *et al.* 2003; Möstl *et al.* 2002; Möstl and Palme 2002; Palme and Möstl 1997; Stead *et al.* 2000; Teskey-Gerstl *et al.* 2000; Wasser *et al.* 2000). Insignificant amounts of cortisol (Ganswindt *et al.* 2003; Stead *et al.* 2000) and only small amounts of corticosterone (Stead *et al.* 2000) are found in the faeces of African elephants. Furthermore, various faecal cortisol metabolites are present and one of the major metabolites is 5β-androstane-3α,11,β-diol-17-one (Ganswindt *et al.* 2003).

Studies on faecal androgens present in specific species show that testosterone as well as androgen metabolites appear in faeces (Billitti *et al.* 1998; Brown *et al.* 1996a; Velloso *et al.* 1998). This is also true for male African (Ganswindt *et al.* 2002) and Asian (see review by Brown (2000)) elephant bulls. Ganswindt *et al.* (2002) showed that testosterone and epiandrosterone are both abundant in faeces of African elephants. In other species, only androgen metabolites and no native testosterone are present (Walker *et al.* 2002).

The time delay from secretion to the detection of metabolites in the faeces is important if one wants to relate behaviour or reproductive events to faecal steroid concentrations (Whitten *et al.* 1998). As the metabolites are essentially excreted in the bile, the intestinal passage time from the duodenum to the rectum determines the time delay (Palme *et al.* 1996; Schwarzenberger *et al.* 1996a). The time delay from release to peak excretion of steroids (see review by Schwarzenberger *et al.* (1996a)) is approximately 12 to 24 hours in ruminants (Kapke *et al.* 1999; Morrow and Monfort 1998; Möstl *et al.* 2002; Palme *et al.* 1996; Thompson *et al.* 1998; Wasser *et al.* 2000), 12 to more than 48 hours in cats (Brown *et al.* 1996a; Brown *et al.* 2001b) and 24 to more than 48 hours in horses (Möstl *et al.* 1999; Palme *et al.* 1996), pigs (Palme *et al.* 1996), rhinoceroses (Brown *et al.* 2001a) and primates (Barrett *et al.* 2002; Foley *et al.* 2001; Wasser *et al.* 2000). In the African elephant, the time delay for peak faecal steroid excretion varies from 30 to 48 hours (Ganswindt *et al.* 2003; Wasser *et al.* 1996; Wasser *et al.* 2000). The slow rate of elimination of cortisol and testosterone in faeces following peak excretion suggests entero-hepatic recirculation (Ganswindt *et al.* 2003).

2.6.3 Applications for assessment of testicular and adreno-cortical function

Faecal steroid monitoring has been applied in various species for various reasons (see reviews by Lasley and Kirkpatrick (1991); Schwarzenberger *et al.* (1996a) and Whitten *et al.* (1998)).

Faecal androgens have been used to study the link between testosterone secretion and dominance or aggressive behaviour in species such as meerkats (Moss *et al.* 2001), primates (Brockman *et al.* 1998; von Engelhardt *et al.* 2000), and other animals (see review by Whitten *et al.* (1998)). Ganswindt *et al.* (2002) monitored faecal androgens in African elephant bulls in order to investigate the relationship between endocrinology and reproductive behaviours such as musth.

Faecal androgens have also been used to assess testicular function, seasonality and reproductive rhythm in males of various species including felids (Brown *et al.* 1996a; Morato *et al.* 2001), wombats (Hamilton *et al.* 2000), meerkats (Moss *et al.* 2001), primates (Barrett *et al.* 2002; Brockman *et al.* 1998; Moreland *et al.* 2001), wolves (Velloso *et al.* 1998; Walker *et al.* 2002), rhinoceroses (Brown *et al.* 2001a; Kretzschmar *et al.* 2004) and deer (Li *et al.* 2001).

Cortico-adrenal status and stress have been assessed using faecal cortisol metabolites in many studies (see review by Whitten *et al.* (1998)). Examples are wild felids (Brown *et al.* 2001b; Wasser *et al.* 2000), domestic livestock (Möstl *et al.* 1999; Möstl *et al.* 2002; Palme *et al.* 2000; Palme and Möstl 1997), ferrets (Young *et al.* 2001), hares (Teskey-Gerstl *et al.* 2000), rats (Bamberg *et al.* 2001), hornbills (Crofoot *et al.* 2003), primates (Wasser *et al.* 2000), rhinoceroses (Turner *et al.* 2002; Wasser *et al.* 2000), deer (Huber *et al.* 2003) and African elephants (Foley *et al.* 2001; Ganswindt *et al.* 2003; Ganswindt *et al.* 2004a; Ganswindt *et al.* 2004b; Stead *et al.* 2000; Wasser *et al.* 2000). ACTH challenges were performed in African elephants by Stead *et al.* (2000) and Wasser *et al.* (2000) to validate the use of faecal cortisol metabolites to measure glucocorticoid secretion. Foley *et al.* (2001) measured faecal cortisol metabolites in African elephant cows to obtain information on stress induced by natural and human disturbances. Assays to measure faecal cortisol metabolites in African elephant bulls were studied by Ganswindt *et al.* (2003) and used to determine patterns of glucocorticoid secretion during musth.

2.6.4 Collection and storage of faecal samples

Faecal samples should be collected as soon as possible after defecation. The time-lapse from defecation to collection and from collection to analysis can influence the steroid concentrations. This is essentially due to bacterial metabolism and oxidation (Whitten *et al.* 1998). Möstl *et al.* (1999) observed an increase in cortisol metabolite concentrations in the faeces of pigs, cows and ponies after storage of samples at room temperature for 1, 4 and 24 hours. Using a different enzymimmunoassay, Möstl *et al.* (2002) observed a decrease in faecal cortisol metabolites in cow faeces after storage at room temperature for longer than four hours. In deer, a decrease in faecal cortisol metabolite concentrations was detected only when samples were collected later than 6 hours post-defecation (Huber *et al.* 2003). Faecal progesterone in cows shows a slow decrease when faeces are stored at room temperature but

values are still reliable for the first four days (Glatzel 1999). A study in fowl showed no effect of storing droppings at room temperature for up to 48 hours (Cockrem and Rounce 1994).

Once collected, the samples should be stored or preserved until analysed. The most reliable option is to freeze samples as soon as possible (Lasley and Kirkpatrick 1991; Lynch *et al.* 2003; Möstl and Palme 2002; Whitten *et al.* 1998). This is the method generally used for samples of domestic or captive animals as well as in the field, where possible (Czekala *et al.* 1994; Fieβ *et al.* 1999; Ganswindt *et al.* 2002; Ganswindt *et al.* 2003; Gual-Sill *et al.* 1999; Schwarzenberger *et al.* 1996b; Stead *et al.* 2000; Wasser *et al.* 1996; Wasser *et al.* 2000). Another viable method is to freeze-dry samples as soon as possible (Lynch *et al.* 2003). Different methods of preservation are possible in the field when equipment is limited (see review by Whitten *et al.* (1998)). Samples can be frozen in liquid nitrogen or dried in an oven or in the sun. They can be stored at room temperature in aqueous ethanol. Preliminary extraction methods, which can be applied in the field, have also been developed. In a comparative study on the storage of cheetah faeces, Terio *et al.* (2002) came to the conclusion that the best alternative to freezing at – 20 ° C is storage in 95 % ethanol at room temperature. Furthermore, lyophilisation was better than heat desiccation using an oven or sun heat. Heat desiccation generally causes some changes in steroid concentrations. Ethanol alters steroid concentrations in faeces after storage for extended periods at room temperature or even at – 20 ° C (Khan *et al.* 2002). Smaller changes, however, are observed if samples are stored at – 20 ° C. The effects of sample storage on hormone concentrations varies between hormones (Khan *et al.* 2002; Lynch *et al.* 2003; Terio *et al.* 2002). Depending on the storage method, storage duration, metabolite studied and assay used, the hormone concentrations can either increase or decrease. Androgen metabolites seem to be very stable compared to other steroids and usually only moderate changes occur (Lynch *et al.* 2003; Terio *et al.* 2002). Storage methods should be validated for each specific hormone (Lynch *et al.* 2003; Terio *et al.* 2002).

Steroids are evenly distributed in faeces of most species (Morrow and Monfort 1998; Thompson *et al.* 1998). However, collecting samples from defined sites in the faecal ball or after mixing the faeces seems to yield better results in horses, pigs (Palme *et al.* 1996) and elephants (Wasser *et al.* 1996). Collection from the central part of a bolus (Ganswindt *et al.*

2002; Ganswindt *et al.* 2003; Wasser *et al.* 1996) or from premixed wet faeces (Brown 2000; Fieβ *et al.* 1999; Ganswindt *et al.* 2003; Stead *et al.* 2000; Wasser *et al.* 1996) provides satisfactory homogenous samples in elephants.

2.6.5 Processing of samples before extraction

Most researchers dry samples before extraction (Brown *et al.* 1996a; Brown *et al.* 2001a; Czekala *et al.* 1994; Fieβ *et al.* 1999; Foley *et al.* 2001; Ganswindt *et al.* 2002; Garnier *et al.* 2002; Hamasaki *et al.* 2001; Hamilton *et al.* 2000; Kapke *et al.* 1999; Morrow and Monfort 1998; Stead *et al.* 2000; Thompson *et al.* 1998; Wasser *et al.* 1996). This allows easy removal of undigested plant material from the faecal powder (Fieβ *et al.* 1999; Gual-Sill *et al.* 1999; Schwarzenberger *et al.* 1996a; Wasser *et al.* 1996). Desiccation also allows correction for the differences in water content between samples (Czekala *et al.* 1994). Observations in several herbivores, however, showed that this is not really necessary (Schwarzenberger *et al.* 1996a). Wet samples can also be used (Asa *et al.* 2001; Kirkpatrick *et al.* 1992; Li *et al.* 2001; Moreland *et al.* 2001; Morelra *et al.* 2001; Paris *et al.* 2002; Velloso *et al.* 1998) and results generally correlate well with those of dried samples (Morelra *et al.* 2001). Dried samples are pulverized or crushed before extraction (Fieβ *et al.* 1999; Gual-Sill *et al.* 1999; Hamasaki *et al.* 2001; Hamilton *et al.* 2000; Thompson *et al.* 1998). In elephants, some authors have made use of lyophilised (Fieβ *et al.* 1999; Foley *et al.* 2001; Ganswindt *et al.* 2002; Ganswindt *et al.* 2003; Wasser *et al.* 1996; Wasser *et al.* 2000) or oven-dried (Stead *et al.* 2000) samples and fibrous material is discarded before extracting the powder (Fieβ *et al.* 1999; Ganswindt *et al.* 2002; Ganswindt *et al.* 2003; Wasser *et al.* 1996). Wet samples have also been used in elephants (Ganswindt *et al.* 2003). It seems that dried samples without the fibrous material gave the best results, but the use of well-mixed wet samples is also valid (Brown 2000).

2.6.6 Faecal extraction methods

Numerous faecal extraction methods have been tested (see reviews by Möstl and Palme (2002); Whitten *et al.* (1998)). Solvents mainly used are methanol (Brown *et al.* 1996a; Fieβ *et al.* 1999; Ganswindt *et al.* 2002; Gual-Sill *et al.* 1999; Huber *et al.* 2003; Kapke *et al.* 1999; Khan *et al.* 2002; Möstl *et al.* 1999; Palme *et al.* 1996; Schwarzenberger *et al.* 1996b; Schwarzenberger *et al.* 2000), ethanol (Brown *et al.* 1996a; Brown *et al.* 2001b; Kapke *et al.* 1999; Morelra *et al.* 2001; Stead *et al.* 2000; Wasser *et al.* 1996; Wasser *et al.* 2000), diethyl

ether (Crofoot *et al.* 2003; Czekala *et al.* 1994; Hamasaki *et al.* 2001; Kirkpatrick *et al.* 1992) and ether (Billitti *et al.* 1998; Brown *et al.* 2001a; Hamilton *et al.* 2000). Various extraction protocols can be followed, and include techniques such as shaking (Hamilton *et al.* 2000; Huber *et al.* 2003; Palme *et al.* 1996; Paris *et al.* 2002; Stead *et al.* 2000; Walker *et al.* 2002; Young *et al.* 2001), vortexing (Fieβ *et al.* 1999; Ganswindt *et al.* 2002; Gual-Sill *et al.* 1999; Hamasaki *et al.* 2001; Kapke *et al.* 1999; Li *et al.* 2001; Walker *et al.* 2002; Wasser *et al.* 2000) and boiling (Brown *et al.* 1996b; Brown *et al.* 1996a; Brown *et al.* 2001b; Morelra *et al.* 2001; Morrow and Monfort 1998; Thompson *et al.* 1998; Velloso *et al.* 1998; Wasser *et al.* 1996; Wasser *et al.* 2000). Boiling in 90 % ethanol: 10 % distilled water has proved to be successful for the extraction of androgen metabolites from the faeces of felids (Brown *et al.* 1996a), rhinoceroses (Brown *et al.* 2001a) and many other species (Brown *et al.* 1997). This method has also been used for the extraction of cortisol metabolites in rhinos (Brown *et al.* 2001a). Extraction by vortexing or shaking in 80- 90 % methanol also gives good results for androgen (Walker *et al.* 2002; Wasser *et al.* 2000) and cortisol (Huber *et al.* 2003; Möstl *et al.* 1999; Wasser *et al.* 2000; Young *et al.* 2001) metabolites in various species. Möstl *et al.* (1999) found that, in ponies and pigs, recovery rates of glucocorticoid metabolites improved as methanol concentrations increased and that the highest recoveries were obtained with 80-90 % methanol. The same was observed for sheep with optimum recovery rates achieved with 80 % methanol (Palme and Möstl 1997). A combination of 90 % methanol, 5 % $NaHCO_3$ and 5 % diethyl ether proved to be very efficient for the extraction of androgen metabolites from rhino faeces (Schwarzenberger *et al.* 2000). In elephant cows, efficient extraction of steroid metabolites was achieved by vortexing samples in 1.2 ml methanol, 1 ml distilled water and aluminium oxide (Gual-Sill *et al.* 1999), vortexing in 80 % methanol: 20 % distilled water (Fieβ *et al.* 1999) or boiling in 90 % ethanol: 10 % distilled water followed by centrifugation and further extraction of the pellet by vortexing with 90% ethanol: 10% distilled water (Wasser *et al.* 1996). In elephant bulls, cortisol metabolites were extracted successfully by shaking faecal samples in 80 % ethanol (Stead *et al.* 2000) or boiling in 90% ethanol: 10% water followed by centrifugation and re-extraction of the pellet by vortexing in 90% ethanol (Wasser *et al.* 2000). Androgen metabolites in elephants were extracted effectively by vortexing in 80 % methanol: 20 % water (Ganswindt *et al.* 2002).

2.6.7 Faecal steroid assays

Faecal steroid metabolites can be measured using radioimmunoassays (RIA) (Brockman *et al.* 1998; Khan *et al.* 2002; Terio *et al.* 2002; Velloso *et al.* 1998; Wasser *et al.* 1996) or

enzyme-immunoassays (EIA) (Fieβ *et al.* 1999; Ganswindt *et al.* 2002; Garnier *et al.* 2002; Gual-Sill *et al.* 1999; Schwarzenberger *et al.* 2000; Walker *et al.* 2002; Young *et al.* 2001). Antibodies used should be specific for metabolites or the parent hormones studied, in order to minimise cross-reactivity with other hormone metabolites (Ganswindt *et al.* 2003; Lasley and Kirkpatrick 1991; Möstl *et al.* 2002). The use of group-specific antibodies can be very efficient since they detect several metabolites from a particular parent hormone and consequently provide a more reliable measure of the secretion of the original hormone (Schwarzenberger *et al.* 1996a; Wasser *et al.* 2000; Whitten *et al.* 1998). Assays should be validated for each hormone and each species because of species-specific differences in steroid metabolites, as well as substances that may interfere with the assay (Lasley and Kirkpatrick 1991; Wasser *et al.* 2000; Whitten *et al.* 1998).

Immunoassays with antibodies to testosterone are generally used to measure faecal androgens (Brockman *et al.* 1998; Brown *et al.* 1996a; Brown *et al.* 1997; Brown *et al.* 2001a; Ganswindt *et al.* 2002; Hamilton *et al.* 2000; Kretzschmar *et al.* 2004; Lynch *et al.* 2003; Moreland *et al.* 2001; Moss *et al.* 2001; Palme and Möstl 1993; Terio *et al.* 2002; Velloso *et al.* 1998; von Engelhardt *et al.* 2000; Walker *et al.* 2002). Antibodies to 17-oxo-androstanes (epiandrosterone) may also be used (Ganswindt *et al.* 2002; Palme and Möstl 1993; Schwarzenberger *et al.* 2000). Ganswindt *et al.* (2002) characterised and measured faecal androgens in African elephant bulls using HPLC and enzyme-immunoassays for testosterone, epiandrosterone and 5α-androstane-17α-ol-3-one. Both testosterone and epiandrosterone assays gave reliable profiles of testicular endocrine function such that either can be used to measure androgen secretion. Epiandrosterone was the main androgen detected in elephant faeces, although epiandrosterone antibodies are probably less specific and cross-react with more androgen metabolites.

Wasser *et al.* (2000) compared a corticosterone RIA with three cortisol RIAs for measuring cortisol excretion in faecal samples. The corticosterone RIA gave the best results in several species including the elephant, because it cross-reacts with various cortisol metabolites. The corticosterone RIA used by Wasser *et al.* (1996) was used successfully by Foley *et al.* (2001) to measure the stress response in African elephants. Group-specific immunoassays for cortisol metabolites can also be used (Möstl *et al.* 2002; Palme and Möstl 1997) and generally detect higher concentrations of cortisol metabolites than cortisol or corticosterone EIAs (Ganswindt *et al.* 2003; Palme and Möstl 1997; Teskey-Gerstl *et al.* 2000). Palme and

Möstl (1997) developed an 11-oxoaetiocholanolone EIA that measures 11,17-dioxoandrostanes in sheep. Compared to a cortisol or a corticosterone EIA, it was much more sensitive in detecting cortisol excretion patterns after cortisol infusion. The same assay has been validated in several species, including horses (Möstl *et al.* 1999), cattle (Palme *et al.* 2000), hares (Teskey-Gerstl *et al.* 2000), primates (Foley *et al.* 2001; Wallner *et al.* 1999) and elephants (Ganswindt *et al.* 2003; Stead *et al.* 2000). Möstl *et al.* (2002) developed another 11-oxoaetiocholanolone EIA that measures 3α,11-Oxo-CM, and is more sensitive because it cross-reacts with a larger number of cortisol metabolites. It was developed in cattle and has also been used in deer (Huber *et al.* 2003) and elephants (Ganswindt *et al.* 2003). Stead *et al.* (2000) compared the cortisol, corticosterone and 11-oxoaetiocholanolone EIA described by Palme and Möstl (1997) in African elephants. The 11-oxoaetiocholanolone EIA gave the best results. Another study by Ganswindt *et al.* (2003) compared the use of these cortisol and 11-oxoaetiocholanolone EIAs to the 11-oxoaetiocholanolone EIA described by Möstl *et al.* (2002) and an 11β-Hydroxy-aetiocholanolone EIA. The results showed that each assay is able to detect glucocorticoid metabolites in faeces. The 11-oxoaetiocholanolone EIA described by Möstl *et al.* (2002) for measuring 3α-11-oxo-cortisol metabolites was, however, the most sensitive for detecting cortisol metabolites and showed no significant cross-reactivity with testosterone metabolites. It is, therefore, the most appropriate assay for measuring cortico-adrenal activity in male elephants, especially when they are in musth.

CHAPTER 3

MATERIAL AND METHODS

3.1 Elephant bulls

Table 1 shows the six bulls and their age, location and behavioural status prior to the primary GnRH vaccination.

Table 1: Elephant bulls vaccinated against GnRH

Animal	Age	Location	Aggressive behaviour[a]
Kinkel	22	Johannesburg Zoo, RSA	+
Thembo	18	Tshukudu Game Reserve, RSA	+
Toto	18	Imire Game Park, Zimbabwe	-
Chaka	27	Imire Game Park, Zimbabwe	-
Makavhuzi	28	Imire Game Park, Zimbabwe	-
Grootvoet	40	Shambala Private Reserve, RSA	+, Musth

[a] Aggressive behaviour at the beginning of the study, before immunization
+ present; - absent; musth, see 3.6 for definition

Kinkel - Johannesburg Zoo

Kinkel was a 22 year-old African elephant bull, housed at Johannesburg Zoo. He was housed together with a 22 year-old female in a relatively large outside enclosure (approximately 3500 m^2) during the day. At night the two elephants were separated and kept in different night rooms. They were also fed in the night rooms in the evening and morning. During the day when they were in the outside enclosure, lucerne was provided. Kinkel had been at the Zoo for two years before the start of the study. During the first year he showed signs of musth for two periods of approximately 10 days each. Increased aggressive behaviour and temporal gland secretion were observed. Urine dribbling was absent. Periods with similar musth signs were repeated four times during the second year. During the six months prior to vaccination, Kinkel showed continual phases of intense aggressive behaviour combined with heavy temporal gland secretion. The aggressive behaviour was essentially directed towards the cow, especially during feeding. He also directed his aggressive behaviour towards people and objects. Before arriving at the zoo, the bull had been kept on a game farm with two elephant cows. At that stage he had also displayed aggressive behaviour and had attacked a few rhinos.

Thembo - Tshukudu Game Reserve

Thembo was an 18 year-old bull kept on Tshukudu Game Reserve. He was free-ranging together with a cow and an older bull on the reserve. During winter, supplementary food was provided for the animals on the reserve. Rhinos, buffalos and elephants foraged together at the same feeding site. Thembo and the cow arrived at Tshukudu as orphans. Both became habituated to people, but were never trained. Prior to arrival of the older male, temporal gland secretion and aggression had been observed. The aggressive behaviour was directed towards the rhinos, and he killed two of them. The older male was a free-ranging bull from the Kruger National Park and broke into the reserve two years ago. Since then, Thembo showed less intense signs of musth. Aggressive behaviour was still directed towards rhinos, especially when food was involved, but no more were killed. He also created a lot of damage to fences and the lodge. Two weeks after the second vaccination, Thembo broke out of the reserve. He was recaptured four weeks later and finally translocated to a reserve close to Moketsi, where he is being trained by Mr. Rory Hensman. The rest of the study was carried out there. For the first month, Thembo was kept in a boma where he was fed and trained. Later he was fed on the reserve during the day, accompanied by his keepers, and kept inside a boma at night.

Toto, Chaka and Makavhuzi - Imire Game Park, Zimbabwe

Toto, Chaka and Makavhuzi were three domesticated elephant bulls kept on Imire Game Park in Zimbabwe. At the beginning of the study, they were approximately 18, 27 and 28 years old, respectively. They were trained daily for safari rides and housed together with an 11year-old cow. Originally there was a fourth bull on Imire Game Park. Sixteen months before the start of the study, however, this 24 year-old bull entered musth. He was extremely aggressive and had to be euthanased.

Toto, Chaka and Makavhuzi were leg-chained outside at night. During the day they fed on the reserve, accompanied by their keepers. Makhavuzi was the dominant bull and had been showing some sporadic mild musth-like signs for a year, with some aggression directed towards Chaka. Two months before the start of the study a fight broke out between Makavhuzi and Chaka. Makavhuzi broke his tusks and Chaka finally backed off. Excepting on some occasions when new keepers were introduced to Chaka, no significant aggressive signs towards people had been observed in any of the bulls.

Grootvoet - Shambala Private Reserve

Grootvoet was a 40 year-old bull free-ranging on Shambala Private Reserve for the last five years. The reserve also housed a group of 9 females and a 25 year-old bull. At the beginning of the study, Grootvoet had been in full musth for 6 months, presenting signs such as urine dribbling, temporal gland secretion and aggressive behaviour towards vehicles and people.

3.2 Vaccination

3.2.1 Vaccine formulation

The GnRH vaccine used in this study was previously described by Oonk *et al.* (1998) and is manufactured by Pepscan Systems (Lelystad, The Netherlands). Briefly, it is a modified GnRH-tandem-dimer-ovalbumin conjugate in which the GnRH molecules are modified by substituting L-glycine in the 6-position with D-lysine to enable conjugation of the peptide to ovalbumin. The vaccine was developed for the immunocastration of male pigs (Oonk *et al.* 1998).

Two different adjuvants were used: Montanide® ISA 51 (Seppic, Paris, France), consisting of manide oleate in mineral oil, and Covaccine™ (Covaccine BV, Lelystad, The Netherlands), which is a proprietary product. The vaccine was mixed with the Montanide® ISA 51 adjuvant as follows: 1.5 ml ISA 51 was added to the vaccine containing 2 mg peptide conjugated to ovalbumin in 1.5 ml PBS buffer. The mixture was emulsified by pushing the vaccine back and forth through a connecting needle with two glass syringes until a stable emulsion was formed. Mixing the vaccine with the Covaccine adjuvant was performed by simply adding 1.5 ml Covaccine to 1.5 ml vaccine and shaking briefly.

3.2.2 Immunization protocol

The dose of GnRH used for each vaccination was 2 mg GnRH–peptide mixed with one of the two adjuvants to provide a total vaccine volume of 3 ml. The bulls were vaccinated three to four times. The vaccination details of each bull are shown in Table 2. The vaccine was administered intra-muscularly in the gluteus muscle group by hand or by means of a dart. Hand injection was performed using 5 ml syringes and 18 gauge 1 inch long needles. The bulls were leg-chained prior to injection. Administration by dart was performed with the Daninject® system (Wildlife Pharmaceuticals, Fort Collins, Colorado, USA), using 5ml darts

and 60 mm barbless needles. The needles were glued to the darts in order to make sure they would fall off with the dart.

Table 2: Immunization protocol showing the adjuvant, administration method and day of each vaccination for the 6 bulls

		Day of vaccination, administration method and adjuvant							
		Primary		1st Booster		2nd Booster		3rd Booster	
Bull	Kinkel	Day 0	Dart ISA 51	Day 21	Dart ISA 51	Day 43	Dart ISA 51	Day 64	Dart Covaccine
	Thembo	Day 0	Dart Covaccine	Day 21	Dart Covaccine	Day 66	Hand Covaccine		
	Toto	Day 0	Hand Covaccine	Day 49	Hand Covaccine	Day 196	Hand Covaccine		
	Chaka	Day 0	Hand Covaccine	Day 49	Hand Covaccine	Day 70	Hand Covaccine		
	Makavhuzi	Day 0	Hand Covaccine	Day 49	Hand Covaccine	Day 70	Hand Covaccine		
	Grootvoet	Day 0	Dart Covaccine	Day 21	Dart Covaccine	Day 50	Dart Covaccine		

3.3 Collection and processing of samples

3.3.1 Faecal samples

Faecal samples from immunized animals were collected for approximately four to five consecutive days prior to the primary vaccination, two weeks after each vaccination and four months after the last vaccination. In certain cases, however, the sampling could not be performed according to the protocol. An additional sample was collected from Grootvoet 3 months before the first vaccination. Time of collection of faecal samples is shown with the results for each bull in Tables 3 and 4.

Each sample was taken from the middle of a faecal bolus. The samples were collected straight after defecation and put in individual plastic bags. At Johannesburg Zoo, however, samples were collected in the morning after the bull had been removed from the night room. Samples were transported in a cooler box on ice and frozen within 30 minutes to 4 hours after collection. They were stored at – 20 ° C until extraction and analysis.

3.3.2 Blood samples

Blood samples were collected from Toto, Chaka and Makavhuzi before the primary vaccination, and from Chaka and Makavhuzi 12 days after the first vaccination and four months after the third vaccination. Blood was collected from the saphenous vein. Single samples were also obtained from Thembo six weeks after the second vaccination and from Grootvoet 3 months before the primary vaccination. These two samples were collected after the bulls had been immobilized.

After the serum had been separated it was stored at – 20 ° C until analysed.

3.4 Hormone assays

Samples were analysed by the Veterinary Wildlife Laboratory (University of Pretoria) (faecal epiandrosterone and serum testosterone) and the Institute of Biochemistry (Vienna, Austria) (faecal cortisol metabolites).

3.4.1 Faecal sample extraction and enzyme-immunoassays

3.4.1.1 Extraction

The first 13 samples collected from Kinkel were freeze-dried before extraction. The dried samples were crushed by hand using a pestle. After removing the fibrous material, the powder was extracted. Extraction of wet portions of these samples was also performed. Assay of these wet and dried samples demonstrated good parallelism. Consequently, further extractions and analyses for Kinkel and the other bulls were performed exclusively on wet samples.

Faecal samples were extracted according to Palme and Möstl (personal communication) as follows:

- 0.5 g wet faeces were mixed with 5 ml aqueous methanol (80 %) (10 ml in case of dried samples) in 12 ml glass test-tubes, shaken for 30 minutes at room temperature and centrifuged for 15 minutes (3 700 rpm, 5 °C)
- 5 ml diethylether and 250 µl 5 % $NaHCO_3$ were added to 1 ml of the supernatant in a new glass test-tube
- After vortexing for 10 seconds, the samples were centrifuged for 15 minutes as above and frozen for 30 minutes at –70 °C

- The supernatant (liquid phase) was transferred to a new glass test-tube and dried under N_2 vapour at 45 °C
- The dried extract was taken up in 0.5 ml assay buffer.

3.4.1.2 Epiandrosterone

Faecal epiandrosterone concentrations have been shown to be a reliable method for monitoring testosterone secretion in the African elephant (Ganswindt *et al.* 2002). The epiandrosterone double-antibody-biotin-streptavidin EIA was validated for African elephants by Ganswindt *et al.* (2002). The EIA is described in detail by Palme and Möstl (1993). The reagents were supplied by the Institute of Biochemistry (Vienna, Austria). Epiandrosterone is used as the standard and 5α-androstane-3,17-dione-thioether as label. Antibodies to 5α-androstane-3α-ol-17-one-HS:BSA were raised in rabbits and showed the following main cross-reactivities: epiandrosterone, 100 %; 5α-androstane-3,17-dion, 80 %; 5α-androstane-3α-ol-17-one, 40 % and 4-androsten-3,17,-dione, 15,6 %.

The EIA was performed as follows:

- The microtitre plate (MTP) was coated with antibody by incubation at room temperature over-night with a coating buffer containing an anti-rabbit antibody raised in sheep
- After discarding the first coating buffer, a second coating buffer containing BSA was added to the MTP
- The MTP was incubated at room temperature for a minimum of 3 hours
- Coated MTPs were washed three times with washing solution before use
- Standards and samples were added to the wells
- Biotin-labelled steroid and the specific anti-steroid antibody were added to each well
- The MTP was incubated overnight at 4 °C on a shaker and, the next day, washed four times with cold washing solution
- Streptavidin peroxidase was added to each well
- The MTP was incubated for 45 minutes at 4 °C on a shaker and washed four times with cold washing solution
- Substrate (Tetramethylbenzedine) solution was added to each well and the MTP was incubated for 45 minutes at 4 °C on a MPT-shaker
- Stop reagent (H_2SO_4) was added

- Absorbance was measured at 450 nm using an iEMS Labsystem plate reader and Genisis software

3.4.1.3 3α,11oxo-cortisol metabolites

Faecal cortisol metabolites were analysed using the 11-oxoaetiocholanolone EIA described by Möstl *et al.* (2002). This biotin-streptavidin EIA has been shown to provide a sensitive measure of adrenal glucocorticoid output in the male African elephant (Ganswindt *et al.* 2003). It measures 3α,11oxo-cortisol metabolites (3α,11oxo-CM). Antibodies to 11-oxoaetiocholanolone-17-CMO:BSA were raised in rabbits. 11-oxoaetiocholanolone (5β-androstane-3α--ol-11,17,-dione) was used for the standards and 11-oxoaetiocholanolone-17-CMO-biotinyl-3,6,9-trioxaundecanediamine as label. The major antibody cross-reactions were: 5β-androstane-3α-ol-11,17,-dione, 100 %; 5β-pregnane-3α-ol-11,20-dione, 37 %; 5β-androstane-3α,1β-diol-17-one, 3.3 % and 5β-androstane-3,11,17-trione, 1.2 %.

This EIA was performed using the method described for epiandrosterone.

3.4.2 Serum testosterone assay

Testosterone concentrations in serum samples were analysed using an RIA kit (Coat-a-Count® Total Testosterone, Diagnostic Products Corporation, Los Angeles, CA). This assay has been validated in domestic species such as pigs (Almond *et al.* 1992), dogs (Reimers *et al.* 1991) and horses (Reimers *et al.* 1991). The Coat-A-Count procedure is a solid phase radioimmunoassay, based on testosterone-specific antibodies immobilized on the wall of a propylene tube. ^{125}I-labeled testosterone competes with testosterone in the standard or sample for antibody sites. To separate bound from free testosterone the tube is then decanted and radioactivity in the tube is counted using a gamma counter. The amount of testosterone present in the patient sample is determined from a calibration curve. The system used was Cobra® Series Auto-Gamma® Counting System (Packard Instrument Co., Meriden, USA)

3.4.3 Analysis of GnRH antibody titres

Blood samples from Chaka and Makavhuzi collected before the primary vaccination and four months after the third vaccination and a blood sample from Thembo collected six weeks after

the second vaccination were analysed for GnRH antibody titres. GnRH antibody titres were measured using an enzyme-linked immunosorbent assay (ELISA). This assay was based on an ELISA used to measure anti-PZP antibody titres in African elephant cows (Fayrer-Hosken *et al.* 1999).

It was performed as follows:

- The plates were coated with GnRH-tandem-dimer-ovalbumin conjugate (2 µg per well) and incubated at 4 °C for 12 hours
- The plates were washed three times with Tris buffered saline (TBS; 0,606g Tris and 0,88g NaCl in 100 ml dH2O)
- 200 µl of TBS and Tween (TBST; TBS with 0,05% Tween-20) with 5 % BSA was added to each well and the plate was incubated at 4 °C for 12 hours
- The TBST was removed and the wells were washed three times with TBS
- 50 µl of sample elephant serum at a dilution of 1: 1 000 was added to each well and the plate incubated for four hours
- The plates were washed three times with TBS
- 50 µl of rabbit anti-elephant IgG antibody (1:1 000 dilution in TBST) was added to each well and the plate incubated for two hours
- 50 µl of goat anti-rabbit antibody with alkaline phosphatase (dilution 1: 2 000 in TBST) was added to each well and the plate incubated for 2 hours
- The plates were washed three times with TBS
- 200 µl of carbonate buffer was added for 5 minutes to each well and then removed
- 50 µl of p-nitrophenyl phosphate in carbonate buffer at pH 9.8 was added to each well
- The colour reaction was stopped 30 minutes later by adding 50 µl of 3 M NaOH per well
- Absorbance was measured at 405 nm and 492 nm nm using an iEMS Labsystem plate reader and Genisis software.

3.5 Behavioural observations

The effect of the vaccine on aggressive behaviour was studied by comparing the frequency of aggressive behaviours before and after vaccination.

When possible, aggressive behaviour was assessed before each vaccination and two weeks and four months after the last vaccination.

Each assessment period consisted of a total of approximately 12 hours of observations collected over several consecutive days during the morning, midday and afternoon. In order to assess aggressive behaviour, observations were recorded using the data sheets shown in Appendix 1, 2, 3 and 4 as follows:

- Behavioural traits relating to aggression, dominance or musth were recorded in the table shown in Appendix 1 each time they were observed. These behaviours were based on behavioural traits previously described by Poole and Granli (2003), Poole (1987) and Garai (1997). The definitions are provided in Appendix 2. Furthermore, the table indicates whether the behaviour was directed towards people, elephants, other species or objects.
- In addition to interactions, which were recorded every time they occurred, the main activity was recorded every 5 minutes. The following code was used:
  - I: interaction with elephants: affiliative, aggressive, dominant, submissive, sexual, investigative or interaction with people.
  - F: feeding/drinking
  - B: sand or mud-bathing
  - R: resting/standing
  - W: walking
  - P: playing
- The distance to people, other animals or elephants was also recorded every 5 minutes.
- Appendix 3 was used for further description of the observed interactions.
- During each observation period, detailed signs of musth other than aggressive behaviour were recorded as described by Poole (1987) (Appendix 4). The presence or absence of temporal gland secretion was noted. The amount of temporal gland secretion was classified in 4 grades:

  1- from the gland to in line with the ventral border of the eye,

  2- half way between the eye and the corner of the mouth,

  3- to the corner of the mouth,

  4- to the base of the lower jaw.

  Temporal gland secretion was further classified as dark (fresh) or light (old), and, where possible, as watery or viscous. Penis discolouration (green penis syndrome: the distal part of the sheath/proximal part of the penis develops a greenish colour) was recorded as absent, slight or marked. Urine odour was described as absent, slight or

strong. The presence of urine dribbling and urination with the penis still sheathed was also recorded.

- Finally, some additional aspects were noted:
  - physical condition: emaciated, very thin, thin, good, fat, very fat.
  - association with female herd, with males or alone

In addition to our observations, animals were monitored by the game rangers and keepers for aggressive behaviour on a continuous basis before and after vaccination, using the data sheet shown in Appendix 5. This allowed us to collect useful supplementary information. For logistical reasons, Grootvoet could not be monitored by us. Behavioural data was only collected by the game manager, using the data sheet of Appendix 5.

Information collected allowed us to:

- Classify bulls as being in musth or non-musth. A musth male was a male exhibiting temporal gland secretion and urine dribbling, or evidence of recent urine discharge (Poole 1987c).
- Compare frequencies of aggressive behaviour before and after immunization. Frequencies were expressed as number of aggressive behaviours per hour (AB/h).
- Determine the dominance hierarchy between bulls.
- Detect any other behavioural or physical changes.

3.6 Data analysis

For each animal, the samples and observations were grouped in the following data sets:

- Stage 1: before primary vaccination
- Stage 2: after primary vaccination
- Stage 3: after second vaccination
- Stage 4: after third vaccination
- Stage 5: after fourth vaccination
- Stage 6: approximately 2 months after last vaccination
- Stage 7: approximately 4 months after last vaccination

The epiandrosterone data was analysed by Repeated Measures of ANOVA.

For each bull, differences in hormone concentrations between different stages were analysed by means of One Way ANOVA with post hoc comparison using the Tukey-Kramer Multiple Comparison Test.

Also, for epiandrosterone, the data for non-musth aggressive and non-musth non-aggressive bulls during Stage 1 were grouped and compared using the two-sample t-test. Mean epiandrosterone values for each bull during Stage 1 were used as data in the t-test.

The non parametric Spearman Rank Order Correlation Test was used to analyse the correlation between epiandrosterone and 3α,11oxo-cortisol metabolites for each bull individually.

The α-level of significance was set at < 0.05 for all the tests.

Statistical analyses were performed using NCSS Statistical Software 2004. Graphs were created using NCSS Statistical Software and Sigmaplot 2001.

The small number of observations did not allow the statistical analysis of behavioural parameters.

CHAPTER 4

RESULTS

4.1 Administration of the vaccine

Vaccination using the Daninject® darting system with barbless 60 mm needles was found to be a practical way to administer the vaccine when mixed with the Covaccine adjuvant. The Montanide ISA 51 adjuvant was unsuitable for dart delivery because the emulsion was too viscous, resulting in incomplete injection of the vaccine. Consequently it was only used for the three first vaccinations of Kinkel.

Hand injection of the vaccine was convenient for trained bulls.

No adverse side effects, such as swellings or granulomas at the injection sites or systemic signs, were observed with either adjuvant, even after booster vaccinations ahd been administered.

4.2 Results of hormone assays

Detailed faecal epiandrosterone and 3α,11oxo-cortisol metabolite results for each individual bull are provided in Tables 3 and 4.

4.2.1 Faecal epiandrosterone results

Repeated measures ANOVA of epiandrosterone levels in the six bulls showed no significant differences between the stages of vaccination ($DF = 6$, $F = 0.70$, $p = 0.65$).

Fig. 1 shows the faecal epiandrosterone concentrations of Kinkel, Thembo, Toto, Chaka, Makavhuzi and Grootvoet during the different stages of vaccination.

Kinkel

Epiandrosterone concentrations during Stage 4 were significantly higher than at all other stages ($DF = 5$, $F = 7.82$, $p < 0.001$).

Thembo

From start to finish there was a downward trend in epiandrosterone concentrations. They were significantly lower during Stages 4 and 7 than Stages 1, 2 and 3, and during Stage 6 compared to Stage 1 ($DF = 5$, $F = 11.029$, $p < 0.001$).

Toto
Epiandrosterone levels during Stage 3 were significantly lower than during Stages 1 and 2 (DF = 3, F = 5.05, p = 0.012).

Chaka
Chaka's epiandrosterone concentrations were low throughout with Stage 7 being significantly higher than Stages 2 and 3 (DF = 3, F = 4.61, p = 0.016).

Makavhuzi
This bull's faecal epiandrosterone concentrations were low throughout, with no significant differences between stages (DF = 3, F = 2.21, p = 0.129).

Grootvoet
There were no significant differences between stages (DF = 3, F = 1.13, p = 0.373).
The faecal sample collected three months before the first vaccination had an epiandrosterone concentration (209 ng/g) approximately 3-fold higher than the samples collected during Stages 1, 2, 3 and 4 (overall mean 62.11 ± 23.53 ng/g). During this preexperimental period, he was in full musth.

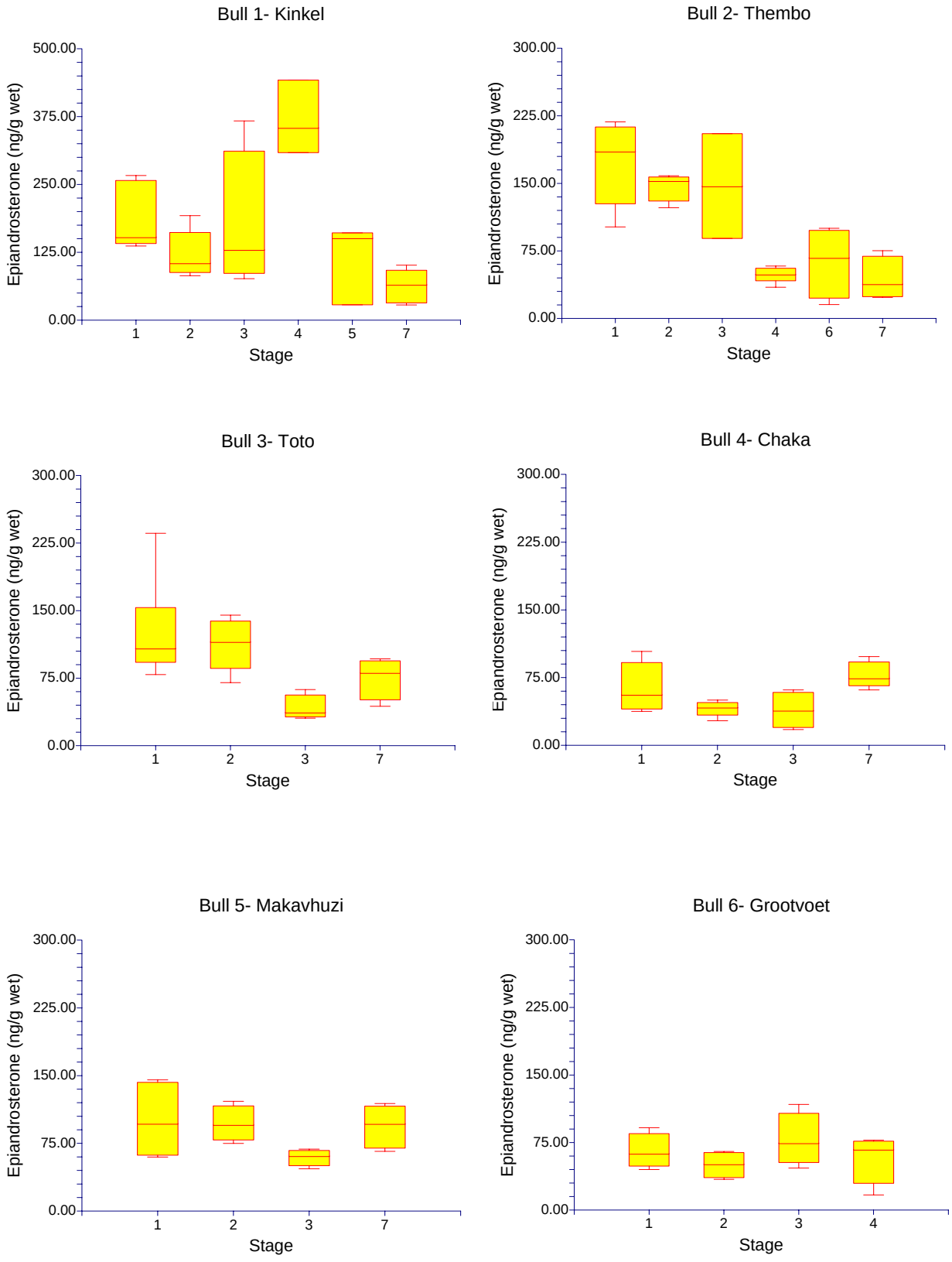

Fig. 1: Grouped concentrations of faecal epiandrosterone for each of the 6 bulls. The boxes represent the median and the upper and lower quartile values of samples collected at different stages of vaccination. The whiskers show the range.

Comparison of epiandrosterone levels of non-musth aggressive bulls vs. non-aggressive bulls during Stage 1

Non-musth aggressive bulls were represented by Kinkel (184.46 ± 59.67 ng/g) and Thembo (173.07 ± 47.05 ng/g). Non-aggressive bulls were represented by Makavhuzi (100.53 ± 37.16 ng/g), Chaka (63.79 ± 26.70 ng/g) and Toto (125.65 ± 56.15 ng/g).

Aggressive bulls (178.76 ± 8.05 ng/g) showed significantly higher ($t = 3.483$, $DF = 3$, $p = 0.04$) epiandrosterone levels than non-aggressive bulls (96.66 ± 31.11ng/g) (Fig. 2).

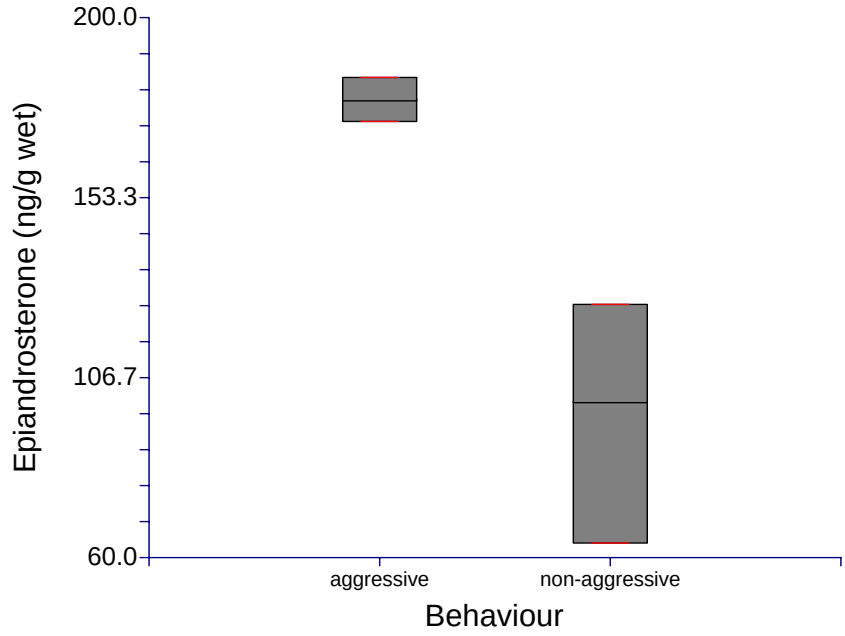

Fig. 2: Grouped epiandrosterone levels of non-musth aggressive and non-aggressive bulls.

Table 3: Faecal epiandrosterone (EA) and 3α,11oxo-cortisol metabolite concentrations (CM) in ng/g wet samples during the different stages of vaccination for Kinkel, Thembo and Grootvoet. Stage 1: before primary vaccination, Stage 2: after primary vaccination, Stage 3: after first booster, Stage 4: after second booster, Stage 5: after third booster, Stage 6: 2 months after last booster, Stage 7: 4 months after last booster. Days on which the samples were collected are also indicated. Day 0 corresponds to the day of the primary vaccination.

Kinkel

Stage 1			Stage 2			Stage 3			Stage 4			Stage 5			Stage 7		
Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM
-28	143	23	16	82	11	36	367	6	60	355	22	79	151	32	175	66	44
-20	143	9	17	192	5	37	76	3	63	442	14	80	161	32	177	101	24
-19	266	32	18	94	11	38	116	5	64	308	16	81	28	22	178	35	41
-5	254	20	19	105	7	42	144	9							179	83	33
-3	137	27	20	131	13										180	28	39
-2	164	28															

Thembo

Stage 1			Stage 2			Stage 3			Stage 4			Stage 6			Stage 7		
Day	**EA**	**CM**	Day	**EA**	**CM**	Day	**EA**	**CM**	Day	**EA**	**CM**	Day	**EA**	**CM**	Day	**EA**	**CM**
-4	207	68	16	153	28	29	205	45	79	49	125	125	44	78	186	75	56
-3	153	48	18	153	24	66	89	57	80	53	94	127	91	27	187	26	48
-2	218	53	19	123	23				81	58	96	128	15	32	188	23	43
-1	186	37	20	158	22				82	35	50	130	100	84	189	50	78
0	102	51							83	49	30						

Grootvoet

3 months before Stage 1			Stage 1			Stage 2			Stage 3			Stage 4					
Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM			
-89	209	11	-4	60	15	16	60	23	46	117	133	67	17	80			
			-3	45	16	17	42	40	47	71	160	68	43	89			
			-2	65	13	18	34	47	48	78	146	69	75	126			
			0	91	18	20	65	57	50	47	68	70	78	100			
												71	67	135			

Table 4: Faecal epiandrosterone (EA) and 3α,11oxo-cortisol metabolite concentrations (CM) in ng/g wet samples during the different stages of vaccination for Toto, Chaka and Makavhuzi. Stage 1: before primary vaccination, Stage 2: after primary vaccination, Stage 3: after first booster, Stage 4: after second booster, Stage 5: after third booster, Stage 6: 2 months after last booster, Stage 7: 4 months after last booster. Days on which the samples were collected are also indicated. Day 0 corresponds to the day of the primary vaccination.

Toto											
Stage 1			Stage 2			Stage 3			Stage 7		
Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM
-7	125.79	84	8	69.94	84	68	62.3	99	192	43.73	70
-6	104.73	94	9	115.5	117	69	36.36	91	193	91.9	87
-5	111.65	135	10	101.64	169	70	37.84	86	194	81.14	139
-4	97.1	120	11	131.77	89	72	30.49	34	195	96.37	93
-2	78.86	105	12	144.96	90				197	57.95	58
0	235.77	18									
Chaka											
Stage 1			Stage 2			Stage 3			Stage 7		
Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM
-7	41	98	8	42	73	68	50	66	192	98	151
-6	87	133	9	27	85	69	17	60	193	74	174
-5	104	111	10	45	137	70	27	55	194	61	142
-4	38	122	11	40	96	72	61	68	195	71	128
-2	50	144	12	50	67				197	86	87
0	63	92									
Makavhuzi											
Stage 1			Stage 2			Stage 3			Stage 7		
Day	EA	CM	Day	EA	CM	Day	EA	CM	Day	EA	CM
-7	104	53	8	122	53	68	62	26	192	73	54
-6	145	71	9	101	67	69	60	33	193	119	69
-5	142	61	11	91	53	70	47	38	194	113	32
-4	63	57	12	75	48	72	69	37	195	97	64
-2	60	64							197	66	42
0	90	46									

4.2.2 Serum testosterone results

Serum testosterone concentrations are represented in Table 5.

Table 5: Serum testosterone concentrations in ng/ml

Bull	3 months before vaccine I	Just before vaccine I	12 days after vaccine I	6 weeks after vaccine II	4 months after vaccine III
Thembo	-	-	-	7.82	-
Toto	-	0.77	-	-	-
Chaka	-	0.20	0.71	-	0.12
Makavhuzi	-	0.63	10.00	-	0.71
Grootvoet	44.04	-	-	-	-

The musth bull (Grootvoet) had a high serum testosterone concentration (44.04 ng/ml) compared to the non-musth bulls (Toto, Chaka, Makavhuzi) (overall mean 0.53 ± 0.30 ng/ml).

4.2.3 Faecal 3α,11oxo-cortisol metabolites results

Fig. 3 shows the patterns of faecal 3α,11oxo-CM concentrations of Kinkel, Thembo, Toto, Chaka, Makavhuzi and Grootvoet during the different stages of vaccination.

Kinkel

3α,11oxo-CM levels were significantly higher during Stage 7 than during Stages 1, 2, 3 and 4 and during Stages 1 and 5 compared to Stages 2 and 3 ($DF = 5$, $F = 16.15$, $p < 0.001$).

Thembo

3α,11oxo-CM levels were significantly increased during Stage 4 compared to Stage 2 ($DF = 5$, $F = 2.64$, $p = 0.05$).

Toto

Wide ranges were observed throughout in this bull and between stages there were no significant differences ($DF = 3$, $F = 0.66$, $p = 0.6$).

Chaka

3α,11oxo-CM levels were significantly higher during Stage 7 than during Stages 3 and 2 and during Stage 1 compared to Stage 3 ($DF = 3$, $F = 7.93$, $p < 0.01$).

Makavhuzi

In Makavhuzi 3α,11oxo-CM levels were significantly lower during Stage 3 than during Stages 1 and 2 ($DF = 3$, $F = 5.15$, $p < 0.05$).

Grootvoet

3α,11oxo-CM levels were significantly higher during Stages 3 and 4 compared to Stages 1 and 2 ($DF = 3$, $F = 18.85$, $p < 0.001$).

There were no significant correlations between faecal epiandrosterone and 3α,11oxo-cortisol metabolites in Kinkel ($R_{26} = -0.216$, $p = 0.29$), Thembo ($R_{24} = -0.235$, $p = 0.27$), Toto ($R_{20} = 0.201$, $p = 0.39$) and Grootvoet ($R_{17} = 0.389$, $p = 0.12$). A positive correlation was found in Chaka ($R_{20} = 0.508$, $p < 0.05$) and Makavhuzi ($R_{19} = 0.482$, $p < 0.05$).

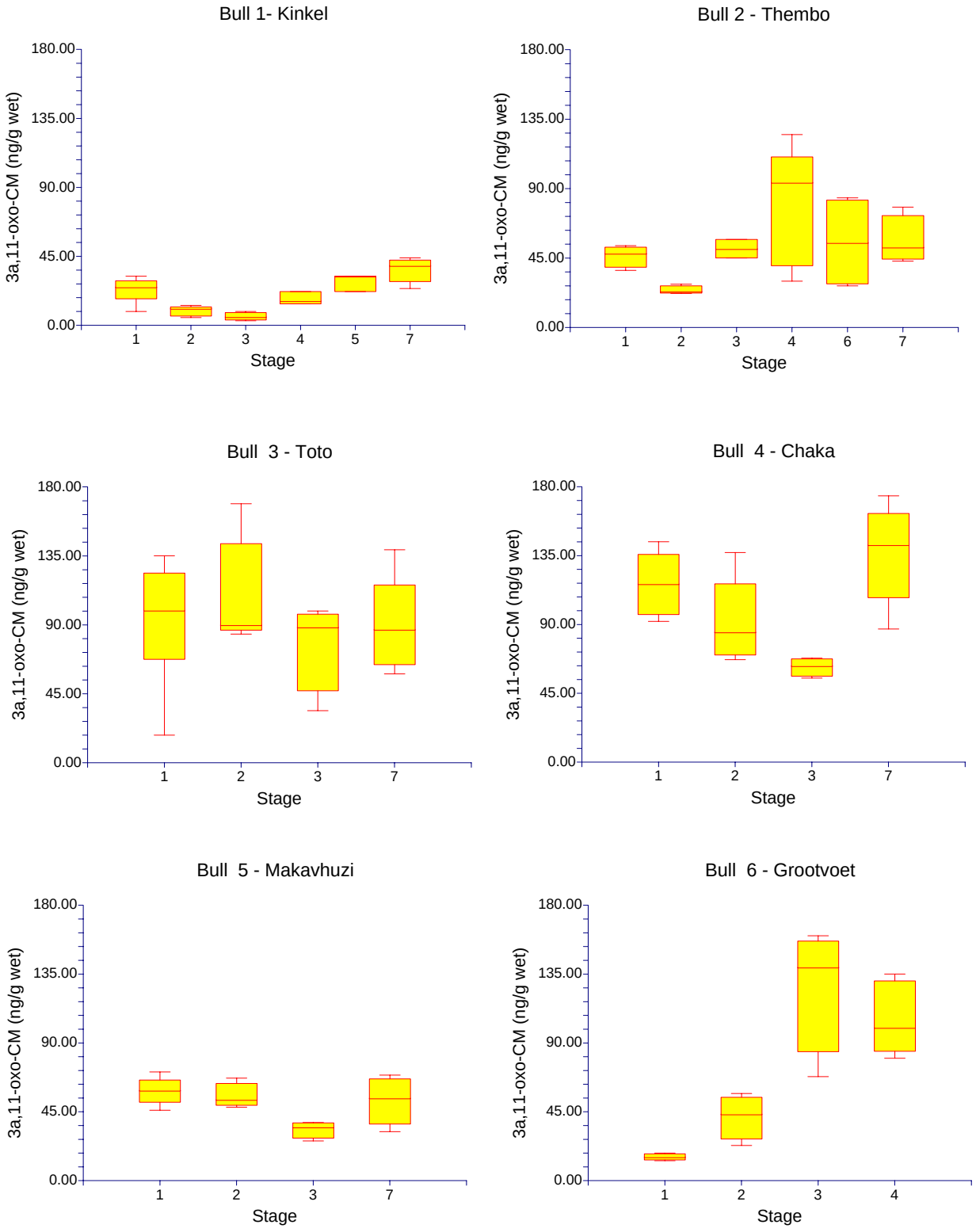

Fig. 3: Grouped concentrations of faecal 3α,11oxo-cortisol metabolites for each of the 6 bulls. The boxes represent the median and the upper and lower quartile values of samples collected at different stages of vaccination. The whiskers show the range.

4.3 GnRH antibody titres

GnRH antibody titres from Chaka, Makavhuzi and Toto are represented as absorbance values in Fig. 4. The absorbancies clearly show positive antibody titres in response to the vaccination.

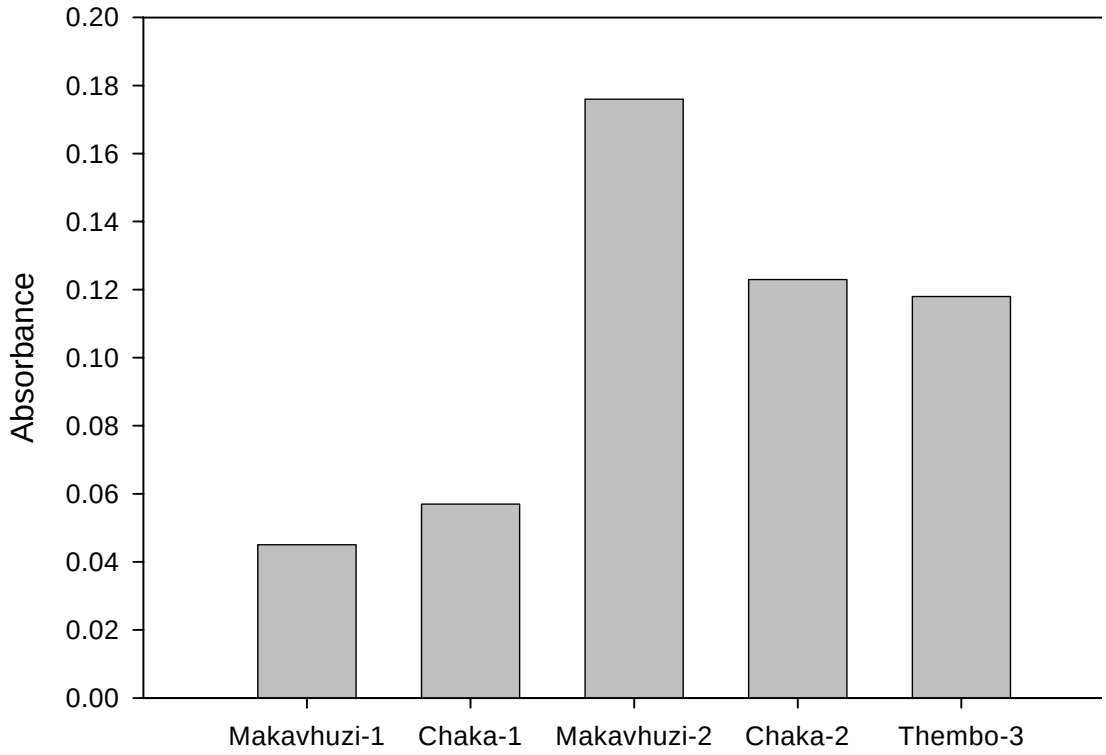

Fig. 4: Results (absorbance) from ELISA for GnRH antibody titres in blood samples from Makavhuzi, Chaka and Thembo (1: Before vaccination I; 2: 4 months after vaccination III; 3: 6 weeks after vaccine II).

4.4 Behavioural observations

Frequencies of aggressive behaviour during the different stages of vaccination are represented in Fig. 5 for Kinkel, Thembo, Toto, Chaka and Makavhuzi. This could not be performed for Grootvoet because data were limited.

4.4.1 Kinkel

- Non-musth state throughout the study.
- Highly aggressive before the first vaccination. Aggressive behaviour was directed towards the cow. Fig. 5 shows that aggressive behaviour towards the cow reduced after the fourth vaccination. The bull was still in a non-aggressive state 4 months after the last vaccination.
- Temporal gland secretion was relatively abundant before the first vaccination, decreased after the first, second and third vaccination and ceased after the fourth vaccination.
- No important changes in main activities and physical condition of the bull were noticed throughout the study.
- The cow was submissive and avoided the bull. No avoidance was observed after the fourth vaccination and the two animals were often seen close to each other.

4.4.2 Thembo

- Non-musth state during Stages 1, 2, 4, 6 and 7. The bull was not in musth at the beginning of Stage 3 before he broke out of the reserve. Information was insufficient to determine if he entered musth after he broke out until his recapture.
- Highly aggressive towards rhinoceros and objects during Stages 1 and 2. At the beginning of Stage 3, the bull broke one of his tusks close to the base, which resulted in the formation of an abscess. Aggressive behaviour towards rhinoceros and objects seemed to be increased during this stage. After he broke out, he was seen with other elephants and caused a lot of damage to the lodges in neighbouring reserves until he was captured 6 weeks after the second vaccination. The abscess was treated while he was under anaesthesia. Fig. 5 shows that no aggressive behaviour was observed after the third vaccination. From then onwards the bull was very relaxed.
- Temporal gland secretion was present during Stages 1 and 2 and ceased after the third vaccination.
- No noticeable changes in physical condition were observed.

4.4.3 Toto

- Non-musth state during the entire study
- Non- aggressive during the entire study.
- Submissive towards Chaka and Makavhzi.

- No changes in main behaviours and physical condition were observed.

4.4.4 Chaka

- Non-musth state during the entire study. Sporadic musth signs (urine dribbling and temporal gland secretion) were observed 8 months after the third vaccination. At the time he also attempted to mount the cow. Chaka came into full musth 10 months after the last vaccination and showed aggressive behaviour towards rhinoceros and people.
- Non-aggressive until 10 months after the last vaccination.
- Submissive towards Makavhuzi.
- No other changes in main behaviours and physical condition were observed.

4.4.5 Makavhuzi

- Non-musth during the entire study.
- Non-aggressive during the entire study.
- Dominant over the two other bulls. Aggressive behaviours shown in Fig. 5 represent sporadic threatening behaviours directed at Toto and Chaka.
- No changes in main behaviours and physical condition were observed.

4.4.6 Grootvoet

- Had been in musth for the last 6 months before the start of the study (temporal gland secretion, urine dribbling, strong urine odour and aggressive behaviour directed towards people and objects). Went out of musth 10 days after the first vaccination.
- Aggressive behaviour ceased completely when he went out of musth.
- No other changes in main behaviours and physical condition were observed.

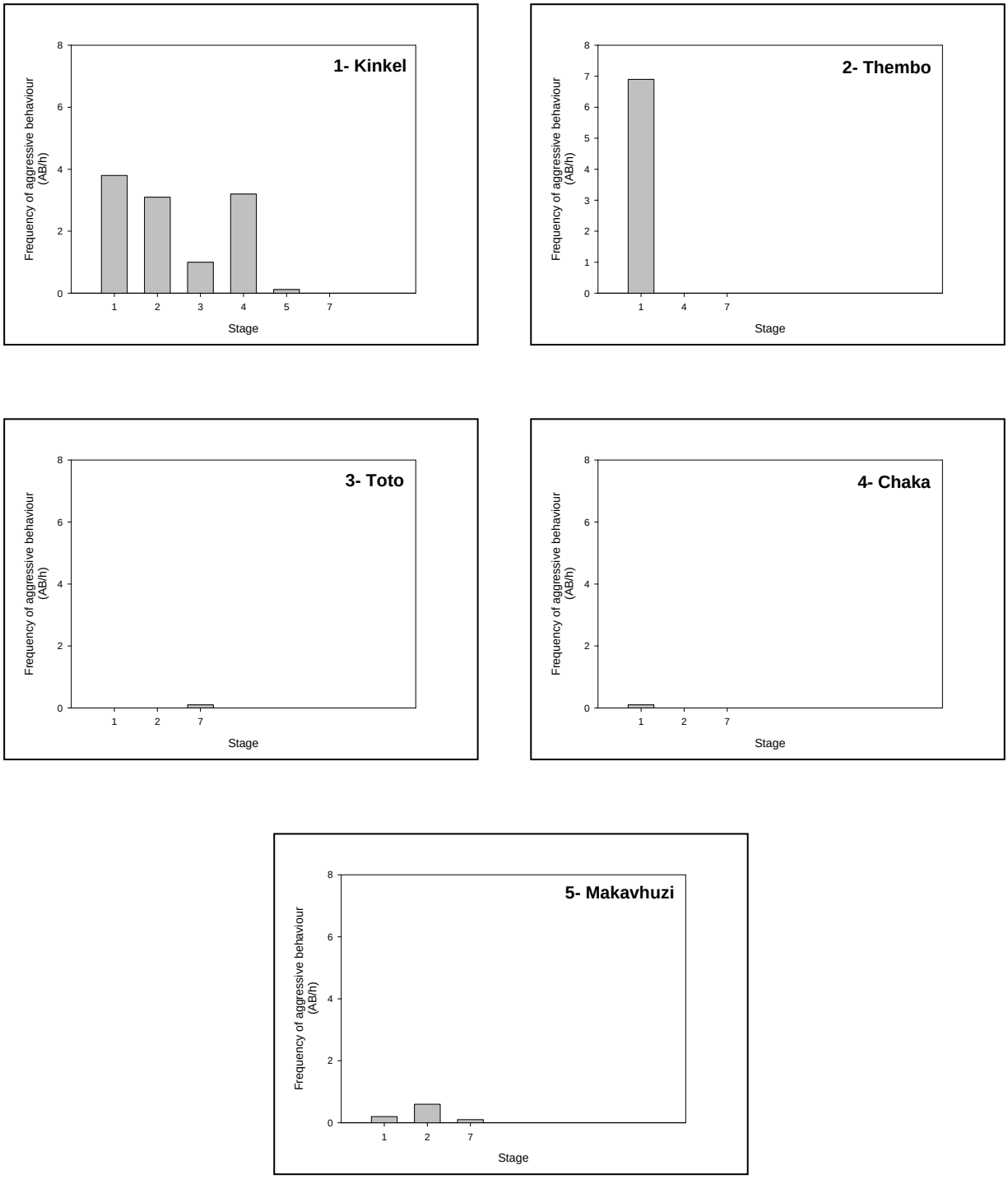

Fig. 5: Frequency of aggressive behaviour as a function of the stage of immunization for Kinkel, Thembo, Toto, Chaka and Makavhuzi. (Stage 1: before vaccination I; 2: after vaccination I; 3: after vaccination II; 4: after vaccination III; 5: after vaccination IV; 7: 4 months after last vaccination). AB/h = number of aggressive behaviours per hour.

CHAPTER 5
DISCUSSION

5.1 Effect of GnRH immunization on androgen secretion and behaviour

Immunization of African elephant bulls against GnRH was expected to suppress androgen secretion after the second or third vaccination. The suppression was expected to be maintained for at least four months after the last booster vaccination. These expectations were based on the patterns of testosterone secretion observed after successful immunization in domestic animals. In young pony stallions immunized with the same vaccine formulation, for instance, the individuals responding properly showed decreased testosterone secretion after two vaccinations (Stout and Colenbrander 2004). The concentrations remained suppressed for 4-5 months. When the pooled data from the six bulls was analysed by repeated measures ANOVA, no effect of vaccination on faecal epiandrosterone levels could be found. Faecal epiandrosterone levels were highly variable between the bulls, however, and Kinkel and Thembo presented initial epiandrosterone levels 1.5 to nearly 3 fold higher than the other bulls. The number of bulls studied was also small. Besides, individual bulls can be expected to react differently or show different degrees of response to GnRH immunization as has been observed in studies on different species (Dalin *et al.* 2002; Lincoln *et al.* 1982; Malmgren *et al.* 2001; Meloen 1995; Miller *et al.* 2000; Schanbacher 1984; Stout *et al.* 2003; Turkstra *et al.* 2002). For the above reasons, individual responses to the vaccine could be masked if data is grouped and analysed altogether. In view of this, the epiandrosterone results were analysed on an individual bull basis.

Kinkel showed no clear pattern as far as a decrease in epiandrosterone levels is concerned taking all the stages of immunization into consideration. There was some doubt, however, as to whether the delivery of the first three vaccinations was complete. Montanide ISA was used as adjuvant for these vaccinations. When emulsified with vaccine it produced a highly viscous mixture, which was expelled with difficulty by the dart. In all probability the bull did not receive the full dose during the first three vaccinations. Accordingly we decided to change to the Covaccine adjuvant for the fourth vaccination. This adjuvant produced an emulsion with the vaccine that was easily expelled from the dart. Subsequently all vaccinations were carried out using the Covaccine adjuvant. When only Stages 1, 5 and 7 were compared, a significant

decrease was observed during Stage 7 compared to Stage 1 (DF = 2, F = 6.94, p = 0.011) (Fig 6). Ganswindt *et al.* (2004a) found that the presence of temporal gland secretion correlates with moderately elevated epiandrosterone levels, which was the case in this bull. Aggressive behaviour decreased after the fourth vaccination and temporal gland secretion ceased, supporting the hormonal changes observed. A decrease in aggressive behaviour occurred two weeks after the fourth vaccination. At that time, androgen secretion was not yet significantly decreased, but Fig. 6 shows that it was already declining.

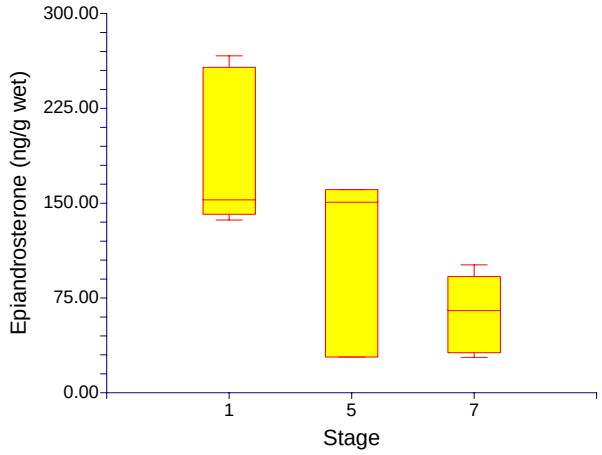

Fig. 6: Grouped faecal epiandrosterone levels observed in Kinkel during Stages 1, 5 and 7.

Thembo showed a significant decrease in androgen secretion after the third vaccination and four months later it was still low compared to Stage 1. The response corresponded to the expected pattern. The third vaccine, however, was administered after the bull had been immobilised with etorphine and azaperone for treatment and relocation. Anaesthesia with etorphine could also have altered gonadotrophin secretion for at least 1-2 days. *In vitro* studies on pituitary cells have shown inhibitory effects of opiates, including etorphine, on gonadotropin secretion (Blank *et al.* 1986; Stojilkovicacute *et al.* 1987). This suggests that *in vivo* exposure to exogenous opiates may suppress reproductive function at the hypophyseal level. The stress of capture could also have affected testosterone secretion. Stress has been found to decrease pulsatile release of GnRH and thus testosterone secretion in many species (see review by Knol (1991)) (Katsiia *et al.* 1989; López-Calderón *et al.* 1991; Norman 1993). To have a significant and prolonged effect, however, stress needs to be chronic. Rises in testosterone have also been observed in some instances (Armario and Castellanos 1984) but most frequently the hypothalamus-pituitary-testis axis is depressed (Knol 1991; Welsh *et al.* 1998). This can occur through inhibition at various levels of the axis. Brown *et al.* (1993)

found basal testosterone concentration of a musth bull to be reduced two days after capture. Sensitivity to anaesthesia and capture seems to be specific to musth bulls since pituitary-testicular function showed no significant change after repeated anaesthesia and capture of non-musth bulls. In contrast, repeated immobilisations of a captive Asian bull in musth did not suppress signs of musth (Kock and Kock 1984). Androgens were not monitored in this bull. Effects of immobilisation on musth are thus unclear. Observations on Thembo could not be performed between the first and second boosters as he broke out of the game reserve. At the time of capture, serum testosterone concentration (7.82 ng/ml) as well as the absence of physical musth signs suggested that he was not in musth during that period. Although the suppression of testosterone secretion by capture cannot be excluded, the fact that the androgen levels remained low thereafter indicate that it was due to the vaccine. Moreover, the measurement of antibody titres clearly showed an antibody response to the vaccine in Thembo. The change in the bull's environment after the second booster made the interpretation of behavioural changes difficult. It is possible, for instance, that the change from free-ranging to being enclosed in a boma in isolation, with no rhinos to compete with at the feeding site, and receiving continuous attention from people, induced an improvement in behaviour. Nevertheless, the bull did not show any signs of irritability and was extremely relaxed compared to the stages before the final vaccination. We attribute this to hormonal changes. Like Kinkel, Thembo's temporal gland secretion ceased when epiandrosterone levels decreased after the last vaccination. The pain caused by the abscess in the broken tusk most likely contributed to increased irritability and aggressiveness observed at the beginning of Stage 3. Bulls with tusk abscesses are known to be irritable and aggressive (Steenkamp, personal communication).

The faecal androgen levels of Toto decreased after the second vaccination. Four months after the last vaccination, however, they were similar to initial levels. Due to circumstances, this bull was only vaccinated twice. This could explain the short duration of the response.

No changes in androgen secretion were observed after GnRH immunization in the three remaining bulls. Serum testosterone concentrations in Chaka and Makavhuzi also did not change significantly after treatment. The antibody titres measured four months after the last booster vaccination were, however, 2-fold and 4-fold higher compared to before the primary vaccination in Chaka and Makavhuzi respectively. This shows that the bulls developed antibodies to GnRH and these lasted at least four months after the last booster vaccination.

The slightly higher serum testosterone concentration of 10 ng/ml observed in Makavhuzi after the first vaccination was not reflected in the faecal samples. A number of species have been shown to exhibit several peaks of testosterone per day and in Asian elephants short-term fluctuations of serum testosterone concentrations with occasional peaks have also been described (Lincoln and Ratnasooriya 1996). The concentration of 10 ng/ml may have been such a peak in Makavhuzi.

The limitations of this study, due to the small number of bulls, some inconsistencies in the vaccination protocols and the difficult circumstances surrounding behavioural observations of two bulls, make the interpretation of results within and between bulls difficult. Similarly to studies in other species, however, there seemed to be a marked individual variation in the response to GnRH vaccination. In most studies, an adequate response failed to occur in 100 % of treated animals (Meloen 1995). It seems that a certain number of non-responders can be expected (Stout *et al.* 2003; Zeng *et al.* 2001; Zeng *et al.* 2002a; Zeng *et al.* 2002b). The reasons for individual variations in response to GnRH immunization are not completely understood. Age has clearly been shown to influence the results with older animals producing greater individual variations, less marked responses and shorter duration of effects. The modified GnRH-tandem-dimer used in this study has been shown to suppress testosterone production completely in domestic pigs (Oonk *et al.* 1998), Chinese pigs (Zeng *et al.* 2001; Zeng *et al.* 2002a), blackbuck, springbok, goats (Turkstra *et al.* 2001) and horses (Stout *et al.* 2003; Stout and Colenbrander 2004; Turkstra 2003), but satisfactory responses with reduction of testosterone concentrations to castrate levels were mostly obtained in young animals. In general, more vaccinations are required to obtain a response in older animals. In piglets, two vaccinations reduced testosterone concentrations to undetectable levels efficiently (Oonk *et al.* 1998) whereas four immunizations were necessary to induce some degree of response in 3-5 year-old boars (Turkstra 2003). Two vaccinations were also effective in nearly mature Chinese pigs (Zeng *et al.* 2001). In immunized blackbuck and springbok, testosterone levels were reduced in young rams but not in adults (Turkstra *et al.* 2001). The modified GnRH-tandem-dimer vaccine combined with Covaccine adjuvant was also tested in horses (Stout and Colenbrander 2004). Two vaccinations were sufficient to suppress testosterone production in young sexually mature pony stallions but further boosters were generally needed in older stallions (Stout *et al.* 2003; Stout and Colenbrander 2004). The bulls in our study varied in age. The three responding bulls happened to be young bulls of 18 - 22 years old and were the youngest animals in the study. Two of the older bulls were shown to produce antibodies, but

the titres may have been too low to induce a response at the testicular level. As in horses (Stout *et al.* 2003), a higher number of boosters may be necessary before a response in the older bulls is seen. Changing the dose of the vaccine could also influence the results. Zeng *et al.* (2002) observed a dose related effect on antibody titres, testis size and serum testosterone but not on serum LH levels in Chinese pigs. Oonk *et al.* (1998) noticed that a very low dose resulted in an increased percentage of non-responders while Finnerty *et al.* (1994) showed an effect of the dose on the extent and duration by which testosterone concentrations in bull calves were reduced. In cats (Levy *et al.* 2004), however, there was no dose effect on antibody titre, testosterone levels and the number of non-responders. Similar results were seen by Ladd *et al.* (1990) in rats where the magnitude of the immune response was not dose-dependent as long as the threshold dose had been exceeded.

There are considerable variations between species in response to specific vaccines (Ferro *et al.* 2004; Ladd *et al.* 1994; Meloen 1995). These could be due to differences in the immune systems (Ferro *et al.* 2004). Dose of antigen, number of boosters, timing of immunizations as well as factors such as carrier protein and adjuvant used can influence the success of immunization (Enright 2003). In African elephant bulls, further work is needed to determine the optimal vaccine formulation and protocol in order to obtain a strong and more consistent response. Species-specific characteristics of the hypothalamic-pituitary-gonadal system may also cause differences in response to GnRH immunization. Very little is known about the hypothalamic-pituitary-gonadal system in elephants. Brown *et al.* (1993) observed differences in the response to GnRH agonists in African elephants compared to some other species. Chronic treatment with a GnRH agonist initially stimulates and then suppresses basal LH and testosterone secretion in elephants, whereas it increases basal testosterone secretion in beef bulls. In elephants, the pituitary is still responsive to exogenous GnRH 20 days after agonist treatment and the testes become hyper-responsive to LH, but this is only apparent after GnRH treatment. This supports the hypothesis that there may be important physiological difference between elephant and cattle, which influence the response to the GnRH vaccine.

Species specific endocrine characteristics of elephants might not only influence the response to GnRH vaccination but also the faecal hormonal changes. There seemed to be no changes in epiandrosterone levels in Chaka, Makavhuzi and Grootvoet, but no conclusions can be drawn with certainty given the lack of knowledge on the reproductive physiology of elephant bulls. One possible explanation is that changes in testosterone levels are masked by adrenal

androgen secretion. In humans, as well as in laboratory and domestic species, the main androgens secreted by the adrenal cortex are dehydroepiandrosterone, dehydroepiandrosterone sulphate and androstenedione (Odell and Parker 1984; Smikle 1999); all can be converted to testosterone. Adrenal androgen secretion seems to be partly controlled by ACTH but also by other ACTH-independent factors (Gonzalez 1999; Odell and Parker 1984). This is based on the lack of association between adrenal androgen and cortisol secretion sometimes observed. In humans, Georgiadis *et al.* (1992) found no correlation between adrenal and testicular androgen production. This showed that the regulation of androgen production in the adrenals and testes is controlled by different factors. Initial epiandrosterone levels in Chaka, Makavhuzi and Grootvoet were low. If testicular testosterone secretion were lower than adrenal secretion, variations in testicular secretion would be difficult to detect, especially when using a method of monitoring that nullifies fluctuations. In addition, the values recorded were highly variable within a stage and the number of samples per stage was small, which would have increased the difficulty of detecting differences between stages. A clear decrease in androgen secretion in response to vaccination would more likely be observed in bulls with high initial androgen levels. Fig. 2 shows that Thembo and Kinkel had higher initial androgen levels than the other bulls. This may be why the decrease in androgen secretion was clear in these bulls. Adrenal androgens in humans (Gonzalez 1999; Smikle 1999), chimpanzees (Cutler *et al.* 1978), dogs (Cutler *et al.* 1978; Schiebinger *et al.* 1981) and rabbits seem to increase with sexual maturation and are consequently lower in young individuals (see below). Toto was younger than Chaka, Makavhuzi and Grootvoet and his adrenal androgen contribution may have been less, allowing changes in testicular testosterone secretion to be more accurately detected. The fact that none of the responding bulls reached undetectable epiandrosterone levels is consistent with the idea that significant amounts of androgens are secreted by a source other than the testicles, like the adrenal cortex, and that maintained baseline levels were independent of testicular secretion. Values in animals are generally lower than in humans but there is a high variability in adrenal androgen levels among species and secretory patterns throughout life (Cutler *et al.* 1978; Gonzalez 1999). In humans, adrenal androgens rise in late childhood, are maximal during the third and fourth decade of life and then decrease again (Gonzalez 1999; Smikle 1999). A similar pattern is observed in chimpanzees (Cutler *et al.* 1978). In dogs and rabbits, an increase after sexual maturation is also observed, but levels are lower than in man (Cutler *et al.* 1978; Schiebinger *et al.* 1981). Adrenal androgen secretory patterns need to be investigated in elephants.

The results seen in Grootvoet were surprising. The bull went out of musth 10 days after the first vaccination although no changes in epiandrosterone levels were observed. A blood sample collected 3 months before the first vaccination, when the bull was in full musth, had a testosterone concentration of 44.04 ng/ml, which is characteristic of musth. This was reflected in the epiandrosterone concentrations in a faecal sample (209.235 ng/g wet) collected at the same time. The week before the first vaccination, androgen secretion had already decreased. Faecal epiandrosterone levels were approximately 3-fold lower (65.53 ± 19.31 ng/g) and similar ($p > 0.05$) to those observed for Makavhuzi (100.53 ± 37.16 ng/g), Chaka (63.79 ± 26.7 ng/g) and Toto (125 ± 56.15 ng/g), all of which were non-musth non-aggressive bulls. Faecal epiandrosterone levels did not change significantly during the rest of the observation period in Grootvoet. This suggests that they were back to baseline levels. The bull was still showing full musth until 10 days after the first vaccination, including aggressive behaviour and urine dribbling. This is surprising because Ganswindt *et al.* (2004a) reported that urine dribbling stopped either before or co-incident with the end of elevated androgen levels in all of the eight animals they studied. Information on the temporal relationship between testosterone secretion and musth signs is limited however, and we cannot exclude the possibility of different patterns in different bulls. It is possible that in Grootvoet the first immunization coincided with the natural end of musth, at least as far as androgen levels are concerned. Musth signs persisted for a few weeks after the end of elevated androgen levels. In addition, epiandrosterone levels were low before vaccination and, as discussed previously, the effect of immunization could therefore have been masked.

The results of this study show that androgen levels in non-musth aggressive animals are higher than those in non-aggressive animals. The two non-musth aggressive animals also displayed temporal gland secretion. This is consistent with the results of Ganswindt *et al.* (2004a) who found that the presence of temporal gland secretion without urine dribbling is associated with moderate elevations in epiandrosterone levels and the likelihood of aggression. As proposed in other studies (Dickerman *et al.* 1997; Ganswindt *et al.* 2004a; Rasmussen *et al.* 1984), these results suggest that androgens do promote aggressive behaviour in elephants.

The most significant finding of the current study was the effect of the GnRH vaccine on behaviour. At least two bulls (Kinkel and Thembo) showing aggressive behaviour prior to treatment, and possibly a third (Grootvoet), showed substantial improvement (Fig. 5).

Temporal gland secretion also ceased. Aggressive behaviour did not recur within the 4-month observation period after the final booster. As expected, the non-aggressive bulls (Toto, Chaka and Makavhuzi) showed no change in behaviour. None of these three captive bulls showed aggressive behaviour until 10 months later when Chaka came into musth. The lack of aggressive behaviour is in itself a positive finding and may well be a consequence of the GnRH antibodies. Vaccination *per se* did not cause aggression or disturbance in any of the bulls and no other side effects were observed.

5.2 Androgen and corticosteroid secretion

The hypothalamo-pituitary-adrenal and hypothalamo-pituitary-gonadal axes influence each other. The causal link between these two endocrine systems differs from species to species (Welsh *et al.* 1998). As discussed previously, stress inhibits the hypothalamo-pituitary-gonadal axis in most species. Results of studies on yellow-pine chipmunks (Place 2000) and rats (Viau and Meaney 2004) showed that high androgen levels can lead to reduced corticosteroid secretion. Ganswindt *et al.* (2004b) reported similar observations in African elephant bulls where high androgen levels during musth seemed to suppress cortisol secretion. On the other hand, in dark-eyed juncos, artificial elevation of plasma testosterone was found to increase plasma corticosterone (Klukowski *et al.* 1997).

In our study, a link between androgen and cortisol secretion was investigated by comparing patterns of excretion of faecal cortisol and androgen metabolites. In the bulls showing significant reductions in androgen levels after immunization (Kinkel, Thembo, Toto), the changes did not significantly correlate with changes in cortisol secretion. Hence it seems that reduced androgen secretion after immunization did not affect cortisol secretion. Patterns of secretion in the musth bull (Grootvoet, Fig. 7) were more consistent with the findings of Ganswindt *et al.* (2004b). Faecal samples collected during musth (three months before the primary vaccination and during Stage 1) contained low levels of cortisol metabolites. The concentrations started increasing after the primary vaccination (Stage 2), which is also when the musth signs disappeared. Faecal cortisol metabolite concentrations were significantly higher after the second and third vaccination. Cortisol secretion was thus reduced when the bull was in musth and, as in 40 % of the cases studied by Ganswindt *et al.* (2004b), remained low for a few weeks after the end of elevated androgens. The mechanisms by which

androgens inhibit cortisol secretion during musth are unknown. In a study on rats, Viau and Meaney (2004) suggested that high androgen levels suppress glucocorticoid levels by reducing plasma and intra-pituitary corticosteroid-binding globulin. This would result in increased glucocorticoid negative feedback on ACTH release. Klukowski *et al.* (1997) found that exogenous testosterone increases plasma corticosterone in dark-eyed juncos, but the mechanisms of action also seemed to involve changes in plasma corticosteroid-binding globulin levels. The same precursors are used for androgen and corticosteroid synthesis (Cheesman 1982). Consequently, limitation of precursors could be an alternative reason for reduced corticosteroid production during musth. No proof of this hypothesis or report against it, however, was found in the literature.

In Kinkel, where only Stages 1, 5 and 7 were taken into consideration, there was also no significant correlation between androgen and cortisol secretion (R_{14} = -0.353, p = 0.21). Faecal 3α,11oxo-CM concentrations were, however, significantly higher 4 months after the fourth vaccination compared to before the primary vaccination (DF = 2, F = 3.96, p = 0.05) while the opposite was observed for faecal epiandrosterone (Fig. 8). Even without a significant correlation, the pattern observed does not rule out an inhibitory effect of high androgen levels on cortisol secretion.

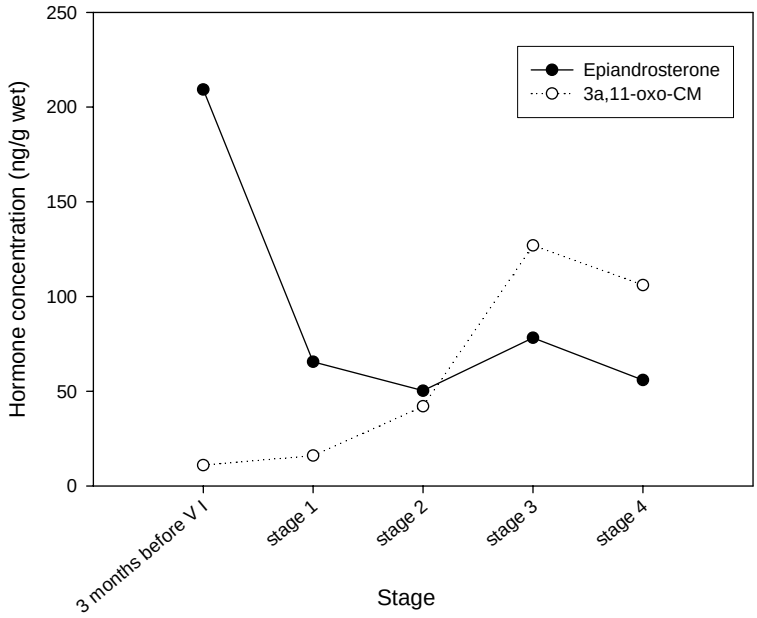

Fig. 7: Mean faecal epiandrosterone and 3α,11oxo-CM concentrations during the different stages of vaccination in Grootvoet.

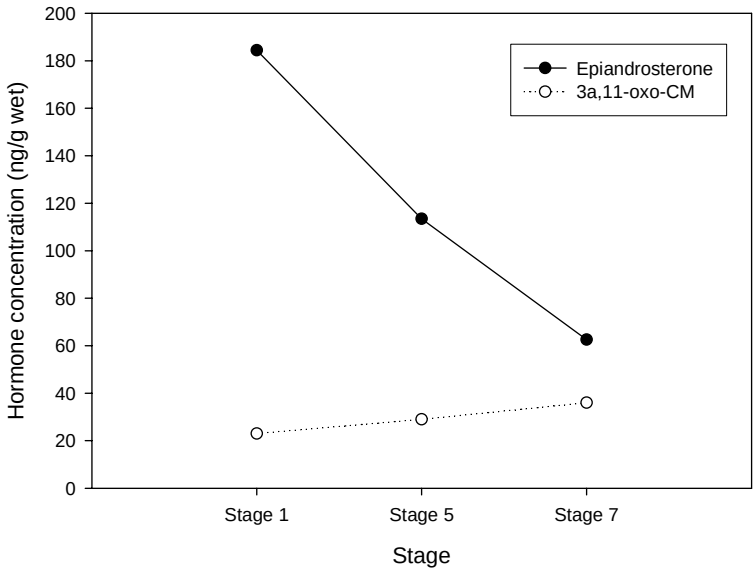

Fig. 8: Mean faecal epiandrosterone and 3α,11oxo-CM concentrations during the Stages 1, 5 and 7 in Kinkel.

In Thembo, the increased faecal 3α,11oxo-CM concentrations observed during Stage 4 as well as the presence of higher peaks of cortisol thereafter (Stages 6 and 7, Fig. 3) may reflect stressful events related to the bull's training and recent change of environment. The bull was captured and moved to a boma two weeks before Stage 4. As discussed previously, stress could have influenced androgen secretion negatively. On the other hand, the bull was extremely relaxed after been moved to the boma.

A positive correlation between the metabolites was observed in Chaka (Fig. 9) and Makavhuzi (Fig. 10). An adrenal origin of the faecal androgens measured in these bulls might explain this observation. As discussed previously, adrenal androgen secretion seems to be partly modulated by ACTH. It would not, therefore, be surprising therefore to sometimes observe coupled androgen and cortisol secretion. If the positive correlation observed in these bulls was due to cross-reactivity of the 11-oxoaetiocholanolone EIA with androgen metabolites, positive correlations would also have been observed in the other bulls, which is not the case. Moreover, Ganswindt *et al.* (2003) showed that the 11-oxoaetiocholanolone EIA used (described by Möstl *et al.*, 2002) does not cross-react significantly with testosterone metabolites in African elephant bulls.

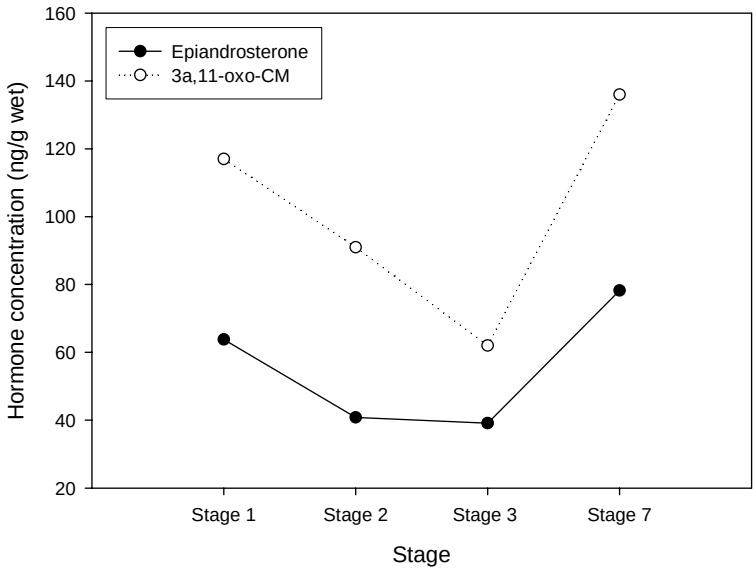

Fig. 9: Mean faecal epiandrosterone and 3α,11oxo-CM concentrations during the different stages of vaccination in Chaka.

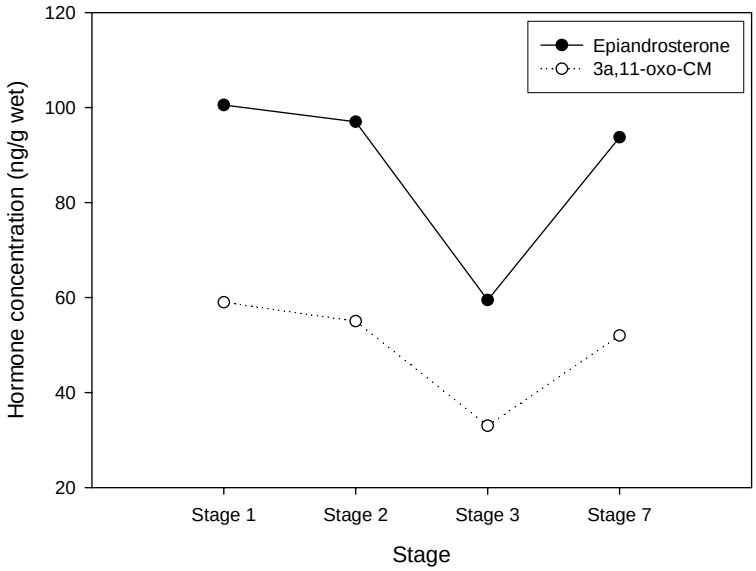

Fig. 10: Mean faecal epiandrosterone and 3α,11oxo-CM concentrations during the different stages of vaccination in Makavhuzi.

CHAPTER 6

CONCLUSIONS

This study suggests that the GnRH vaccine used (modified GnRH-tandem-dimer-ovalbumin conjugate, Pepscan Systems, Netherlands) can alter testosterone secretion and improve behaviour in aggressive African elephant bulls. The vaccine can be delivered remotely by means of drop-out darts, which means that the administration can be carried out under field conditions. Although the number of animals studied was small and a great variation in response between individuals was observed, the results are promising withregard to the use of this vaccine to control musth and aggressive behaviour.

Results on androgen and cortisol secretion suggest that a substantial amount of androgens may be secreted by the adrenal glands, and that this may have influenced the results.

Reduced androgen secretion after immunization does not seem to influence cortisol secretion. Our results support the idea that cortisol secretion is suppressed by high androgen levels during musth in African elephants.

This study also supports the positive role of androgens in aggressive behaviour of African elephant bulls and demonstrates that aggressive behaviour in combination with temporal gland secretion is associated with moderate elevations in androgen levels. At least in one bull, it was observed that musth-like urine dribbling could persist for a few weeks after the end of elevated androgens.

Finally, this study found no evidence of adverse side effects due to GnRH immunization in elephant bulls, indicating that GnRH imuunization could constitute a safe way to suppress aggressive behaviour and/or musth.
Further research is needed, to investigate whether complete suppression of testosterone secretion, and more consistent results, can be achieved. It may be that changes to the vaccination protocol will solve the problems. Moreover, longer-term observations and vaccination of additional bulls are necessary before final conclusions can be drawn on the safety and reversibility of the vaccine.

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APPENDIX 1

Data sheet (1) used to record aggressive behaviour and musth in African elephant bulls

Date…/…/….. Time start ….:…. Location: …….
Time stop ….:…. Animal: ……..

Behavioural traits of aggression, dominance or musth	TIME					
	1 hour		2 hours		3hours	
(1) Head-high						
(2) Chin-in						
(3) Head-shake						
(4) Head-jerk						
(5) Head-toss						
(6) ear-spreading						
(7) Ear-folding						
(8) Ear-flapping						
(9) Ear-wave						
(10) Ears-tense						
(11) Musth-walk						
(12) Forward-trunk-swing						
(13) Tusk-ground						
(14) Turn-towards						
(15) Intensive-look						

Date…/…/….. Time start ….:….
Time stop ….:….

Behavioural traits of aggression, dominance or musth	TIME 1hour	2 hours	3 hours
(16) Look-back			
(17) Advance-towards			
(18) Run-after/chase			
(19) Bow-neck			
(20) Mock-charge			
(21) Real-charge			
(22) Pursuit			
(23) Escalated contest			
(24) Dueling			
(25) Pushing			
(26) Kick			
(27) Trunk-whip			
(28) Tusking			
(29) Throw-debris			

Date…/…/….. Time start ….:….
Time stop ….:….

Behavioural traits of aggression, dominance or musth	TIME 1 hour	2 hours	3 hours
(30) Redirected			
(31) Bush-bash			
(32) Marking			
(33) Urine dribbling			
(34) Snort			
(35) Rumble			
(36) Trunk over back			
(37) Tusks over back			
(38) Push-drive			
(39) Submission/fear from other elephant			
Main activity			
Distance from neighb			
Distance from people			

APPENDIX 2

Definitions of behavioural traits used to record aggressive behaviour in African elephant bulls

HEAD-HIGH (Poole and Granli 2003)
Standing or moving with head held well above shoulders, chin is tucked in, elephant gazes at an adversary with Eyes-Open and ears maximally forward or Ear-Spreading. The animal appears to increase in height and sometimes will deliberately stand upon an object such as a log or anthill in order to increase its height - a tactic often used by males when they are sizing one another up. Elephants normally stand or move about with their eyes cast down. A direct gaze with Eyes-Open is a component of many displays. This posture is used primarily to threaten other elephants.

CHIN-IN (Poole and Granli 2003)
A male in musth standing or walking with his head held high, above his shoulders, and with his chin or jaw tucked in. Chin-In, Head-High and Ears-Tense are jointly components of the Musth-Walk.

HEAD-SHAKE (Poole and Granli 2003)
An abrupt shaking of the head, which causes the ears to flap sharply and dust to fly. It is a sign of an individual's annoyance with or disapproval of an individual or circumstance. The Head-Shake usually starts with the head twisted to one side; the head is then rapidly rotated from side-to-side. The ears slap against the side of the face or neck making a loud smacking sound. Can also be used in play to feign annoyance.

HEAD-JERK (Poole and Granli 2003)
A small single rapid upward movement of the head that then returns down slowly. In a more pronounced version of the Head Jerk the head is first lowered and then pulled up sharply so that the tusks describe a wide arc. This display is often observed as elephants crash through bushes to make a dramatic display to an adversary or predator. This display is often seen during play when Bush-Bashing.

HEAD-TOSS (Poole and Granli 2003)
As performed by a musth male raising and lowering the head or lifting and swinging head and trunk with vigor, sometimes in figure-eight movement. This display is occasionally seen in combination with a Trunk-Curl. In its most intense form the elephant bends his back legs and lowers the hind portion of his body causing the head and trunk to be raised even higher. Less exaggerated forms may be observed in non-musth elephants. The Head-Oscillation may be a threat display often directed at human observer or toward an elephant adversary.

EAR-SPREADING (Poole and Granli 2003)

EAR-FOLDING (Poole and Granli 2003)
Forcing the lower half of ear under and back so that a prominent horizontal ridge or fold appears across the ear. This aggressive display may be used in combination with a variety of other threats such as Head-High, Looking-At, Advance-Toward, etc. to emphasize that an elephant "means business".

EAR-FLAPPING (Poole and Granli 2003)
Elephants may flap their ears hard against the body in aggressive circumstances in association with Ear-Folding and/or Bow-Neck.

EAR-WAVE
At irregular intervals, the inner and upper portion of one ear is waved forcefully forward allowing the lower and outer portion to follow behind (Poole 1987c). This creates a wave diagonally across the ear. Some males seem to prefer one or the other ear for the ear-wave, while a few individuals sometimes show double ear-waves (Kahl and Armstrong 2002). This ear-wave seems to occur more often during aggressive interactions between males or during musth rumbling and the purpose may be to waft the scent from the temporal glands towards a rival (Poole 1987c).

EARS-TENSE (Poole and Granli 2003)
A male in musth standing or walking with his head held high, above his shoulders, and with his chin or jaw tucked in. Chin-In, Head-High and Ears-Tense are jointly components of the Musth-Walk.

MUSTH-WALK (Poole 1987c)
Heads high, well above shoulders, and held at such an angle that chin looked tucked in, ears held tensely, spread and carried high. Walk with a controlled swinging motion to the head and tusks.

FORWARD-TRUNK-SWING (Poole and Granli 2003)
A swinging or tossing of the trunk in the direction of an adversary typically while blowing forcefully out through the trunk. Elephants swing their trunks at other smaller animals (e.g. egrets; ground-hornbills; warthogs, people) to frighten them away or simply for amusement. A high-intensity version of the Forward-Trunk-Swing, the Aggressive-Whoosh is made by musth males, who toss or swing their trunk in an exaggerated manner in the direction of an adversary while blowing loudly through the trunk with a loud "whooshing".

TUSK GROUND (Poole 1987c)
Males get down on their knees and tusk the ground. Sometimes they tusk vegetation and throw bushes and other objects at vehicles or other elephants. This behaviour is always observed during fights between males in musth.

TURN-TOWARDS (Poole and Granli 2003)

INTENSIVE-LOOK

LOOK-BACK (Poole and Granli 2003)
Standing or walking away while looking back over the shoulder. As an elephant Looks-Back it may flatten an ear against its body in order to see what is behind it. This is primarily observed in a retreat situation although depending upon the context and the facial expression it may also be a Threat, as in "Watch it - I can see you!"

ADVANCE-TOWARDS (Poole and Granli 2003)
Purposeful, directed walking toward another with hostile intent that may result in avoidance behaviour or counter-threat. Sometimes all it takes is one step in the direction of the other to

cause a reaction. Advance-Toward may be associated with other aggressive postures such as Head-High, Ear-Spreading, or Ears-Folded etc.

RUN-AFTER/CHASE (Poole and Granli 2003)

BOW-NECK (Poole and Granli 2003)
An aggressor lowers its head by bowing neck downward and simultaneously tilting head upward so that tusks are approximately horizontal. The Bow-Neck may be associated with Ear-Flapping and/or Ear-Folding. This posture may be held at a fast walk or during a Mock-Charge and/or Real-Charge, especially when subject of charge is of smaller stature than the elephant. In a sense the aggressor brings head/tusks down to victim's level.

MOCK-CHARGE (Poole and Granli 2003)
A rushing toward an adversary or predator Standing-Tall and Ear-Spreading that stops short of its target; an elephant may Forward-Trunk-Swing or aggressively Kick-Dust as it abruptly stops. A Mock-Charge is often associated with a shrill Trumpet-Blast.

REAL-CHARGE (Poole and Granli 2003)
Rushing toward a predator or other adversary while Ear-Spreading, head raised or lowered with the apparent intention of following through. The trunk may be tightly curved under so that tusks can make contact first. A Real-Charge is usually silent.

PURSUIT (Poole and Granli 2003)
A persistent, prolonged and aggressive follow at a fast walk by one individual (usually in musth) toward another (usually in musth). A pursuit often follows an escalated contest; when one male has signalled defeat by fleeing the victor initially Runs-After and then Pursues the defeated male for up to several kilometres.

ESCALATED CONTEST (Poole and Granli 2003)
The term Escalated Contest covers all displays that may be seen within the context of a serious battle between two males; in other words in addition to Dueling the suite of displays that occur around and between Dueling bouts. These may include: Dueling, Tusk-Ground, Parallel-Walk, Ear-Folding, Ear-Waving, Musth-Rumbling, Throw-Debris, Bush-Bashing, Trunk-Blocking, Head-Toss, Trunk-Bounce (drag), Trunk-Curl, Run-After, Run-Away and others.

DUELING (Poole and Granli 2003)
Two elephants rushing toward one another, head to head, attempting to gore, tusk, push or interlock tusks to lever an opponent down to the ground or manoeuvre him into a position where he can be gored. Dueling almost inevitably involves two contesting musth males. Dueling males exhibit Head-High, Tail-Raising, Ear-Spreading, Ear-Folding, and Trunk-Blocking (to reduce any blow).

PUSHING (Garai 1997)
Pushing a partner with any part of trunk or the body.

KICK (Garai 1997)
Kicking a partner with a foot.

TRUNK-WHIP (Garai 1997)
Hitting a partner with the trunk.

TUSKING (Poole and Granli 2003)
Poking an opponent with the tip of the tusks. This is a "less polite" form of Pushing another out of the way.

REDIRECTED (Poole and Granli 2003)
Aggression directed toward an individual that is irrelevant to current situation. When the tendency to attack is thwarted, for some reason (e.g. fear of opponent), the individual may redirect his/her aggression to some other animal or object, such as vegetation. This may involve trashing bushes, trees (Bush-Bashing) or throwing sticks, grass (Throw-Debris). Or attacking (Tusk/Turn-Toward/Advance-Toward/Mock-Charge, etc.) other, lesser elephants or smaller animals, humans in the vicinity.

THROW-DEBRIS (Poole and Granli 2003)
Throwing sticks, grass.

BUSH_BASH (Poole and Granli 2003)
Running back and forth through bushes or long grass beating up vegetation. An elephant may lift up vegetation and Throw-Debris or Head-Toss and stare out at "imaginary enemies" while Standing-Tall and Tail-Raising. May charge in Bow-Neck posture or Run-Away in feigned fear.

MARKING (Poole and Granli 2003)
A (usually) musth male rubbing temporal gland against tree trunk or the ground (when mud wallowing). Marking may be so vigorous that the male departs with bark and debris on the side of his face. The dissected temporal glands have been found to have pieces of bark embedded deep inside them. Marking may be seen by non-musth elephants but occurs more often in musth.

URINE DRIBBLING
Males in full musth exhibit a continuous drip of urine (Poole and Moss 1981). The amount of urine discharged can vary from slow discrete drops to a wide stream and changes frequently, depending on the male's activity (Poole 1987c). Urine dribbling can sometimes cease for a while.

MUSTH-RUMBLE (Poole 1987c)
This vocalization is a low pulsating sound of up to 108 decibels with frequencies as low as 14 Hz. Musth rumbles may be performed in association with an ear-wave or an ear-fold. Males in musth rumble under specific contexts like for instance during agonistic encounters with other males, during marking behaviour or prior to copulation.

TRUNK OVER BACK (Garai 1997)
Placing the trunk on a partner's back.

TUSKS OVER BACK (Garai 1997)
Placing the tusks on a partner's back.

PUSH-DRIVE (Garai 1997)
Placing the trunk along the spine of a partner, both animals in locomotion.

SUBMISSION/FEAR FROM OTHER ELEPHANTS
This can be detected by behaviours such as:
GIVE AWAY (Garai 1997)
Moving out of an approaching individual's way or being displaced from a spot without contact.
TURN AWAY (Garai 1997)
Turning away from an approaching partner.
PRESENTING (Garai 1997)
Standing in front of a partner with the posterior or walking backwards towards a partner.

Dominance can be determined by behaviours such as trunk over back, tusks over back, pushing, push-drive and submissive behaviours from other elephants (Garai 1997).

APPENDIX 3

Data sheet (2) used to record aggressive behaviour and musth in African elephant bulls

Date…./…/… Time start…… Location…….
Time stop…… Animal……..

Comments

General impression

APPENDIX 4

Data sheet (3) used to record aggressive behaviour and musth in African elephant bulls

Date…./…/… Time start…… Location…….
Time stop…… Animal……..

Additional information concerning musth

- Temporal gland secretion: - absent – slight – abundant / level 1 – 2 – 3 – 4
  - dark-light:
  - watery – viscous:

- Temporal gland swelling: 1- No swelling
  2- slight swelling
  3- obvious swelling
  4- extreme swelling

- Green penis: - absent – slight – strong

- Odor: - absent – slight – strong

- Urinate: normally / with penis still sheathed

Additional observations

- Physical condition: emaciated – very thin – thin – good – fat – very fat
- Association with female herd – with males – alone:

APPENDIX 5

Data sheet used by rangers to record aggressive behaviour in African elephant bulls

Date …/…/… Location …….

Animal ……...

Aggressive behaviour:

Behavioural traits of aggression	Directed towards people	Directed towards elephants	Directed towards other species	Directed towards objects	Comments
(1) head high- chin tucked in / shaking head					
(2) ear-flapping/waving					
(3) forward trunk swing					
(4) pushing/kicking					
(5) charging/ advance towards					
(6) grabbing, throwing, destroying objects					
(7) tusking					
(8) rumbling					
(9) urine dribbling					

Other signs of musth:

- Temporal gland secretion: absent – slight – abundant
  watery - viscous
- Temporal gland swelling : No swelling- slight swelling – obvious swelling- extreme swelling
- Green penis: absent – slight -strong
- Odour: absent – slight - strong

Additional observations:

- Physical condition: emaciated - very thin – thin – good – fat - very fat
- Association with female herd –with males – alone
- Dominant towards other males- back-off when confronted to other males

Comments