Gonadotropin-Releasing Hormone (GnRH). Production, Structure and Function
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CONTRACEPTIVE APPLICATIONS OF GNRH-ANALOGS AND VACCINES FOR WILDLIFE MAMMALS OF SOUTHERN AFRICA: CURRENT EXPERIENCE AND FUTURE CHALLENGES

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ABSTRACT

Contraception is an essential tool for controlling reproduction in both captive and free-ranging large carnivores. Under captive conditions (e.g., zoos and wildlife sanctuaries), many species including lions, tigers, and cheetahs are prolific breeders; with limited locations to accept surplus animals, careful management of reproduction is critical. Free-ranging lions on smaller fenced reserves in South Africa breed extremely well and, with limited competition from other lion prides or threats from spotted hyena, a population can potentially double every four years. This places intense ecological pressure on prey species, and increases the probability of lions breaking out of reserves and raises the prospect of human vs. wildlife conflict situations. There are a number of sanctuaries in southern Africa which house so-called trained elephants for tourism purposes. A large percentage of these elephant are bulls and, as these animals become sexually mature, circulating androgen levels increase until they develop their first musth cycle. Musth is driven by testosterone so during these periods concentrations of this steroid hormone are extremely high. Typically, raised testosterone levels are associated with increased aggression that can endanger the lives of humans and other animals. GnRH-based treatments have yielded good results in down-regulating androgen production to mitigate testosterone-related behaviour, and could be used to control aggression in captive bulls and even problematic free-ranging bulls in smaller fenced

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reserves. This chapter summarises current contraceptive research among captive and free-ranging mammals in South Africa using GnRH-analogs administered as subcutaneous implants. Application of GnRH-vaccine therapies for contraception in elephants and other mammals is also presented. In addition, veterinary challenges for GnRH-analogs and vaccines in animal control and contraception are discussed.

INTRODUCTION

Until some 50 years ago, wildlife population control was barely considered an issue. As a result of indiscriminate hunting and continued loss of habitat, many species were driven to the limits of survival or even extinction (e.g., Quagga; Smithers, 1983). However in recent decades, attitudes towards wildlife in southern Africa have changed considerably. Wildlife conservation and ecotourism is thriving, and many private game and smaller provincial or national game reserves have been established. To prevent animals from entering surrounding farmlands or human settlements smaller reserves (<60,000 ha) must be fenced. Under these conditions it has become obvious that some species require management. An overabundance of large predators leads to unsustainable losses of prey species or breakouts into surrounding communities, potentially resulting in stock losses or even human fatalities. In contrast, an excess of herbivores can lead to habitat destruction (due to confinement within fenced areas).

Overabundance of wildlife species is not restricted to range countries, but may also occur in zoos throughout the world. For centuries, mammals (and other animals) have been housed in zoos for public viewing, and the practice probably dates to pre-Roman times. Until fairly recently, the primary objective of such institutions was simply to maintain animals as exhibits. During recent years, however, the emphasis has shifted towards conservation and, where possible, *in situ* conservation. Zoos of today seldom buy and sell animals; instead, they typically trade surplus animals for a different species, or the same species if they have a need for genetic diversity. Many mammalian species breed well in zoo environments and, with a limit to the number of institutions that can take excess animals, there is a need to control breeding. Inbreeding in species that breed well in captivity is also a real danger.

So what options are there for population control of large carnivores, given the paucity of available space for both captive and free-ranging animals? Hunting is certainly a possibility, although this option has come under severe criticism during the last few years. The main reason for this has been the practice of ~~trophy hunting, where~~ of hunting an animal ~~is kept~~ in a confined area to increase the likelihood of the hunter obtaining a kill ("canned hunting"). This practice is now banned in South Africa. The abolishment of canned hunts was achieved legislatively in the "National Norms and Standards for Hunting in South Africa" (Government of South Africa, 2011). In any case, trophy hunting of any type is a poor method for population control because it is both highly selective and does not follow the principles of natural genetic selection in free-ranging animals. Since the acquisition of new wildlife areas is unlikely and there is limited space for captive animals, the only remaining options are to slow down the reproductive rate, or to stop animals from breeding altogether.

In southern Africa, over the past 30 years or so, a number of elephant bulls have been captured as calves or subadults, especially during culling operations. Elephants are easy to train in captivity, particularly in groups, and very quickly adopt their keepers as part of their new family.

Normally, the keepers would be regarded as the dominant individuals within such a family. However, bulls in particular, may start to challenge this hierarchy as they get older and approach puberty. Following a number of incidents in South Africa and Zimbabwe, owners soon realised that either androgen-related behaviour needed to be controlled or, as happened to a number of bulls, problem animals had to euthanized. During puberty testosterone concentrations start increasing, causing bulls to become more assertive, difficult to handle, and even aggressive. The ultimate expression of testosterone-driven behaviour in elephant bulls is musth, and bulls in musth are generally impossible to handle—and very often dangerous. Free-ranging African elephant bulls are seldom a problem in larger reserves, but in small reserves the likelihood of encountering a bull in musth is probably increased. Elephants are highly intelligent and quickly realise that 'vehicles' are scared of them and often terrorise people on game drives.

So what role does musth play in the complex social structure of the elephant? The likelihood of a bull mating a cow in estrus is largely dependent on body size and reproductive behaviour. The latter is driven by testicular androgens (mainly testosterone). For elephants, the ultimate reproductive state attributed to very high levels of testosterone is musth (Kaewmanee et al., 2011), and in adults of both species of elephants this occurs in a cyclic pattern once a year; each bull has his own peculiar cycle (Eisenberg et al., 1971; Ganswindt et al., 2010). A thirty-year study which determined the paternity of calves in Amboseli showed that bulls 45 years and older (underscoring the importance of body size) sired 50% of all calves. It also showed that 75% of calves were sired by bulls that were in musth (highlighting the role of androgens) at the time of mating (Hollister-Smith et al., 2007). Musth is thus a normal physiological phenomenon and the aggression and dominant behaviour associated with high testosterone levels increase the chances of a bull mating a cow in estrus. The foul-smelling pheromones present in urine and temporal gland secretion of musth bulls, and which appear from about the age of 35 years, is attractive to cows in estrus although this repels younger bulls (Rasmussen and Riddle, 2002). The control of testicular androgen secretion is a well-known process (see Figure 1) but the factors that control the musth cycle remain incompletely understood. Good body condition and a positive metabolic state are probably important for the initiation of musth (Poole, 1999; Rasmussen and Perrin, 1999; Ganswindt et al., 2010). Aggressive behaviour and musth, while important from a breeding point of view and social structure of elephant, are inconvenient when it comes to the management of captive bulls, or in some instances, bulls in smaller game reserves.

Reproductive Control of Wildlife – Possible Options

In most cases, a reversible method of contraception is required and preferably one that is safe and does not interfere with key behaviours of the target species in question. This means that surgical methods are automatically excluded even though it is possible, if not practical, to reverse a vasectomy. There is, therefore, a need to find better and safer methods of contraception for carnivores. Depending on the reproductive behaviour of the species, there will also be differences in whether to treat males, females, or even both sexes (Kirkpatrick et al., 2011).

According to Jöchle (2008), the only form of contraception practiced on domestic animals for thousands of years was surgical castration.

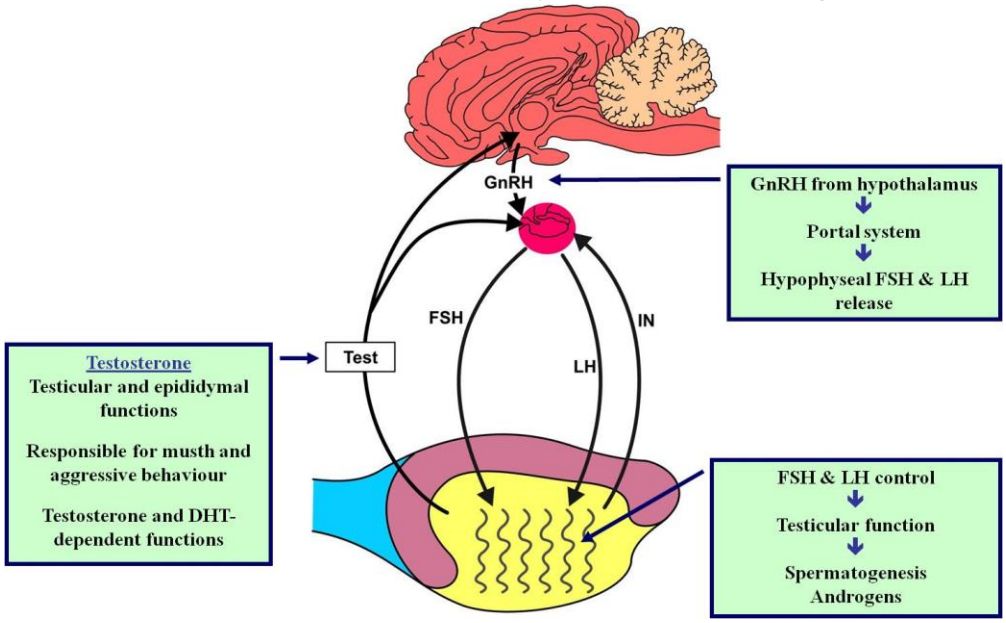


Figure 1. Hormonal control of reproduction, aggression and musth in the elephant bull.

Records of male castration date back at least to 6000 B.C. The early development of orchidectomy is not surprising, given that the gonads of domestic species are situated extra-abdominally. After all, open castration without anaesthesia is still practiced in male piglets today. Ovariectomy, surprisingly, is quoted in Aristotle's writings before 300 BC (Jöchle, 2008).

Non-surgical contraception in women, in the form of contraceptive medications and abortifacients also date back some 3000 years (Finch and Green, 1963). However, no information has been found for the use of similar products on domestic animals. The possible use of IUDs in animals may however date back as far as 3000 years ago, when nomadic people placed pebbles into the uteri of camels (see Figure 2) to prevent conception during their long treks through the desert (Sloane, 2002). It was only in the second half of the 20th century, however, that non-surgical methods for contraception of dogs and cats were widely adopted (Jöchle, 2008).

In dogs, the first oral contraceptive (medroxy-progesterone acetate, MGA) became available in 1963. Oestrogen compounds were the first abortifacients to be used in dogs in 1936 and, despite serious side-effects that may follow after treatment, are still commonly used in many countries. The first use of prostaglandin $F_{2\alpha}$ to abort pregnancy in bitches in was reported by Jöchle et al. (1973). The development of 'modern' contraceptives such as the GnRH super-agonists and immunological tools such as porcine zona pellucida and GnRH vaccines did not begin until 20-30 years after the launch of the first progestins for canine fertility control. Long-acting silicon implants impregnated with progestins (e.g., MGA) have been widely used to down-regulate female reproduction in wild felids, such as lions and tigers, and some wild canid species. Although highly successful as contraceptive agents, their continuous use resulted in a number of side-effects, some of which were life-threatening (Munson et al.,

2002; Munson et al., 2005). Provided no serious pathology had occurred, the effect of the progestins could be reversed by removing the implant. Without removal the tailing-off period is long.

Figure 2. According to legend travelling nomads placed pebbles into the uteri of camels to prevent pregnancies during long treks across the desert (Museum of Contraception, University of Montpellier).

During the late 1990's, a new hormonal method of contraception became available in the form of the GnRH analogue deslorelin, a so-called GnRH super-agonist. Peptech Animal Health (Sydney, Australia) designed a slow-release formulation that released deslorelin for periods of 6 to 12 months or even longer. On the basis of results obtained in domestic dogs and cats (Munson et al., 2001; Trigg et al., 2001; Wright et al., 2001; Junaidi et al., 2003), the subcutaneous biocompatible implant appeared to be an ideal agent for controlling reproduction in large predators. High continuous release of deslorelin acts by down-regulating the release of FSH and LH at the level of the anterior pituitary (see Figure 3). This in turn leads to down-regulation of endocrine stimulation of gonadal activity. It thus has the potential to work in both males and females. Once most of the deslorelin has been released from an implant, the target animal would revert to normal reproductive function. As a result of the successes achieved in male and female dogs and cats, preliminary trials were conducted in lionesses, and male and female cheetahs, wild dogs and leopards (Bertschinger et al., 2001). With the exception of wild dog females, the contraceptive efficacy achieved was 100%.

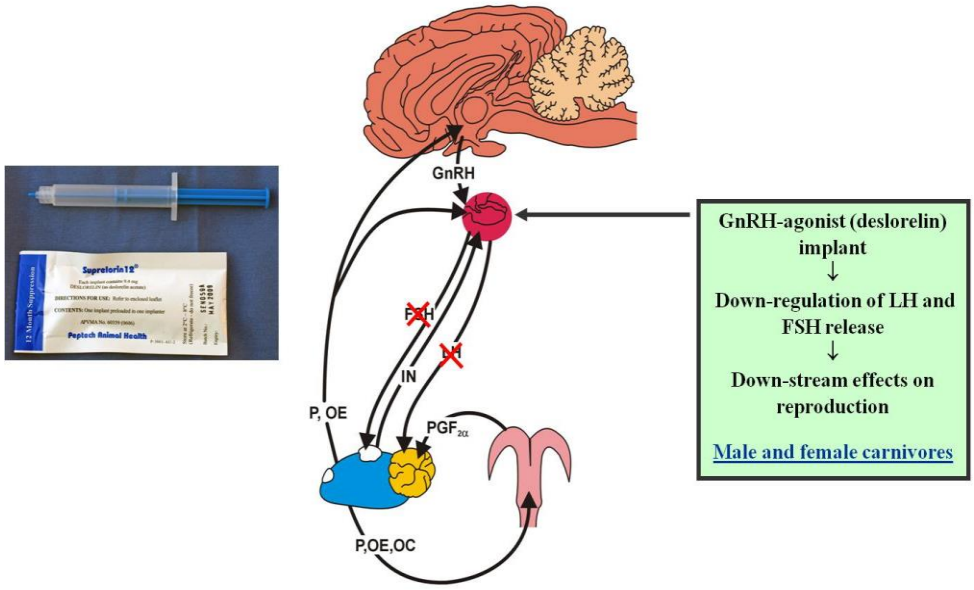


Figure 3. Down-regulation of reproduction in wild carnivores with the long-acting deslorelin implant (Suprelorin®, Virbac, Carros, France). The GnRH super-agonist inhibits the release of the gonadotrophic hormones FSH and LH resulting in down-stream down-regulation of of female and male gonads.

Immunocontraception relies on carefully selecting target proteins involved in critical steps of reproduction. Including such a protein as an antigen in a vaccine stimulates the production of antibodies that neutralise the endogenous molecule or block a particular reproductive process.

Examples of antigens are the native porcine zona pellucida (pZP) and GnRH vaccines. The antibodies produced to the pZP vaccine bind to zona proteins on the oocyte of the targeted females. The binding of antibodies to ZP_3 is thought to block sperm-zona binding and thereby prevent fertilisation from taking place. pZP-immunocontraception has been used extensively for population control of wild horses (Kirkpatrick et al., 1985; Liu et al., 1989; Kirkpatrick and Turner 2002; Turner et al., 2002) but also white tailed deer (Rutberg et al., 2004) and several other zoo mammal species (Frank et al., 2005; Kirkpatrick et al., 2011). pZP immunocontraception has been used successfully to control fertility in free ranging elephant cows in South Africa and today approximately 230 cows in various game reserves are being treated with the vaccine (Bertschinger et al., in press).

The GnRH vaccines, consisting of modified GnRH peptides conjugated to a foreign protein to increase antigenicity, induce antibodies that neutralise GnRH in the target animal. If the anti-GnRH antibody titre is sufficient, the antibodies neutralise hypothalamic GnRH and thereby block the ability of this releasing hormone to stimulate gonadotropin release from the pituitary (see Figure 4).

In theory, the contraceptive vaccine should be effective in both sexes. GnRH immunocontraception was originally developed for the immunocastration of domestic cattle (Hoskinson et al., 1990), although additional research led to vaccines as an alternative to surgical castration to control the problem of boar taint in pork (Oonk et al., 1998; D'Occhio,

1999; Dunshea et al., 2001; Zeng et al., 2001). GnRH vaccines have also been used to control fertility or reproductive behaviour in male feral pigs (Killian et al., 2006), stallions (Dowsett et al., 1996; Turkstra et al., 2005; Burger et al., 2006; Janett et al., 2009), rams (Janett et al., 2003) and bison bulls (Miller et al., 2000).

GnRH vaccines have also been used to successfully down-regulate fertility and sex-related behaviour in domestic and wild mares (Goodloe et al., 1997; Killian et al., 2004; Botha et al., 2010; Schulman et al., 2013).

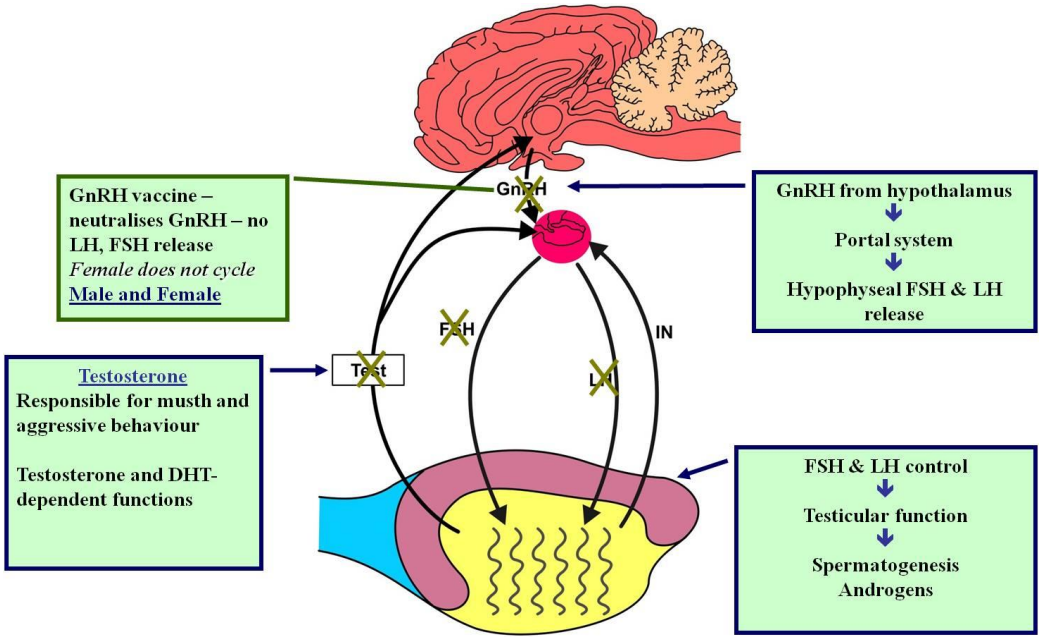


Figure 4. Down-regulation of male and female reproduction using GnRH vaccines. The antibodies provoked by the vaccine neutralise endogenous GnRH and with that the release of gonadotropic hormones FSH and LH is blocked. The lack of gonadotropic stimulation of the gonads leads to anoestrus in the female and down-regulation of spermatogenesis and androgen synthesis and release in the male.

Contraceptive Strategies for Wild Carnivores in Southern Africa

Contraceptive experience in lions, tigers, and cheetahs has mainly been obtained from long-acting biocompatible subcutaneous implants produced by Peptech Animal Health (Sydney, Australia) and, more recently, by Virbac (Carros, France). The implants are marketed as Suprelorin® and Suprelorin12® which contain 4.7 and 9.4mg deslorelin, respectively. In the male domestic dog, down-regulation of FSH and LH lasts approximately 6 and 12 months, respectively. Following implantation, there is an initial surge of FSH and LH release followed by down-regulation of these two gonadotropic hormones.

Cheetah

During the first week following treatment using 4.7 mg deslorelin implants, female cheetahs attract males for 1-2 days. This probably reflects the initial FSH and LH "flare" following deslorelin treatment. Mating, however, has never been recorded and certainly no pregnancies have resulted from this short stimulatory period. The subsequent anestrus following the short period of stimulation lasts at least 18 months; contraception treatments should be maintained at annual intervals (Bertschinger et al., 2001 and 2002).

At least 35 male cheetahs have been treated with 4.7 mg deslorelin implants on an annual basis and some for up to 10 years without any side effects being recorded.

Down-regulation of fertility in males takes longer than in females, as viable sperm are noted for up to six weeks post-treatment. Unlike the gametogenic cycle in the female, spermatogenesis is a continuous process and sperm already present in the cauda epididymides may remain viable for weeks. During the first two years of annual treatment there was a rapid decrease in testicular volume to 61.1% and 40.3% of original volume, respectively, reflecting a rapid reduction in spermatogenesis (Bertschinger et al., 2006). This was followed by a slower decline to reach 28.6% of the original volume after 7 years of annual treatment (see Figure 5). Figure 6 shows a histological section of the testis of a cheetah male that had been treated annually for three years (Bertschinger et al., 2006).The male died as a result of acute capture stress 12 months after the last treatment. The effect of down-regulation of the GnRH analog is quite apparent. Only two to three layers of spermatogenic cells are present, very few spermatids are visible, and no further stages including sperm are present in the tubules (Figure 6). Down-regulation of testosterone synthesis was rapid, and by six weeks basal levels were recorded. Appearance of penile spikes, which are androgen dependent, proved to be a good subjective means of determining the presence of androgen activity.

Figure 7 shows the comparison of penile spikes between an untreated male (A) and a male that had been treated for 4 consecutive years with 4.7 mg deslorelin implants (B). We encountered two males that appeared to be more resistant to the effects of the implant. One year after the first implant both still had viable sperm albeit some reduction in testicular size. As a result we decided to use two 4.7 mg implants for each these males during the annual treatment (Bertschinger et al., 2001, 2002 and 2006). In cheetahs, both sexes should be treated annually using the 4.7 mg implants. Treatment of male cheetahs annually for up to ten years has produced no side effects.

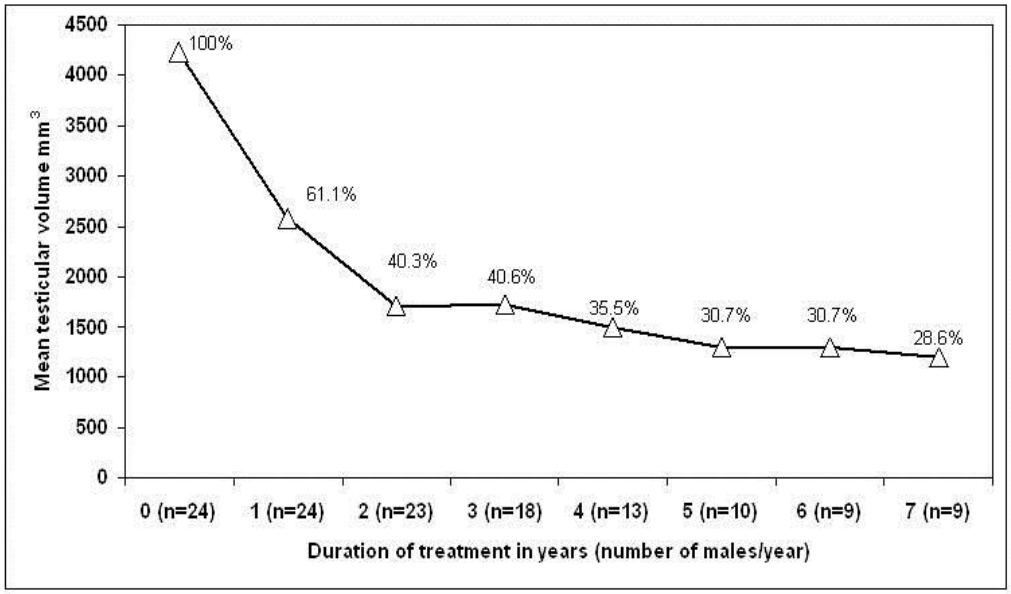
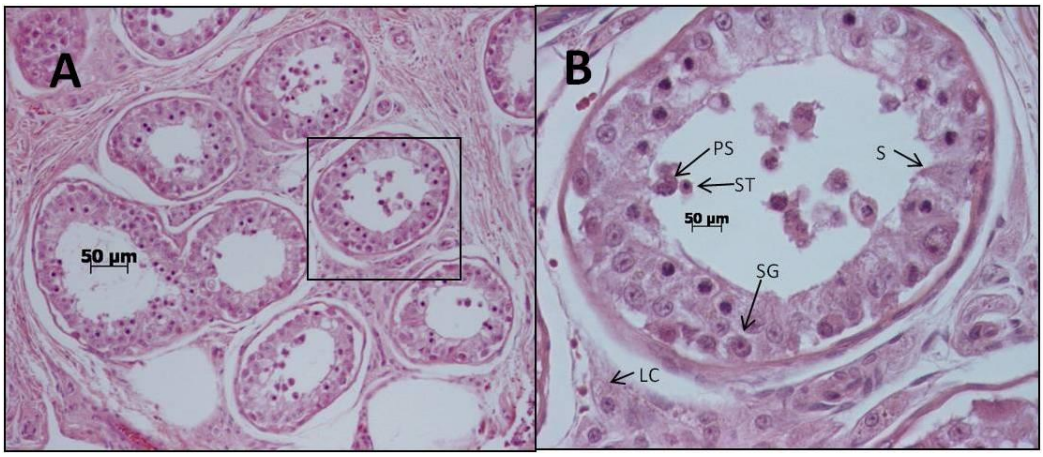
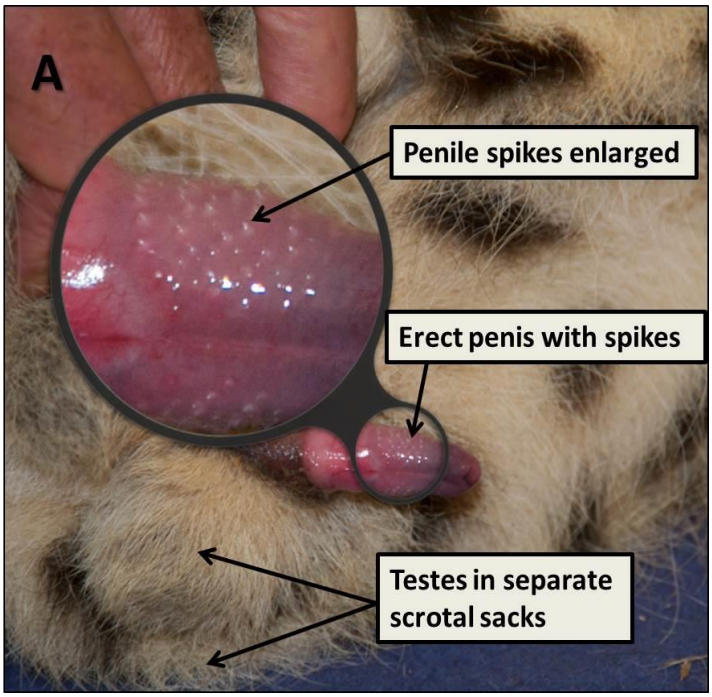


Figure 5. The effect of sequencial annual Suprelorin® treatment (4.7 mg deslorelin) on testicular volume in male cheetahs for up to 6 years.



LC = Leydig cell; S = Sertoli cell; SG = spermatogonium; PS = primary spermatocyte and ST = spermatid.

Figure 6. Histological sections (H and E) of the testis of a cheetah exposed to 3 annual treatments of deslorelin. The last treatment was administered 12 months prior to death of the male. A: Several seminiferous tubules showing limited spermatogenesis with occasional spermatids seen as the final stage. B: Enlargement of the tubule in the rectangular area shown in A. Modified from Bertschinger et al. (2006).



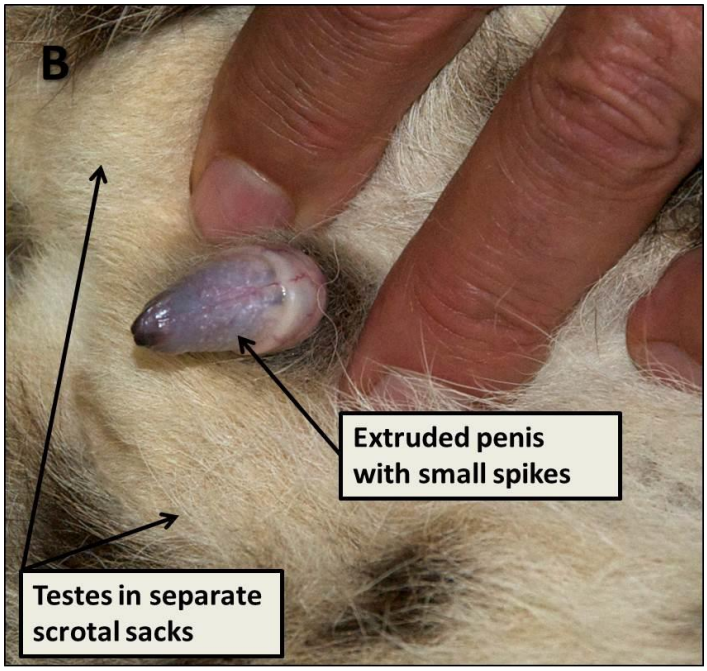


Figure 7A. Penis of a cheetah without deslorelin treatment showing well developed androgen-dependent penile spikes.

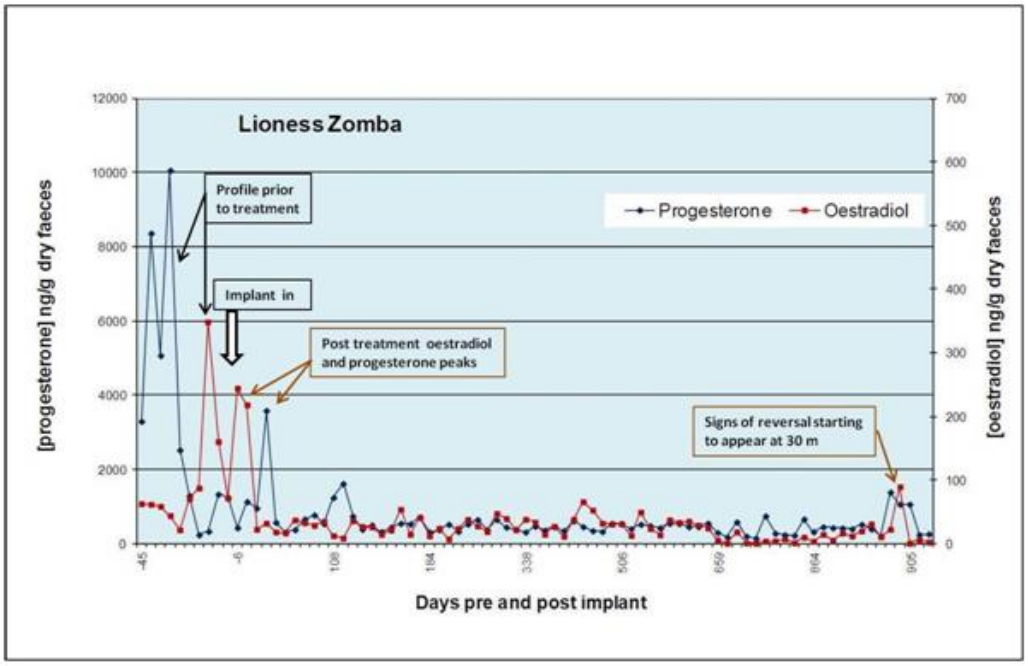
Figure 7B. Penis of a cheetah after 4 annual treatments with deslorelin implants (4.7 mg) showing poorly developed penile spikes.

Lions and Tigers

Male lions (*Panthera leo*) were not targeted for contraception with deslorelin treatment. Although likely to be effective, the treatment would certainly have resulted in loss of androgen-dependent manes. In free-ranging animals, treatment is also likely to affect territorial behaviour which has been shown to be androgen dependent in some species (Maestripieri, D. 1992). Between 1999 and 2007, over 200 treatments on at least 80 lionesses and four female tigers were carried out. However, only 67 of these females could be followed to provide reliable data (Bertschinger et al., 2008). The captive lionesses (*n*=23) in South Africa were 18 months to 8.5yrs of age while the free-ranging lionesses (*n*=40) ranged from 18 months to 13yrs. The free-ranging lionesses were housed in fenced reserves ranging in area from 1500 to 22000 ha. The captive tigers (*n*=4) were 2-4 yrs of age. The animals were treated with one of the following combinations: 3x4.7 mg, 2x4.7 mg, 9.4 mg, or a combination of 1x4.7 and 1x9.4 mg deslorelin implants. Some lionesses (*n*=31) were treated more than once. Another two captive African lionesses that were housed with a vasectomised male in a zoo in Brazil were treated with 1x9.4 mg each and faecal progesterone and estradiol monitored. The monitoring period commenced well prior to treatment and continued for approximately 30 months thereafter. Faecal samples were lyophilised and extracted with 90% ethanol for radioimmunoassay of the two steroid hormones (Brown et al., 1993, 1994 and 1996; Brown and Wildt 1997; Viau et al., 2005; Bertschinger et al., 2008).

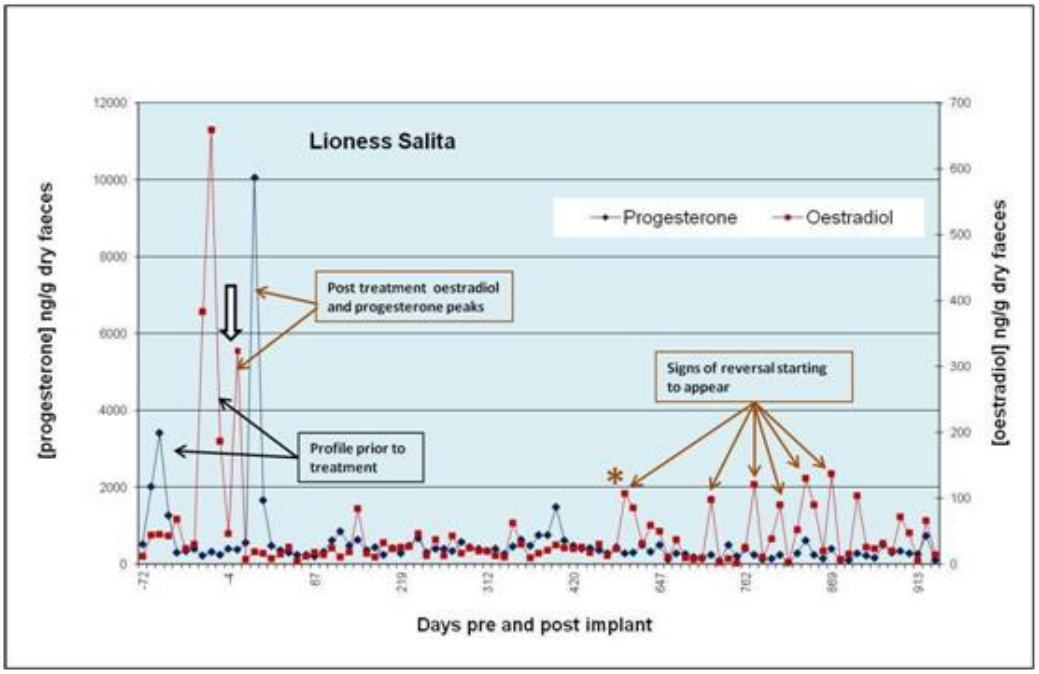
All combinations of deslorelin were successful at inducing anestrus. In females that were not retreated, reconception was approximately 30 months after treatment. With the exception of weight gain in some lionesses (see below), no side effects occurred, although a number of the lionesses were treated repeatedly for up to ten years. Deslorelin (Suprelorin®) is a safe and effective means of controlling reproduction in captive lions and tigers or free-ranging populations of lions. Where contraception is to be maintained we recommend a second treatment after 12-18 months and thereafter at 24-month intervals. The intervals between treatment ranged from 11 to 60 months, and some of these were on treatment for up to 10 years. Preliminary data indicate that the 9.4mg implants probably last longer with the first post-estrus being observed 30 months after treatment. As in cheetahs, lionesses attracted males for a couple of days during the first week after the primary treatment. This short period of activity was associated with an estradiol peak and subsequent brief rise progesterone in the faeces of the Brazilian lionesses (see Figure 8A and B). Once again there were no negative consequences associated with this period of sexual activity. Three South African lionesses showed short periods of heat (± mating) between 2 and 18 months (lioness 1 after 67 and 97 days, lioness 2 after 12 months, and lioness 3 after 18 months) following treatment. Lioness 1 was immobilised after each observed heat, was found to be non-pregnant and on both occasions had baseline blood progesterone concentrations indicating that ovulation had not occurred (lions are reflex ovulators). None of these heats resulted in pregnancy nor did they affect the duration of the contraceptive period.

Current practice in South Africa is to retreat lionesses at 24-month-intervals. One question that arises is where more than one treatment has been carried out, will lionesses reverse as expected after a single treatment after about 30 month? Table 1 shows the breeding and treatment history of two free-ranging lionesses in the Mabula Game Reserve (1,500ha). Both females were treated once after their first litter of cubs with 2x6mg deslorelin and reconceived after 29 and 32 months, respectively.



A

Figure 8. (Continued).



B

[a]modified from Bertschinger et al., 2008.
[b]modified from Bertschinger et al., 2008.

Figure 8. A: Faecal oestradiol and progesterone treatment of Lioness Zomba (housed with a vasectomised male) before and after a single deslorelin implant (9.4 mg). Post-treatment peaks of faecal hormones during the first 3 weeks after implant insertion are visible. Small peaks of oestradiol, not associated with rises in progesterone, start to appear around 900 days (30 months) post treatment probably signifying the start of implant reversal. B: Faecal oestradiol and progesterone treatment of Lioness Salita (housed with a vasectomised male) before and after a single deslorelin implant (9.4 mg). Post treatment oestradiol and progesterone peaks are also visible. In this lioness signs of reversal started earlier. The 1st of these was around Day 530 when she allowed mating (asterisk). No increases in faecal progesterone were associated with the oestradiol peaks.

Female 26 was treated three more times starting in January 2003. Treatment was discontinued following the May 2006 dose. In December 2007 the owners requested removal of the implants as they wanted another litter from the 13-year old female. She started cycling soon after the removal but only conceived 12 months later (27 months from last Suprelorin implant). Female 60 lost her first litter post treatment to a bush fire, conceived again soon after that, and gave birth. After another litter she was also treated with three annual doses from January 2003 to May 2006. Contrary to Female 26, no previous implants were removed and her first post treatment estrus was seen in August 2009, which is 39 months after the final Suprelorin treatment. This case study reveals a few things. First, after a single treatment reconception takes place at about 30 months but only after previous infertile cycles had occurred. Also, four treatments over periods of 6-7yrs duration produced no detectable side effects. Moreover, there seems to be some evidence of residual effect of previous repeated implants on the return to heat and conception after the current or last implant. The female where implants were removed reconceived after 27 months whereas the other female only showed behavioural heat for the first time after 39 months.

Table 1. Casey histories of two free-ranging lionesses treated repeatedly with deslorelin to control reproductive rate

Lioness 26 (DOB 9/1994)		Lioness 60 (DOB 9/1994)	
Date	Treatment/observation	Date	Treatment/observation
June 97	1st litter of 2 cubs	June 97	1st litter of 4 cubs
Nov 99	2 x 6 mg Deslorelin (1)	Oct 98	2 x 6 mg Deslorelin (1)
May 01	1st post treatment heat after 18 months	June 00	1st post treatment heat after 20 months
July 02	1st post treatment litter of 3 cubs – reconception after 32 months	Aug 01	1st post treatment litter of 3 cubs – reconception after 29 months Cubs died in a bush fire
Jan 03	3 x 4.7 mg Deslorelin (2)	Dec 01	2nd post treatment litter of 1 cub
Dec 04	1 x 9.5 mg Suprelorin (3)	Sept 02	3rd post treatment litter of 4 cubs
May 06	1 x 9.5 mg Suprelorin (4)	Jan 03	3 x 4.7 mg Deslorelin (2)
Dec 07	Implants removed	Dec 04	1 x 9.5 mg Suprelorin (3)
Dec 08	1st post treatment litter of 2 cubs – reconception after 27 months	May 06	1 x 9.5 mg Suprelorin (4)
		Aug 09	1st post treatment heat after 39 months

DOB = date of birth; (1) = sequential number of individual deslorelin treatments.

Whereas the captive and free-ranging lionesses in South Africa were only monitored for sexual behaviour and production of litters, the Brazilian lionesses were monitored for both sexual and longitudinal changes in faecal steroids. These steroid profiles provide additional information to explain the observations recorded in South Africa (see Figure 8A and B). The first is the appearance of an estradiol peak around day 4-7, respectively. These peaks were not associated with heat or mating, but corresponded to the period of sexual attractiveness observed in cheetah. A small progesterone peak of short duration followed the oestrogen peak. The second is the appearance of minor estradiol peaks in lioness Salita (see Figure 8B) after 17 months. Around the time of the first peak (about day 530), the lioness was mated by the male but faecal progesterone remained baseline indicating that no ovulation had occurred. Once again, it explained the behaviours observed in 3 lions from South Africa. Perhaps the oestradiol peaks are a reflection of the tailing-off of the deslorelin in the implant. Also as in the South African lions, the 9.4mg implants lasted longer than the 30-month period observed with the 4.7 and early 6mg implants.

The only side effect observed in lionesses was a tendency to gain weight. This was more evident in captive than in free-ranging females. Weight gain was not observed in male or female wild dogs and cheetahs (Bertschinger et al., 2001, 2002, and 2006).

Control of Aggressive Behaviour and Musth in African Elephant Bulls

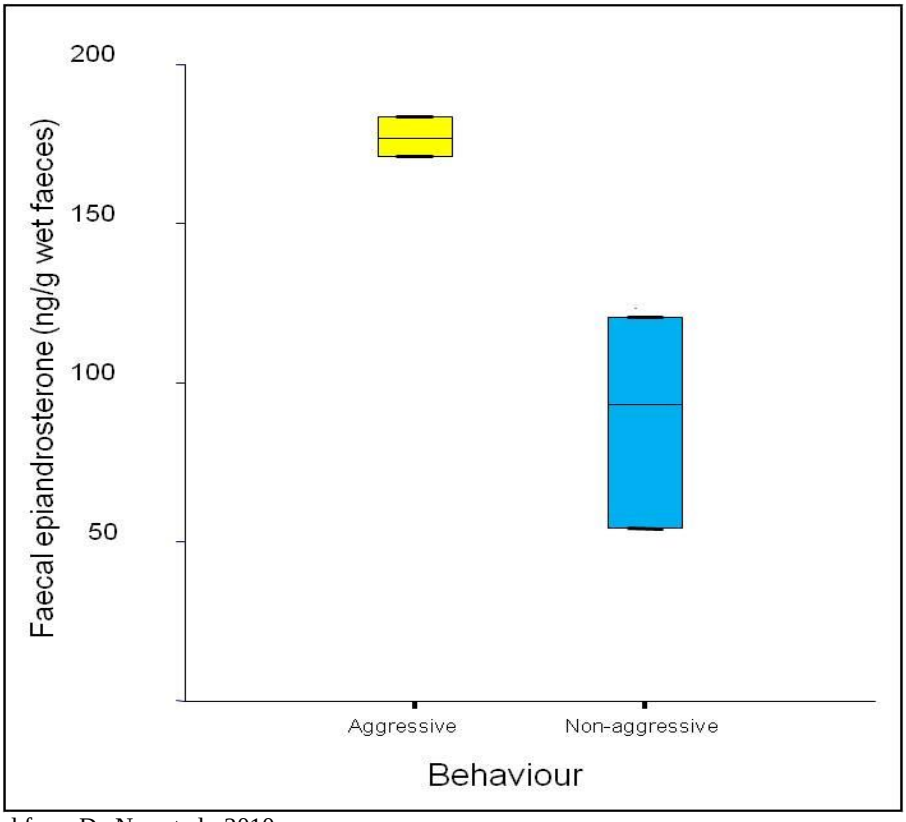
Previously, musth and its associated aggression in zoo and captive elephants have largely been controlled using management/segregation methods. In zoos, bulls of both species (African and Asian elephant) are either housed permanently 'hands-off' in robust enclosures, or are moved to separate facilities during periods of aggression and musth. Working Asian elephants in musth are usually leg-chained in the forest and receive a reduced feed intake during such periods, since dietary restriction is thought to shorten the duration of musth. Generally free-ranging African elephant bulls enter musth when they are in good body condition (Poole, 1999; Rasmussen and Perrin 1999; Ganswindt et al., 2010). Also during musth, elephant bulls can travel great distances during which time they spend little time feeding, which results in loss of body mass. Occasionally, surgical castration has been performed in Asian elephants, and castration of a bull in Zimbabwe (now in South Africa) has resulted in an animal that has been tractable for over 15 years since the surgery (R. Hensman, personal communication). For free-ranging bulls gonadectomy would be very expensive and, of course, irreversible. Anti-androgens (Niemuller et al., 1998) and GnRH agonists (de Oliveira, 2004) have previously been used to ameliorate aggression in elephant bulls, but success in controlling aggression has been limited. The GnRH agonist, Leuprolide, was used repeatedly in an attempt to down-regulate musth in an Asian elephant, but the results were unconvincing (de Oliveira, 2004). In this respect, GnRH agonists such as deslorelin are not able to down-regulate male reproductive function in cattle (D'Occhio, 1999), horse (Stout and Colenbrander, 2004) or donkey (Bertschinger, unpublished). In summary, there is currently no practical and affordable method of controlling androgen-related behaviour in elephant bulls, particularly if a reversible method is required.

In domestic animals, there is one method that stands out because it has been so successful at down-regulating aspects of male reproduction function in a wide range of species. The method involves the use of GnRH vaccines that stimulate the production of anti-GnRH

specific antibodies. The antibodies neutralise endogenous GnRH and, in so doing, suppress down-stream endocrine mechanisms that stimulate reproductive function in the male (see Figure 4). The main end target is testosterone, the steroid hormone mainly responsible for aggressive behaviour and, in elephants, the phenomenon of musth. In theory, GnRH vaccination is reversible and remote delivery is possible, which is important for the treatment of especially free-ranging bulls but useful in captive bulls as well. If effective, there would be tremendous scope for using it to control aggressive behaviour and, if possible, musth in captive and even wild elephant bulls. During the 1990's and early 2000's a number of papers on the use of GnRH vaccines to control reproduction and sex-related behaviour in a variety of species appeared (D'Occhio et al., 1999; Ladd, 1993; Ladd et al., 1994; Malmgrem et al., 2001; Miller et al., 2000; Oonk et al., 1998; Stout and Colenbrander 2004). Accordingly, in 2003 we embarked on the first study which made use of GnRH vaccine to control aggressive behaviour and musth in African elephant bulls (De Nys et al., 2010). The vaccine (Pepscan Systems; Lelystad, Netherlands) was a modified GnRH-tandem-dimer-ovalbumin conjugate. L-glycine in the 6-position was substituted with D-lysine for conjugation of GnRH to ovalbumin. Covaccine™ (Covaccine B.V.; Utrecht, The Netherlands) was used as adjuvant. Four captive and one free-ranging bull were vaccinated 3 times each at intervals of 3-7 weeks. A fifth captive bull was treated with an emulsion of vaccine and Montanide ISA 51VG (Seppic; Paris, France) three times. The emulsion proved to be too viscous for dart-injection, so the bull was given a fourth treatment with the Covaccine™ adjuvant. Prior to the start of treatment, before each booster and at intervals after the last treatment, faecal samples were collected on 4 or 5 consecutive days to determine epiandrosterone concentration using an enzyme immunoassay (EIA) validated for African elephants (Palme and Möstel, 1994).

Faecal epiandrosterone is known to reflect circulating blood testosterone concentrations in African elephant bulls (Ganswindt et al., 2002).

The results of this first trial were not entirely conclusive as only two of the five captive bulls showed aggressive behaviour before treatment commenced. Nevertheless, there were interesting results. Fecal androgen concentrations and aggressive behaviour were well correlated prior to treatment (see Figure 9). Fecal epiandrosterone concentration of the two aggressive bulls (179±8ng/g) was significantly higher ($p < 0.05$) than that of the three non-aggressive bulls (97±31ng/g). Aggressive behaviour ceased after 2nd booster in the one bull (Thembo), and this coincided with a decrease in faecal androgen concentration (see Figure 10). The other aggressive bull (Kinkel, see Figure 10) showed the same pattern but only after the 3rd booster where Covaccine was used as adjuvant. The effectiveness of the previous vaccine deliveries may not have been ideal due to the high viscosity of the preparation, when ISA 51 was used as adjuvant. The free-ranging bull went out of musth 10 days after the first treatment. All bulls remained non-aggressive for the remainder of the observation period 4 months after the final vaccination.



Modified from De Nys et al., 2010.

Figure 9. Association between aggressive (n=2) and non-aggressive (n=3) behaviours and grouped fecal epiandrosterone concentrations in captive African elephant bulls prior to the start of GnRH vaccination.

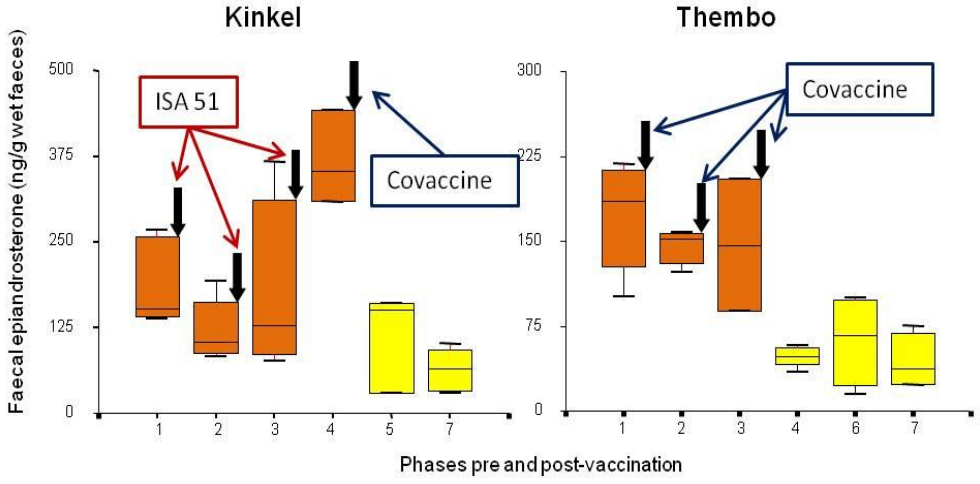


Figure 10. Grouped fecal epiandrosterone concentrations of bulls Kinkel and Thembo prior to the start of GnRH vaccination and before and after each vaccination (solid black arrows). Kinkel only responded after the third booster when Covaccine was used instead of ISA 51. Thembo responded after the second booster (modified from De Nys *et al*, 2010).

However, four of the captive bulls continued to be monitored with notable results. One captive elephant bull (age 27 yrs) maintained on a game farm in Zimbabwe, entered musth 10 months after the last vaccination. This animal had to be euthanized because he killed a rhino bull on the property. Two other elephant bulls on the same property (ages 28 and 18) received no further treatments but became problematic in 2007 and 2009, respectively. They were treated with a GnRH vaccine (Improvac, Pfizer; Sandton, South Africa) in October 2007 and February 2009, respectively with boosters every 5 months. Since the resumption of treatment they have been tractable and no further problems have been experienced to date. These animals are now 38 and 28 years old, ages when musth should occur, and certainly in a 38-year-old. Bull Thembo (18) was moved to a new sanctuary in 2005 and treatment with the Pepscan vaccine resumed in June 2005 every 5-6 months. In August 2006 the Pepscan vaccine was replaced with Improvac. He remains in captivity, is tractable and cooperative despite being 28 years old now. Since the availability of Improvac, more than 35 captive bulls have been treated and mostly with good results as far as testosterone-related behaviour is concerned. Where problems have been experienced, they have been the result of irregular use of the vaccine.

Figure 11 shows the fecal epiandrosterone profile of a captive 35 year old African elephant bull housed at Bowmanville Zoological Park (Canada). This bull had a regular musth cycle and was difficult to manage during periods of musth whereas in between, he was tractable and used for public displays outside of his enclosure. The last musth cycle prior to the start of GnRH vaccine treatment (Pepscan Systems; Lelystad, Netherlands) ended at the beginning of August 2005. He started showing signs of aggression in October 2006 at which time GnRH vaccination commenced. He was treated 6 times during the next seven months which effectively prevented a musth cycle from occurring. In February 2008 (30 months later), by which time a musth cycle still had not occurred the bull was euthanized as a result of sever pododermatitis (W Korver, C Niemuller and HJ Bertschinger, personal communication).

An interesting observation, even in bulls on regular treatments every 6 months, is a period of hypersexual activity in some of the animals in the days immediately following re-treatment.

These bulls develop erections, mount other bulls and, on one or two occasions, ejaculation has been observed. The phase of hypersexual behaviour ceases within a week of treatment. We can find no physiological explanation for these observations since the binding sites of the GnRH molecules contained in Improvac are bound to a carrier protein. Because elephant bulls cannot be bled at regular intervals to obtain samples for testing we are using stallions to find an explanation for the observations. Based on the observation recorded in some bulls, we now recommend five-month treatment intervals, and this has largely solved the problem.

Another side effect, which may well be permanent in bulls where treatment is initiated at a young age, is the effect on penis size. Figure 12A compares three young bulls of similar age (16-18 years). The two bulls Chishuru and Moya had been treated for 32 months when the photo was taken. Chova (a stable mate) on the other hand had never received GnRH vaccine therapy. The difference in size of the extruded penises is quite apparent (see Figure 12B). Another side effect which could be put to good use for contraception of elephants is the effect of GnRH vaccination on spermatogenesis. We are currently conducting a trial to establish the effects of Improvac on the fertility of ten captive and two free-ranging elephant bulls.

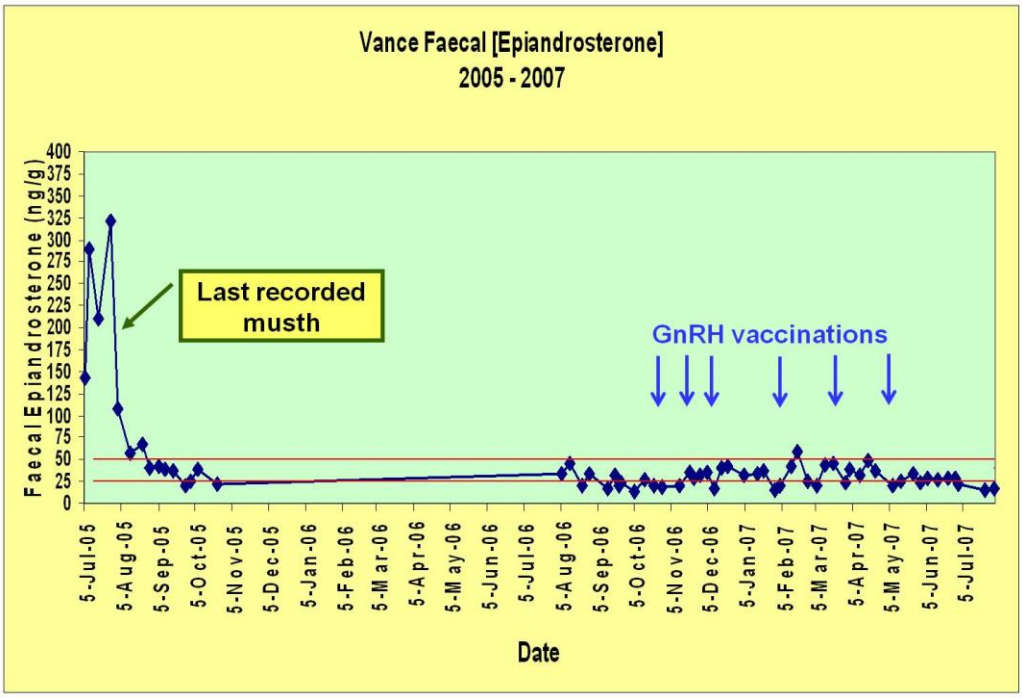


Figure 11. Fecal epiandrosterone profile of African elephant bull Vance (Bowmanville Zoological Park, Canada) from the end of the last recorded musth (beginning of August 2005) and continuing for another two years. Signs of the onset of a new musth were noted in October 2006 at which time GnRH vaccine (Pepscan Systems; Lelystad, Netherlands) treatment was started. Musth was postponed and had not occurred by 19 February 2008 (30 months), when the bull was euthanized as a result of severe pododermatitis (W Korver, C Niemuller and HJ Bertschinger, personal communication).

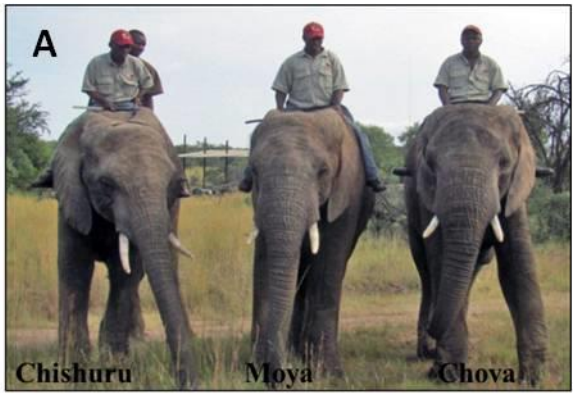


Figure 12 A. Three African elephant bulls of similar age (16 to 18 years old) and size.

Figure 12 B. Effect of the GnRH vaccine (Improvac) on penis size of the two treated bulls (Chishuru and Moya) compared to that of the untreated bull (Chova) 32 months after the start of regular treatment.

The reproductive organs are being examined and measured by means of ultrasound and semen collected by means of either trans-rectal massage or electro-stimulation. Multiple semen parameters are tracked including concentration, motility and morphology.

To date, the results have been encouraging: 12 months after the first treatment no sperm have been identified in any bull receiving GnRH treatment for ≥12 months.

Of note, blood testosterone concentrations in bulls treated for ≥6 months are at baseline level or undetectable (Lüders et al., 2011). Thus it would seem that GnRH vaccines like Improvac could be used for population control of elephants as an alternative to pZP immunocontraception. This method would be particularly well suited to small populations (< 50) with where the number of bulls of breeding age is small. Reversibility and recovery of fertility potential requires further study.

GnRH Vaccine for Control of Androgen-Related Aggression in Other Male Wildlife Species

Encouraging results have been recorded from a limited number of behavioural observations in other species including giraffe bulls (*n*=3), warthog male (*n*=1), rhino bull (*n*=1) and African buffalo bull (*n*=1). The fact that the Improvac vaccine is ready for use and can be delivered remotely via injectable dart makes it a good tool for the control of sex-related behaviour in wildlife males.

Potential for Use of GnRH Vaccine to the Control Estrous Cycle and Fertility

We tested the ability of the GnRH vaccine Improvac to control estrous cycles of domestic horses during the breeding season. Fifty-seven cycling mares were treated twice at an interval of 35 days, and oestrous cycles were monitored using blood progesterone concentrations (Diagnostic Products Corporation; Los Angeles, CA). After the first and second treatments anestrus was induced in 80% and 100% of mares, respectively (Botha et al., 2010). In a second study, we investigated contraceptive reversibility in 51 of the 57 mares. The mares were placed in 3 age categories (<4 years, *n*=25; 5-10 years, *n*=16; >11 years, *n*=10). By Day

720, all but 4 young mares (7.8%, and all were in the youngest age classification) had resumed cyclic activity. Reversibility was good although significantly affected by age, with the youngest mares taking longer than the two other groups to return to oestrous cyclicity (Schulman et al., 2013).

The satisfactory results observed in mares led to similar studies of Improvac use to induce anoestrous in free-ranging African elephant cows. Ten cows were treated with the vaccine and oestrous cycles were compared to that of five control cows using fecal progestin concentrations (Benavides Valades et al., 2012). The results were inconclusive, partly because the initial dose of Improvac was too low (3ml vs. 5ml, as currently in use) as well as the length of the monitoring period. Probably the most important finding of this study was the fact that free-ranging African elephant cows do not show an oestrous cycle year round as previously thought. During the dry season with limited availability of resources, many cows enter anestrus for a few months. Hence, monitoring cycles for only a year, as in our study, is insufficient if the objective is to establish the effects of vaccines like Improvac. In the meantime, in an uncontrolled study of four cows in Zimbabwe we have established that a dose of 5ml Improvac given at 5-month intervals is able to control the oestrous cycle of elephant cows.

More recently, the successful use of another GnRH vaccine (Repro-BLOC®) to induce anestrus in a 59-year-old Asian elephant cow for medical reasons has been reported (Boedeker et al., 2012). However, a series of treatments (primary plus four boosters) were required before the cow was finally down-regulated.

FUTURE DEVELOPMENTS

It would be naïve to believe that testosterone is the only hormone controlling aggressive behaviour in animals. In humans and laboratory animals, it appears that the wiring for dominance and behaviour is established early in perinatal life (Yu and Shi, 2009), and testosterone acts by stimulating existing pathways following puberty. Subjectively, it would seem that the same applies to elephant bulls. This variation is demonstrated by elephant bulls that can produce blood testosterone levels at 150nmol/l yet only some behave aggressively towards motor vehicles, humans, or other animals. We are also convinced that life experiences and the influence of adult elephants play an important role in these intelligent animals. Many elephant bulls that were removed from the influence of their natal herd too early and then grew-up in the absence of adult bulls later became problem animals. An example of this conditioning influence is apparent in subadult bulls relocated from the Kruger National Park to Pilanesberg Game Reserve (Slotow et al., 2000). A number of these bulls made unprovoked attacks on white rhinos, and many of the victims were killed. On the other hand, being threatened or hurt (e.g., shot at with buck-shot) by humans, may lead to elephant-human conflict situations—elephants truly do not forget.

Additional areas which could be researched for aggression control are other steroid hormones, neuropeptides and neurotransmitters. Testosterone itself does not stimulate aggressive pathways. It is first converted to 17ß-oestradiol by the enzyme aromatase in the target cells and the latter hormone then stimulates the pathways (Naftolin et al., 1975). Neurotransmitters such as serotonin (deficit associated with impulsivity and aggression),

noradrenalin (modulates aggressive responses) and dopamine (attention and rewards behaviours) are involved in promoting aggression (Ferrari, 2005), while GABA has also recently been correlated with aggression (Caramaschi et al., 2008). The neuropeptides ocytocin and vasopressin are said to play key roles in complex social behaviours (Campbell, 2008). So, for example, some additional areas of intervention in the elephant bull could be the use of aromatase inhibitors, serotonin re-uptake inhibitors and increased dietary intake of tryptophan (also to increase serotonin levels) (Shea et al. 1990). The elephant has an extremely well developed vomeronasal organ that may also be involved in the control of social behaviour and aggression. In mice protein pheromones have been identified that promote aggressive behaviour by stimulating specific vomeronasal neurons (Chamero et al., 2007).

The current GnRH vaccine (Improvac) available in South Africa appears to produce a short period of suppression lasting 5 to 6 months. In terms of practicality, particularly for free-ranging elephant bulls a longer duration of 1-2 years would be far better. Thus there is a need to develop formulations that produce a longer lasting antibody response or a sequential release of antigen from a depot-site which may also achieve this goal.

As far as Suprelorin implants are concerned, a remote delivery system for the treatment of wild carnivores would be of major benefit.

It would obviate the need to immobilise animals for treatment which would reduce the cost and improve practicality of the method. We have already tested the delivery of Suprelorin implants in captive lions (Bertschinger et al., 2008). The remote delivery and duration of contraceptive effect has also been tested in kangaroos (Herbert et al., 2012).

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