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The effects of two formulations of deslorelin on the reproduction of male African wild dogs (*Lycaon pictus*)

By

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DECLARATION

I, Annie Newell-Fugate, 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 ....
Annie Newell-Fugate

Date ....

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LIST OF ABBREVIATIONS

CITES: Convention on Trade in Endangered Species

CPV: Canine parvovirus

De Wildt: De Wildt Cheetah and Wildlife Centre

EWT: Endangered Wildlife Trust

FSH: follicular stimulating hormone

GnRH: Gonadotropin releasing hormone

IUCN: The World Conservation Union

Kruger: Kruger National Park

LH: Luteinizing hormone

SanParks: South African National Parks

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Abstract

The African wild dog (*Lycaon pictus*) is the second most endangered carnivore in Africa. Although several high-profile endangered species are imperiled due to poor fertility, inadequate genetic diversity, and a reliance on specific niches, the wild dog is threatened by decreasing land space and human hunting practices. Wild dogs are highly fertile with an average litter size of nine at De Wildt Cheetah and Wildlife Centre. Wild dog males have 3 million to 900 million sperm per ejaculate and 45-75% progressive motility during the breeding season. Wild dogs released into small nature reserves in South Africa experience increased survival rates due to sizeable litters, abundant prey, and increased hunting success along fence lines. Furthermore, the current demand for captive-bred wild dogs is low due to lack of demand by overseas zoos and the inability of nature reserves to accommodate more dogs. Long-acting GnRH analogues have been used for fertility control in many wildlife species. However, dosing and efficacy differ among species and individual animals. This study assessed the efficacy of the GnRH analogue, deslorelin, on reproductive parameters of male African wild dogs. Seasonal effects on reproduction were also evaluated.

Captive male African wild dogs housed at the De Wildt Cheetah and Wildlife Centre were administered either a 4.7 mg deslorelin implant (Suprelorin,® Peptech Animal Health (Pty) Ltd, Sydney, Australia; n = 10), an experimental 9.4 mg deslorelin injection (n = 11) or a placebo injection (n = 6). Treatment was administered during the non-breeding season (Month 0), and dogs were assessed at Months 3, 5, 6, and 7. Reproductive parameters evaluated before and after treatment included: serum testosterone, testicular and prostatic volume, and semen quality. Serum testosterone was assessed with a previously validated double antibody DSL testosterone radioimmunoassay kit (Diagnostic Systems Laboratories, Inc, Webster, TX). Testicular volume was calculated from testicular dimensions measured with a calliper and prostatic volume from dimensions obtained by trans-cutaneous ultrasound. Data were analyzed with ANOVA.

Although the 4.7 mg deslorelin implant was safe for use in male wild dogs, there was wide variation in efficacy among dogs. The serum testosterone of implant dogs did not decrease to baseline after treatment and only half the dogs administered an implant became azoospermic post-treatment. The experimental long-acting deslorelin injection was ineffective for contraception of male African wild dogs. All three groups of dogs experienced an

improvement in reproductive parameters during the months of February through May, the rainy season in northern South Africa and the period during which female African wild dogs enter oestrus, suggesting that a breeding season not only exists in the female African dog but also in the male. Testis and prostatic volume increased, serum testosterone concentrations and semen quality improved during that time of year.

Further studies of deslorelin in male wild dogs are warranted to determine the appropriate dose, pay-out pattern, delivery method, and season of delivery necessary for adequate contraception in this species.

CHAPTER 1

INTRODUCTION

The African wild dog (*Lycaon pictus*) is a highly-social, cooperative breeder that lives in packs of 6-20 individuals (Skinner and Chimimba, 2005; Creel and Creel, 1995; Fuller *et al.*, 1992). Wild dog packs are comprised of an unrelated breeding pair, non-breeding adults that are either offspring or siblings of the breeding pair, and the breeding pair's young. New packs form when same-sex siblings disperse, up to 250 km in some cases (Fuller *et al.,* 1992b), from their natal pack and encounter another, unrelated sibling pack of the opposite sex. Thus, newly formed packs are comprised of members with different genetic compositions which prevents inbreeding (Skinner and Chimimba, 2005; McCreery and Robbins, 2001; McNutt *et al.,* 1996; Creel and Creel, 2002). The alpha male and female account for the majority of offspring produced in a pack, however, up to five individual males can sire an alpha female's litter (Monieux *et al*., 2006; Girman *et al.,* 1997). Furthermore, beta females will whelp litters but their young are: suckled by the alpha female, abandoned to die of starvation, or victims of infanticide (Creel and Creel, 1998).

Captive female wild dogs become sexually mature between 18 and 24 months of age (Van Heerden and Kuhn, 1985; personal observation). For both the eastern and southern subpopulations of African wild dog, litters are born during the months of April to September, with a peak during the months of June, July and August (Creel and Creel, 2002; Maddock, 1989). This period coincides with the end of the impala rut, resulting in an abundance of poor condition rams, and with a concentration of ungulates around water sources (Skinner and Chimimba, 2005). Wild dogs enjoy a high hunting success rate (45 %) when compared with other carnivores (lions average 30 %; Creel and Creel, 2002) Furthermore, they have large litters (average litter size is eight pups; Creel and Creel, 1995; Van Heerden and Kuhn, 1985). The survival rate of young to adulthood (two years) varies by ecosystem and from year to year; however, generally African wild dogs enjoy a high pup survival rate (Selous: 64 %, Kruger 56 %, Serengeti 24 %, Masai Mara 43 %; Creel and Creel, 2002; Mills and Gorman, 1996).

In 2001, in an effort to conserve the endangered African wild dog in South Africa, the Endangered Wildlife Trust and the South African National Parks established an African wild

dog meta-population in several small reserves. Wild dogs in small reserves experienced even higher survivorship of their young. The pup survival rate (to adulthood) in the Venetia reserve was 88 % for two consecutive years (EWT, 2006). Wild dogs in small reserves use fence lines, rivers, and waterholes to their advantage in hunts, which increases their already high hunting success rate (Rhodes and Rhodes, 2004). In the metapopulation, the high survival rate of young is attributed to increased hunting success and decreased depredation of young by lions and hyena. Thus, wild dog meta-populations experience a high population growth rate and inflict a large prey depredation rate.

All reserves in South Africa are fenced to protect surrounding farms from depredation by wildlife and to safeguard wildlife from poachers. Small fenced reserves interfere with wild dog natal dispersal behavior and encourage adults to remain within their natal pack. This behavior places the pack at risk for in-breeding. During the 2004-2005 breeding season in Hluhluwe-Umfolozi reserve a mother-son breeding resulted in a viable in-bred litter (Szkyman-Gunther, personal communication). Dispersal renders dogs vulnerable to depredation by larger carnivores and death due to inter-pack fights. The inability to disperse not only results in the dilution of genetic diversity but bolsters the survival rate of adult dogs, both of which result in low mortality rates and high population growth rates.

De Wildt Cheetah and Wildlife Research Center (De Wildt) has bred and raised over 400 wild dogs during the last 30 years with genetic lines from South Africa, Namibia, Botswana and Zimbabwe (De Wildt, records unpub.). In 1995, De Wildt averaged nine pups/litter as compared with the world-wide average in captivity, 3.04 pups/litter (De Wildt, records unpub.). During the late 1990's, many of De Wildt's captive bred wild dogs were released to the wild. Furthermore, De Wildt contributed wild dogs to the EWT meta-population project. However, the last release of captive wild dogs to any national park in South Africa was in 2002 at Marakele National Park (EWT and De Wildt, records unpub.). Despite their endangered status, demand for release of wild dogs into protected reserves in South Africa is dramatically low at present. Although currently breeding of wild dogs is contraindicated, the genetic lines in captivity at De Wildt are valuable to species survival and diversity.

Consequently, in light of the wild dog overpopulation in captivity and in small reserves, there is an urgent need for a safe, reversible contraceptive for this species. This contraceptive

should be easily administered by remote delivery to facilitate its use in free-ranging wild dogs in small reserves.

This is the first study to develop a long-acting contraceptive injection of deslorelin for use in male African wild dogs. This study aims to develop an effective, reversible contraceptive for use in captive and free-ranging male African wild dogs. The objectives of this study were:

- To contracept 10 male African wild dogs with deslorelin implants, 11 male African wild dogs with deslorelin long-acting injections and six male African wild dogs with injectable placebo
- To determine the effectiveness of the two treatments as compared with the control through the measurement of serum testosterone, testicular volume, prostatic volume, preputial gland size, presence of spermatozoa and semen quality
- To determine the safety of the two treatments as compared with the control through the measurement of body mass, serum chemistries and haematology before and after treatment
- To determine whether male African wild dogs experience improved reproductive traits during a particular season in South Africa

CHAPTER 2

LITERATURE REVIEW

2.1 African wild dog natural history and population status

2.1.1 Natural history

The African wild dog (*Lycaon pictus*) is an obligate cooperative breeder that lives, hunts and reproduces in small packs of six to 20 individuals (Creel and Creel, 1995; Fuller *et al.,* 1992). Pack structure is formed by male and female dominance hierarchies. The pack alpha female is usually the oldest female, whereas the alpha male tends to be younger and in his prime (Creel and Creel, 2002). The alpha male and female breeding pair account for over 90 % of offspring produced in a pack and the subordinate members of the pack help to feed and guard litters of pups (Creel and Creel, 1995; Fuller *et al.,* 1992). Although it has been assumed for years that superfecundation (more than one sire for the same litter) is prevalent in the wild dog (Girman *et al.,* 1997), it was only proven recently when Mouiex *et al.,* (2006) showed that up to five males can sire a single litter by an alpha female. Subordinate pack members hunt in a highly coordinated manner that requires several individuals (usually four to six) to bite and pull at the prey species (Creel and Creel, 1995; Courchamp and Macdonald, 2001). Pups eat first at a kill, followed by yearlings and then adults (Creel and Creel, 2001).

Typically, a pack is comprised of a group of related males (litter mates) and a group of related females (litter mates), with no relationship between the group of males and the group of females (McNutt *et al.,* 1996; McCreery and Robbins, 2001). Yearlings may stay in their natal pack or disperse from the pack to form a new pack with another dispersal group. Although many adult wild dog populations have a sex-ratio biased toward males, the sex ratio in litters may be biased toward males, females or without bias (Creel and Creel, 2002), depending on the parity of the dam (Creel *et al.,* 1998). If the alpha female is primiparous she predominantly produces sons, whereas, if she is multiparous she predominantly produces daughters (Creel *et al.,* 1998).

2.1.2 Historical population status on African continent

The wild dog formerly ranged throughout sub-Saharan Africa and the central Sahara, living in primarily savannah and desert-like terrain, and was probably absent from low-lying rainforest and severely dry desert areas (Sillero-Zubiri *et al.,* 2004). The estimated population size 100 years ago was 300-500 000 dogs (Painted Dog Conservation Project-Zimbabwe, 2006). However, African wild dog populations have declined dramatically over the last 40 years (Woodroffe *et al.,* 1997). This species, which once ranged across 39 countries, now inhabits only 25 countries. Since the World Conservation Union (IUCN) wild dog survey in 1990, the species has disappeared from the Serengeti and populations have declined dramatically in the Luangwa Valley, Zambia (Woodroffe *et al.,* 1997).

2.1.3 Current population status on African continent

Currently, the wild dog is the second most endangered large carnivore on the African continent (Fuller *et al.,* 1992; Woodroffe *et al.,* 1997). With the exception of wild dog populations in the Selous Game Reserve in Tanzania, Chobe National Park in Botswana, Hwange National Park in Zimbabwe, Kruger National Park (Kruger) in South Africa, and Northern Namibia (Figure 1), most remaining wild dog populations consist of less than 100 individuals (Creel and Creel, 1998; Woodroffe *et al.,* 1997). These six populations listed, although currently viable, are fragmented and confined primarily to protected areas (Fanshawe *et al.,* 1991). Seven years ago, 3 000-5 000 African wild dogs remained in the wild, mostly distributed throughout southern and eastern Africa (Woodroffe and Ginsberg, 1999). Wild dogs no longer range within most of western and central Africa, with the possible exception of Cameroon and Sénégal (Woodroffe *et al.,* 1997). There are small but potentially viable populations in Kenya, Ethiopia, Malawi, Mozambique, and Zambia (Woodroffe *et al.,* 1997).

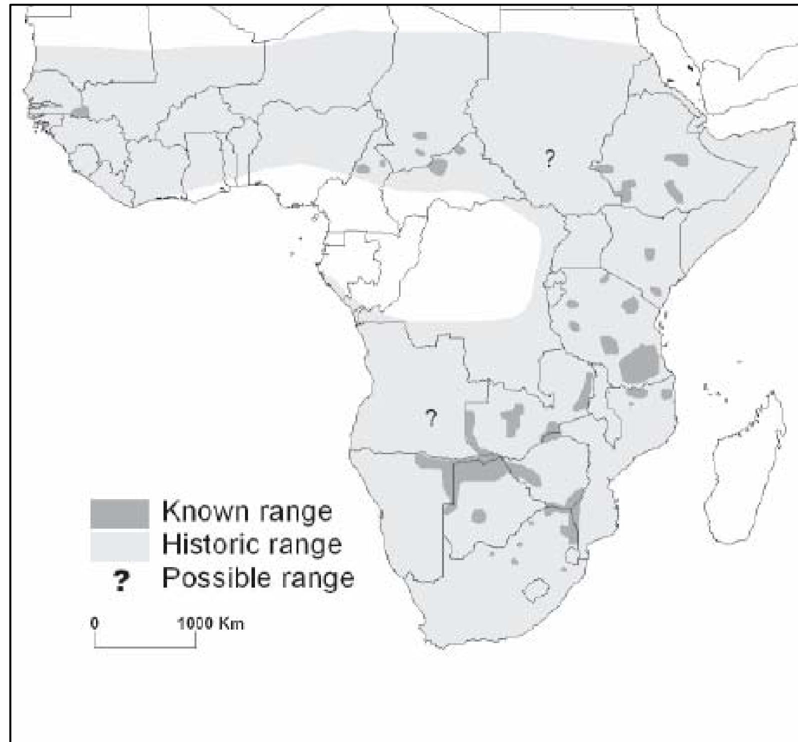

Figure 1: Distribution of the African wild dog.
(Woodroffe *et al.*, 1997)

2.1.4 Causes for population decline

Causes for the severe decrease in wild dog population numbers in the last 40 years include: interspecific competition and predation, intraspecific competition, infectious disease, and the effects of human activities (Figure 2; Creel and Creel, 1998).

2.1.4.1 Interspecific competition and predation

Across all ecosystem types, the density of wild dog populations is inversely correlated with the density of other large carnivores in the same area, particularly lion and hyena (Carbone *et al.,* 1997; Woodroffe *et al.,* 1997; Creel and Creel, 2002; Creel and Creel, 1996). In Kruger, wild dogs avoid high density prey areas because of the large number of lion in these areas (Mills and Gorman, 1997). In contrast to the density of lion and hyena in Kruger (approximately 100/1 000 km^2; Maddock and Mills, 1993), wild dogs exist at a density of merely 16.7/1 000 km^2. The diets of lion, hyena, and wild dog overlap substantially and lion and hyena will steal kills from wild dogs (Woodroffe and Ginsberg, 1999; Creel and Creel,

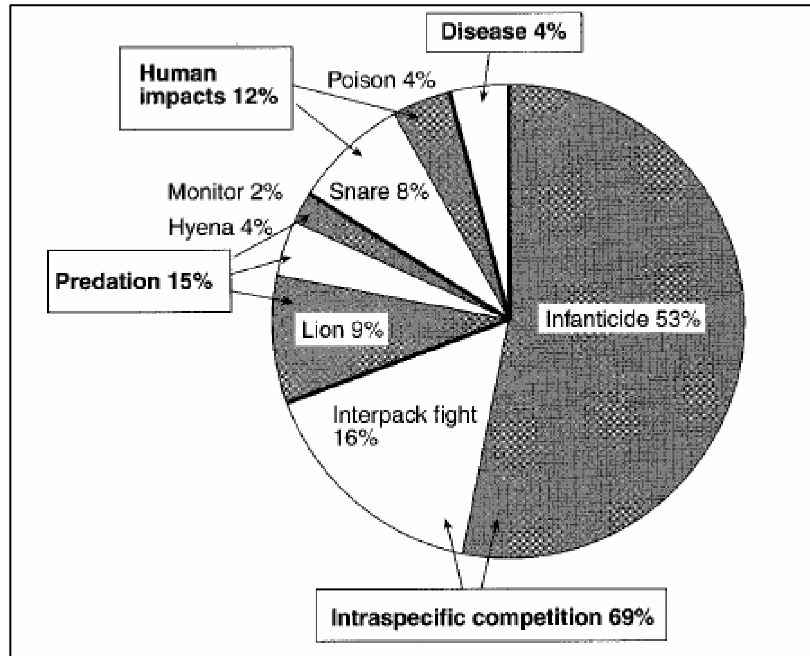

Figure 2: Known causes of death for wild dogs in the Selous, Tanzania. (Creel and Creel, 1998)

1998; Creel and Creel, 1996). Larger wild dog packs are better able to defend kills against these two competitors (Courchamp and Macdonald, 2001; Vucetich and Creel, 1999; Creel and Creel, 1998). Wild dog packs with pups consume a large amount of food (~4 kg meat/dog/day); if hyenas effectively steal carcasses and decrease the amount of food a pack with pups is able to ingest, it is possible that the pack will abandon the pups, thus decreasing pack recruitment (Fuller and Kat, 1990). Furthermore, wild dogs in Kruger require an average home range size of 537 km^2 to avoid persecution by other large carnivores (Mills and Gorman, 1997).

Although hyenas rarely cause direct mortality of healthy wild dogs (Creel *et al.,* 1995), lion predation is probably one of the most important causes of natural mortality to this species (Woodroffe and Ginsberg, 1999). In Kruger lions account for 39 % of pup and 43 % of adult dog deaths (Mills and Gorman, 1997).

2.1.4.2 Intraspecific competition

Intraspecific competition exists within and between wild dog packs. Competition for resources within packs has evolved the strategy of dominance hierarchies and reproductive

suppression of social subordinates within hierarchies. With the possible exception of the beta female, subordinate females within a wild dog pack do not breed (Creel *et al.,* 1996; Creel *et al.,* 1997); however, recently it has been shown that subordinate males within a pack do mate alpha or beta females (Mouiex *et al.,* 2006). Furthermore, pups born to the beta female are killed or, alternatively, acquired by the alpha female to be raised with her own litter. In one study in the Selous, infanticide of pups caused 53 % of known-cause deaths in pups (Creel and Creel, 1998). Another source of intraspecific competition is fighting between neighbouring packs (Creel and Creel, 2002; Creel and Creel, 1998). Creel and Creel (1998) showed that on one occasion, 16 % of known-cause deaths in the Selous were due to fighting between packs.

2.1.4.3 Infectious diseases

Disease outbreaks are considered a minor source of mortality to the wild dog metapopulation (Woodroffe and Ginsberg, 1999). However, the local extinction of wild dog populations in the Masai Mara National Reserve and the Serengeti plains in the early 1990's was attributed to an outbreak of canine distemper coupled with a high background rate of rabies infection (Ginsberg *et al.,* 1995a; Kat *et al.,* 1995; Alexander and Appel, 1994). Additionally, a general epizootic of anthrax in Luangwa, Zambia killed dogs in several packs (Turnbull *et al.,* 1991). In southern Africa, disease is of limited importance to wild dog populations since protected areas are subject to stringent fencing, which prevents access to them by domestic canids. Van Heerden *et al.,* (1995) found that no wild dogs in Kruger were seropositive for *Bacillus anthracis* toxin (n=12), canine distemper virus (n=43), canine parvovirus (n=43), or rabies virus (n=31). However, it was found in the same study that dogs in Kruger had a high seroprevalence to adenovirus (84 %), coronavirus (65 %) and *Toxoplasma gondii* (100 %). A well-documented outbreak of rabies took place in the 1990's in Madikwe Game Reserve when 20 dogs from a pack of 23 died as a result of the infection. The source of infection was a rabid black-backed jackal (Hofmeyr, personal communication). Canine parvovirus (CPV) may cause significant mortality in wild dog pups; litter sizes of packs in Selous were smallest the years during which CPV titres were highest (Creel *et al.,* 1997). Due to the large territories over which wild dogs range (up to 3 800 km^2 per pack depending on location), packs seldom interact with one another and, therefore, outbreaks, of diseases other than the prey-transmitted anthrax, generally do not spread through entire wild dog populations (Woodroffe and Ginsberg, 1999). In summary, different populations of wild dogs experience different diseases and susceptibilities to diseases.

2.1.4.4 Human impacts

Direct persecution by humans over the last century has dramatically decreased wild dog numbers. In order to hunt and to avoid competition with other predators, wild dogs range over large areas. This behaviour renders them likely to come into contact with humans. In the late 1800's and early 1900's government-funded hunting of wild dogs contributed to initial population declines in this species (Fuller *et al.,* 1992). Presently, wild dogs that wander outside of protected areas are still shot as they are considered a threat by livestock and game farmers. Persecution by humans is the number one cause of mortality in wild dogs, accounting for more than 27 % of recorded deaths (Woodroffe and Ginsberg, 1999). However, the perception that wild dog cause large losses to livestock herds is debatable. In the one depredation study, only 26 of 3 132 head of cattle were killed by wild dogs and all of these were calves (Rasmussen, 1999). Furthermore, if prey is available to wild dog, they typically do not kill livestock (Fuller and Kat, 1990). If, however, wild prey is scarce, wild dog will repeatedly kill livestock (Woodroffe *et al.,* 2005). All parks and reserves within South Africa are fenced in order to keep predators, like the wild dog, within the park boundaries. Dogs that manage to get through park fences are likely to be shot by nearby ranchers (Strachan, personal communication).

Snaring practices also adversely affect wild dog populations. Snares usually set for other game species can be tripped by wild dogs. Snaring accounted for 11 % of known-cause deaths in Selous (Creel and Creel, 1998), 18 % in Kruger (van Heerden *et al*., 1995), and 29 % in Hwange (Ginsberg *et al.,* 1995b). Two wild-caught dogs (out of a total of 85 dogs) at De Wildt Cheetah and Wildlife Centre required leg amputations as a result of snare injuries (Strachan, personal communication).

When in competition with lions and hyenas, wild dogs require large territories to survive; therefore, human-induced habitat fragmentation also has had a negative impact on wild dog population numbers (Vucetich and Creel, 1999; Woodroffe and Ginsberg, 1999). Furthermore, wild dogs only exist outside protected areas where human densities are low (Woodroffe *et al.,* 1997). Thus, many wild dog populations are isolated from one another in small reserves, with minimal or no emigration or immigration into or out of the pack.

2.1.5 Population status in South Africa and causes for local population explosion

Kruger contains the only viable wild dog meta-population in South Africa (Lindsey *et al.,* 2004; Woodroffe *et al.,* 1997). As there are no other large, protected areas (Kruger is 21 353 km^2) to accommodate wild dogs in South Africa, in 2001, the Endangered Wildlife Trust (EWT) and the South African National Parks (SanParks) decided to establish a second wild dog meta-population distributed over several smaller reserves (Lindsey *et al.,* 2004; Davies-Mostert, personal communication). As the average home range size of wild dog packs in Kruger is 537 km^2, parks included in the EWT meta-population release program are similar in size; Hluhluwe-Umfolozi Park (960 km^2), Madikwe Game Reserve (750 km^2), Marakele National Park (900 km^2), Pilanesberg National Park (500 km^2), and Venetia Limpopo Nature Reserve (Figure 3).

EWT hopes to use the wild dog meta-population project to assess the effectiveness of this technique for medium to long-term conservation of this species. Two detailed studies of wild dog ecology and behaviour were carried out by EWT at Venetia-Limpopo Nature Reserve (Venetia) and Marakele National Park (Marakele). The pack released into Venetia in 2002 was comprised of wild-caught and captive-born individuals. Post-release the alpha female had two litters with a high average pup survival rate (to 12 months of age) of 88 % (EWT, 2006). Release of wild dogs into smaller parks may affect the viability of prey populations in these areas due to several factors: 1. At an average of 45 %, wild dogs have a higher hunting success rate than other large social carnivores (lions-30 % success rate on group kills; Creel and Creel, 2002). 2. Wild dogs in smaller reserves routinely use fence lines, rivers, large waterholes, and tourist camps as barriers while chasing their prey. This behaviour dramatically increases their hunting success rate, as they do not need to chase the prey to exhaustion level prior to killing it (Rhodes and Rhodes, 2004). Furthermore, packs are able to expend less energy to catch larger prey items when barriers are used for hunting (van Dyk and Slotow, 2003; Kruger *et al.,* 1999). 3) a high reproductive success rate (as shown above) can be attributable to increased hunting success and, therefore, availability of food, which results in a higher population growth rate and prey depredation rate.

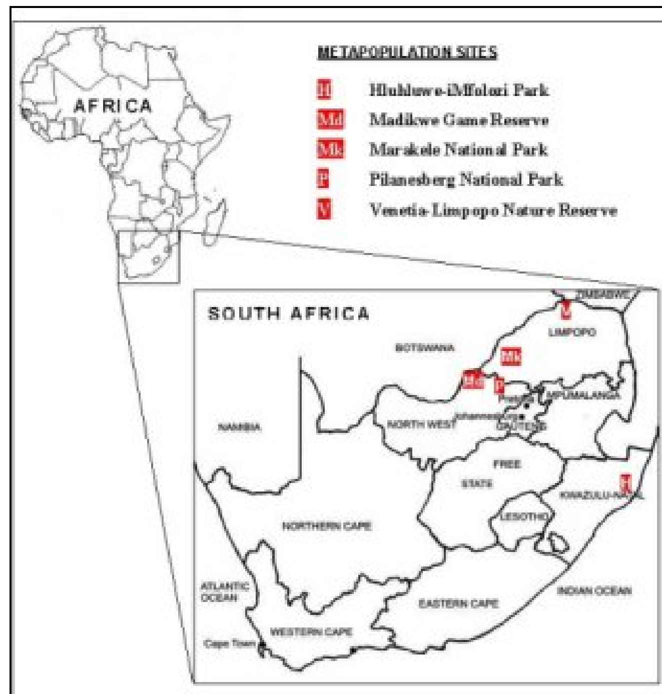

Figure 3: Location of African wild dog meta-population sites in South Africa (EWT, 2001)

Many areas in northern South Africa which previously contained livestock operations are now engaged in privately-managed game lands which accommodate the hunting needs of free-ranging wild dogs with few negative repercussions (van der Waal and Dekker, 2000). Additionally, private game lands are favoured locations for wild dog release due to the potential ecotourism value of this species (Woodroffe *et al.,* 1997; Davies and du Toit, 2004). However, dogs released onto private game lands are tolerated only as long as their cost to the prey species is at or near zero (Lindsey *et al*., 2005). Smaller public or private reserves are suitable wild dog habitats as long as: prey density is high, wild dog numbers are actively controlled by translocation, and prey populations are supplemented (Lindsey *et al*., 2004). When lions are incorporated on a small reserve, the space allotted for a wild dog pack must be increased due to predator avoidance behaviours; however, lion populations limit the voracious hunting success of wild dogs. A heavy lion presence on a small reserve ensures that wild dogs select their preferred habitat distant to lion prides, and hence, distant to areas with optimal prey densities (Lindsey *et al.,* 2004). Furthermore, as wild dogs have extremely high daily energy expenditure rates (Gorman *et al*., 1998), they require large home range areas to meet their daily food requirements (Fuller and Kat, 1990; Creel and Creel, 1995; Fuller *et al.,* 1995).

As stated above, the EWT and SanParks wish to establish a second metapopulation of wild dog over a number of smaller reserves in South Africa (Section 2.1.5). Irrespective of whether lion and hyena occur on these reserves (see Section 2.1.4.1), inbreeding and overpopulation threaten the envisaged metapopulation of wild dog. All reserves within South Africa are fenced in order to protect livestock and people on surrounding farms from large predators. However, African wild dog population survival mandates the dispersal of sub-adults from the natal pack at one to two years of age. In small, fenced reserves, wild dogs may be unable to disperse from their natal pack. Dogs that remain with their natal pack, particularly male dogs, may rise through the dominance hierarchy rank and become alpha male. In such a situation, there is a good chance that the alpha male will be related to the alpha female. One litter has been produced by such a pairing during the May breeding season of 2004 in Hluhluwe-Umfolozi (Szkyman-Gunther, personal communication). Furthermore, dispersal of wild dogs increases their natural mortality rate (Creel and Creel, 2002). Therefore, the inability to disperse, coupled with ample prey and barriers along which to chase prey, will permit low mortality within wild dog populations in small reserves and increased population growth.

2.1.6 Captive breeding in South Africa

African wild dogs breed easily and, in the wild, produce an average of 9.1 pups/litter (Creel and Creel, 2002). Between 1954 and 1997, 787 wild dogs were held in captive breeding programs at seven South African institutions: Cango Croc Ranch and Cheetahland, Oudtshoorn; Hoedspruit Cheetah Project, Hoedspruit; Johannesburg Zoological Gardens, Johannesburg; Tygerberg Zoopark, Kraai; De Wildt Cheetah and Wildlife Research Centre (De Wildt), Pretoria; National Zoological Gardens, Pretoria; Hartebespoort Dam Snake and Animal Park, Hartebespoort (Frantzen *et al.,* 2001). Of these, De Wildt is the only breeding institution that is approved by the Convention on International Trade in Endangered Species (CITES). The De Wildt dogs have the lowest Inbreeding Coefficient (F=0.031) of any institution (Frantzen *et al.,* 2001). Beginning in 1977, wild dogs were bred proactively at De Wildt and other locations in South Africa. Over the last 20 years, over 500 pups were born at De Wildt (De Wildt records, unpublished). Despite the historical captive breeding success at De Wildt and other locations within South Africa, a disproportionate number of offspring were born from a small percentage of the founder population (Frantzen *et al.,* 2001). One dog at De Wildt contributed 21 % of the total genetic material of the entire captive wild dog

population; whereas, at the other extreme, six founders (three De Wildt, two Hoedspruit and one Johannesburg Zoo) only contributed 2 % of the captive genetic material (Frantzen *et al*., 2001).

In 1995, captive breeding results world-wide showed an average of 3.04 pups/litter (De Wildt records and communication, unpublished), whereas, the average at De Wildt in South Africa that year was nine pups/litter (De Wildt records, unpublished). De Wildt attributes its breeding success to enlarged enclosures and an optimal diet. During the late 1990's there was high demand for captive dogs to be used for release into small reserves in South Africa. Several of these attempts failed due to pack dissolution after release, disease epidemics such as rabies in Madikwe Game Reserve in 1995; (De Wildt records, unpublished) or failure to hunt successfully (Frantzen *et al*., 2001). Successful attempts include all the current parks involved in the wild dog meta-population project. The last release of dogs in South Africa took place in the Marakele National Park in 2002 (EWT and De Wildt records, unpublished). Demand for wild dogs both for zoological parks and for release to the wild is low at present. Therefore, in order to combat potential local overpopulation of this species in captivity and to prevent inbreeding within wild populations on small reserves in South Africa, there is high demand for a reversible contraceptive. Remote delivery of such a contraceptive to facilitate its use in free-ranging wild dogs would be ideal.

2.1.7 African wild dog reproduction and fertility control at De Wildt prior to 2005

In 2004, prior to the start of this study, 13 study dogs were contracepted with 9.4 mg implants. Six of these 13 dogs (38 %) sired litters during 2005 (De Wildt, unpublished data). One of these six dogs sired two litters by the same bitch in May 2005. At the time of treatment administration in these 13 dogs, the average concentration of serum testosterone was 0.84 ng/ml and the average total testicular volume ($\pi LxWxW/6$) was 19 603 mm^3 (De Wildt, unpublished data). Furthermore, at the time of the 2004 contraception, five of the six dogs responsible for siring litters in that year had an average serum testosterone of 1.3 ng/ml and an average testicular volume of 22 977 mm^3 (De Wildt, unpublished data). The average dog age at the time of contraception in 2004 was 3.6 years. Furthermore, the average age of 2004-2005 sires (n=5) at the time of 2004 contraception was 4.4 years (De Wildt, unpublished data).

2.2 Fertility control in carnivores

Methods employed for control of fertility in captive and free-ranging carnivores include: husbandry techniques, gonadectomy, vasectomy, hormonal contraception, and, to a lesser extent, immunological contraception. The first four categories will be discussed in this section.

2.2.1 Husbandry techniques

Captive carnivore management may include husbandry techniques used to prevent breeding. The breeding of solitary carnivores, like felids, is managed more easily by husbandry techniques, because individual separation of animals is a natural and ethical means of captive management. Cheetahs, for example, are easily managed for captive breeding in solitary enclosures for the females and group enclosures for the males (Meltzer, 1999; Strachan, personal communication). Some carnivore species such as wild dogs, lions, pinnipeds and hyena are social and, for ethical reasons, should not be housed solitary (Gage, 2003; Creel and Creel, 2002; Wack, 2003; Fernandez-Moran, 2003; Denver, 2003; Ramsay, 2003). Spontaneous ovulators, like canids, can be separated by sex once signs of imminent oestrus become apparent, although this managerial technique is time consuming and fallible due to human error in oestrus assessment. It is appropriate for zoos and other captive-breeding institutions to house social carnivores in same-sex enclosures (Strachan, personal communication) as this technique allows simultaneous socialization and good public display of the animals while avoiding breeding activity. Management of certain carnivore species, like wild dog, with same-sex housing is difficult, because animals of the same sex fight for dominance (personal observation).

2.2.2 Gonadectomy

Gonadectomy is the most reliable method of breeding control in carnivores. This procedure has been used routinely by companion animal veterinary practitioners for control of the pet population, to prevent reproductive tract diseases, such as mammary neoplasia, and to help mitigate undesirable behaviours in the dog and the cat (Howe, 2006; Van Goethem *et al.,* 2006; Spain *et al*., 2004; Howe *et al*., 2000; Stockner, 1991). Techniques that have been used for permanent sterilization in carnivores include ovario-hysterectomy or ovarioectomy via a

ventral midline or flank incision of the abdomen, or via laparoscopic exposure, early-age gonadectomy and castration (Howe, 2006; Howe *et al.,* 2000). In the male, castration removes the main source of testosterone h, 5-dihydrotestosterone and estradiol-17beta (Kiley, 1976; Knol and Egberink-Alink, 1989; Martin and Lindsay, 1998). The loss of sex steroids associated with castration has multiple effects on the animal. After castration, a male initially experiences an increase in gonadotropin releasing hormone (GnRH) pulses, which ultimately leads to decreased stored GnRH in the hypothalamus, decreased luteinizing hormone (LH) pulses, and decreased production of testosterone (Martin and Lindsay, 1998). Local effects of the loss of testosterone include a decrease in the size of the prepuce, penis and prostate, and the bulbourethral glands in cats. In carnivores, the systemic loss of testosterone can result in loss of male odour, dominance behaviour, a decrease in the degree of shoulder and neck muscling, and, in lions, loss of the mane (Martin and Lindsay, 1998; personal observation). Adverse side effects of castration in domestic cats and dogs include weight gain and lethargy (Nguyen *et al.,* 2004; Kanchuk *et al*., 2003; Martin *et al.,* 2001; Fettman *et al.,* 1997). It is rare for gonadectomy to be used in a zoo or wildlife setting because the procedures are expensive and, more importantly, irreversible (Munson, 2006). An exception to this may be ovario-hysterectomy in older felids that have been treated repeatedly with melengestrol acetate (MGA) and are at risk for uterine pathology (Wack, 2003; Asa, 1999; Harrenstien *et al.,* 1996). Captive and free-ranging wild carnivores generally have genetic value in terms of the meta-population and reversible contraceptive methods should be used.

2.2.3 Surgical vasectomy

Vasectomy is a viable sterilization option that does not affect the secondary sex characteristics of the animal and is potentially reversible (Asa, 1999). This technique has been utilized infrequently in small animal medicine due to the variable ability to reverse it and the potential for adverse side effects (Péréz-Marín *et al.,* 2006; Roberts *et al.,* 2002; Fallick *et al.,* 1997). This sterilization technique has been attempted in captive lions (Nayak *et al.,* 1997; George *et al.,* 1995) and considered as a means of population control in free-ranging grey wolves (Haight *et al.,* 1997). Problems associated with vasectomy include: testicular degeneration, ductus deferens dilatation, epididmytitis, sperm granuloma and spermatocoele (Péréz-Marín *et al.,* 2006; McDonald, 1996). In South Africa vasectomy has been used on at least three free-ranging lions as a means of population control (Bertschinger, personal communication). Although it worked as a birth control measure, the continuous cycling of the females led to

fighting between sexes. Eventually the females broke out of the reserve and were mated by fertile males in the neighbouring game park. A similar problem was noted in a female that had been sterilized by means of partial salpingectomy (Bertschinger, personal communication).

2.2.4 Hormonal Contraception

2.2.4.1 Progestins

Progestins are synthetic progesterone derivatives that can be administered orally, by injection or by means of subcutaneous implants. Progestins have been most commonly used in contraception of female carnivores, especially felids, in the zoo setting (Asa, 1999). This class of hormonal contraceptives does not effectively arrest spermatogenesis in male carnivores (England, 1997). The exact means by which progestins disrupt reproduction in females is unknown but several mechanisms have been suggested: 1. A negative feedback loop with the pituitary-hypothalamic axis that results in down-regulation of GnRH, LH and follicle stimulating hormone (FSH) and failure of follicle development and ovulation, 2. abnormal motility of the tubular tract which interferes with oocyte transport and fertilization, and 3. altered function of the endometrium resulting in implantation failure (Munson, 2006). However, Colon *et al.* (1993) demonstrated that oral megestrol acetate does not suppress basal GnRH in anoestrus bitches nor does it suppress the elevated secretion of LH and FSH, which occurs in ovarioectomized bitches. These authors presume that the ability of progestins to effectively contracept canids rests with their prevention of a rise in GnRH above basal levels or perhaps a direct effect at the oocyte level. Progestins directly effect folliculogenesis in rats, rabbits and primates rather than exerting their effects on the ovary via the indirect inhibition of GnRH secretion (Peluso, 2006; Setty and Mills, 1987). In bitches, short-term administration of progestins for contraception purposes will result in an anoestrus period similar in duration to an endogenously induced anoestrus.

When progestins are administered at a dose of 2 mg/kg (megestrol acetate) during anoestrus or within one to three days after the initiation of proestrus, they inhibit oestrus. Specifically, when administered in proestrus, progestins block ovulation, prevent corpus luteum (CL) formation and induce an anoestrus period (Beijerink *et al.,* 2007; Wanke *et al.,* 2006). Progestins administered during an artificially induced oestrus period may decrease pituitary response and increase ovarian response to exogenous GnRH (Sung *et al.,* 2006).

Furthermore, progestins have been used in the past to block oestrus induction in response to the GnRH analogue, deslorelin (Wright *et al.,* 2001). Progesterone also is known to relax the uterus and support endometrial growth (Feldman and Nelson, 2004).

By contrast, two separate studies (Kazensky *et al*., 1998; Baldwin *et al.,* 1994) have demonstrated that synthetic progestins do not alter folliculogenesis in felids, suggesting that this contraceptive class may work in these species by disruption of uterine motility and endometrial receptivity. Because progestins do not inhibit folliculogenesis in felids, contraception with this class of hormones still permits the physical and behavioural signs of oestrus. One of the most commonly used progestins for contraception of captive felids is melengestrol acetate (MGA) implant (Asa, 1999; Wack, 2003; Munson, 2006). Other progestins commonly used for carnivore contraception can be found in Table 1 (Munson, 2006; Hori *et al*., 2005; Jöchle and Jöchle, 1975).

Despite being highly effective and cost-efficient for contraception of carnivores, progestins are not without adverse side effects. The same properties of progestins that are used for contraception also cause uterine and ovarian pathology, especially when administered over long periods (Munson, 2006). Additionally, because females contracepted with progestins may continue to cycle, the endogenous progesterone in their systems compounds the deleterious effects of the contraception. MGA has been associated with the following reproductive organ diseases in both domestic and wild carnivores: pyometra and endometrial hyperplasia (Munson *et al*., 2002; Bellenger and Chen, 1990; Henik *et al.,* 1985), hydrometra, uterine carcinomas and leiomyosarcomas (Munson *et al.,* 1995; Linnehan and Edwards, 1991), fibroadenomatous mammary gland hyperplasia (Loretti *et al*., 2005; Hayden *et al*., 1989; Romatowski, 1989) and malignant mammary neoplasia (Misdorp, 2002; Herrenstien *et al*., 1996; Misdorp, 1991). Progesterone's effects on felid endometrium are so potent that megestrol acetate will even induce endometrial hyperplasia in prepubertally ovarioectomized kittens (Bellenger and Chen, 1990). Levonorgestrel in a slow-release injection has been applied in felids by Looper *et al,* (2001) as an alternative to MGA implants.

Although the adverse effects of MGA have been primarily described in felids (Jewgenow *et al.,* 2006; Munson, 2006), a case of pyometra following MGA implant in an African wild dog was also documented at the Cincinnati Zoo (Herrick, personal communication) and a single case of pyometra and uterine adenocarcinoma in a coati was published (Chittick *et al.,* 2001).

A wild dog female, given a single dose of proligestone (Delvosterone, Intervet) for contraceptive purposes, developed pyometra four weeks later and died (van Heerden, personal communication). Furthermore, efforts toward development of other safer hormonal contraception methods have dominated the contraception literature in recent years.

2.2.4.2 Antigestagens and luetolytic agents

Pregnancy termination with antigestagens or luteolytic agents is used as a last resort when other contraceptive methods have failed. Pregnancy termination can be achieved pre-implantation with steroids or steroid analogues, like the application of oestrogen post-mismating in the dog (Concannon and Meyers-Wallen, 1991). However, oestrogen preparations used before implantation in bitches are thought to increase the risk of pyometra and can potentially suppress the bone marrow in dogs by a mechanism not completely understood (Feldman and Nelson, 2004).

Antigestagens or luteolytic agents used after implantation terminate pregnancy through the disruption of progesterone concentrations in the endometrium (Jewgenow *et al*., 2006). Antigestagens have a higher affinity for progesterone receptors than endogenous progestogens and, thus, displace endogenous hormones (Jewgenow *et al.,* 2006). These compounds have been effective in pregnancy termination in dogs (mifepristone, aglepristone; Eilts, 2002; Concannon *et al*., 1990), cats (mifepristone, aglepristone; Eilts, 2002), and bears (R956; Jewgenow *et al.,* 2001). These drugs must be given between zero and 45 days of gestation to be effective and are ethically unacceptable in the second half of pregnancy (Feldman and Nelson, 2004).

Prostaglandins and anti-prolactinic agents are also luteolytic in carnivores. In many carnivore species pregnancy is progressively prolactin dependent and multiple doses of dopamine agonists alone or in combination with prostaglandin $F_2\alpha$ will safely and effectively terminate pregnancy in the last “trimester” (Eilts, 2002; Lengwinat *et al.,* 2001; Jöchle, 1997; Onclin and Verstegen, 1996; Onclin *et al*., 1995; Verstegen *et al.,* 1993; Post and Jöchle, 1988). Prolactin is the primary luteotropic hormone in dogs and inhibition of its synthesis will result in luteal arrest, decreased progesterone synthesis, and loss of conceptus late in pregnancy (Feldman and Nelson, 2004). Verstegen *et al.* (1993) demonstrated that queens, similar to bitches, depend on the secretion of progesterone from the corpus luteum in order to maintain

pregnancy to term. Dopamine agonists block prolactin production, which results in loss of the corpra lutea and suppression of milk production. These functions make dopamine agonists useful in cases like pyometra, mammary tumours, unwanted pregnancies, pseudopregnancy and cessation of milk production post-partum (Gobello, 2006; Nöthling *et al.,* 2003, Gobello *et al.,* 2001). Prolactin secretion is controlled directly at the pituitary by inhibition of dopamine and indirectly at the hypothalamic level by serotonin, which inhibits dopamine secretion (Feldman and Neslon, 2004). In at least one carnivore species, the red fox, administration of a dopamine agonist must be timed to mid-pregnancy in order to be effective (Marks *et al.,* 2001). The most commonly used dopamine agonists are cabergoline, bromocriptine and metergoline. Cabergoline and bromocriptine are luteolytic through direct action on the D_2-dopamine receptors in the pituitary gland (Verstegen *et al.,* 1999; Concannon *et al.,* 1987), but metergoline is a serotonin agonist that becomes dopaminergic at high doses (Gobello, 2006). Despite the ability of metergoline to decrease the plasma progesterone concentrations below 8 nmol/l, Gerstenberg and Nöthling (1995) demonstrated that this dopamine agonist, administered at 0.4-0.5 mg/kg twice daily during mid-gestation in the bitch, is not effective for abortion-induction without $PGF_{2\alpha}$. However, metergoline, administered alone at 0.6mg/kg twice daily during the three weeks of gestation, is sufficient to induce complete luteolysis and abortion once plasma progesterone concentrations dip below 4.8 nmol/l (Nöthling *et al.,* 2003).

Prostaglandins help dopamine agonists to terminate pregnancy by two methods: 1. direct effect of prostaglandins on inhibition of progesterone synthesis by the corpora lutea 2. indirect effect secondary to interruption of prolactin secretion (Feldman and Nelson, 2004). In the African wild dog, a protocol for pregnancy termination has been devised using only the PGF2α product dinoprost tromethamine (Lutalyse, Pfizer Animal Health, Exton, PA). The protocol was devised by Henk Bertschinger and is as follows: Day 1: 50 µg/kg (roughly 1000 µg for an average size dog) IM with a pole syringe, Day 2: 100 µg/kg (roughly 2000 µg for an average size dog) IM, Day 3: 100 µg/kg IM, Day 4: 100 µg/kg. Abortion occurs on the fourth day.

2.2.4.3 Androgens

Administration of androgens to female carnivores may result in anoestrus for up to two years with continuous administration (Kutzler and Wood, 2006). Androgens may be administered

orally (methyltestosterone or mibolerone; Asa, 1999; Burke *et al.,* 1977; Gannon, 1976) or intramuscularly (testosterone proprionate; Gannon, 1976). Androgens block LH release and, thus, testicular or ovarian function (Plumb, 2002). Mibolerone (Cheque drops®, Pfizer Animal Health, Exton, PA) has been used successfully in numerous carnivore species, including dogs, cats, wolves, jaguars, lions and leopards (Gardner *et al.,* 1985; Burke *et al.,* 1977). Its use, however, is discouraged in felid species because it is hepatotoxic and thyrotoxic (Plumb, 2002). Side effects of this class of contraceptives in females include: masculinization (mane growth in lionesses) and increased aggression (Asa, 1999; Gardner *et al.,* 1985), clitoral hypertrophy, vaginitis, thickening of the cervical dermis and epiphora (Plumb, 2002; Burke *et al.,* 1977). It is recommended that androgens not be used for oestrus control for periods longer than 24 months (Kutzler and Wood, 2006).

Androgens are mildly effective for long-term suppression of spermatogenesis in male carnivores (Kutzler and Wood, 2006). In one study (England, 1997), one subcutaneous dose of testosterone esters (testosterone proprionate, testosterone phenyl proprionate, testosterone isocaproate, testosterone decanoate) caused decreased sperm motility three weeks post-injection for up to three months. Furthermore, oral administration of methyl-testosterone on a daily basis results in decreased sperm output (Freshman *et al.,* 1990). However, it has not been shown that androgens can render a male carnivore completely azoospermic.

2.2.4.4 Gonadotropin releasing hormone agonists

Gonadotropin releasing hormone agonists are the newest and potentially most promising category of hormonal contraceptives. GnRH agonists are synthetic analogues of endogenous GnRH. During the last 30 years over 2000 analogues of GnRH have been developed (Padula, 2005). Synthetic GnRH agonist peptides are resilient to degradation by peptidases, which increases their half-life in circulation and facilitates their binding properties with GnRH receptor sites (Herbert and Trigg, 2005). Additionally, chemical differences in the structure of GnRH agonists render them 200 times more potent than natural GnRH and result in their anti-fertility effects (Padula, 2005; Karten and Rivier, 1986). GnRH agonist amino acid structure, receptor affinity and function depend upon substitution of L-isomers with D-isomers; more specifically, the potency and binding affinity of a GnRH agonist depends upon the amino acids conserved and substituted within the structure (Sealfon *et al.,* 1997; Figure 4).

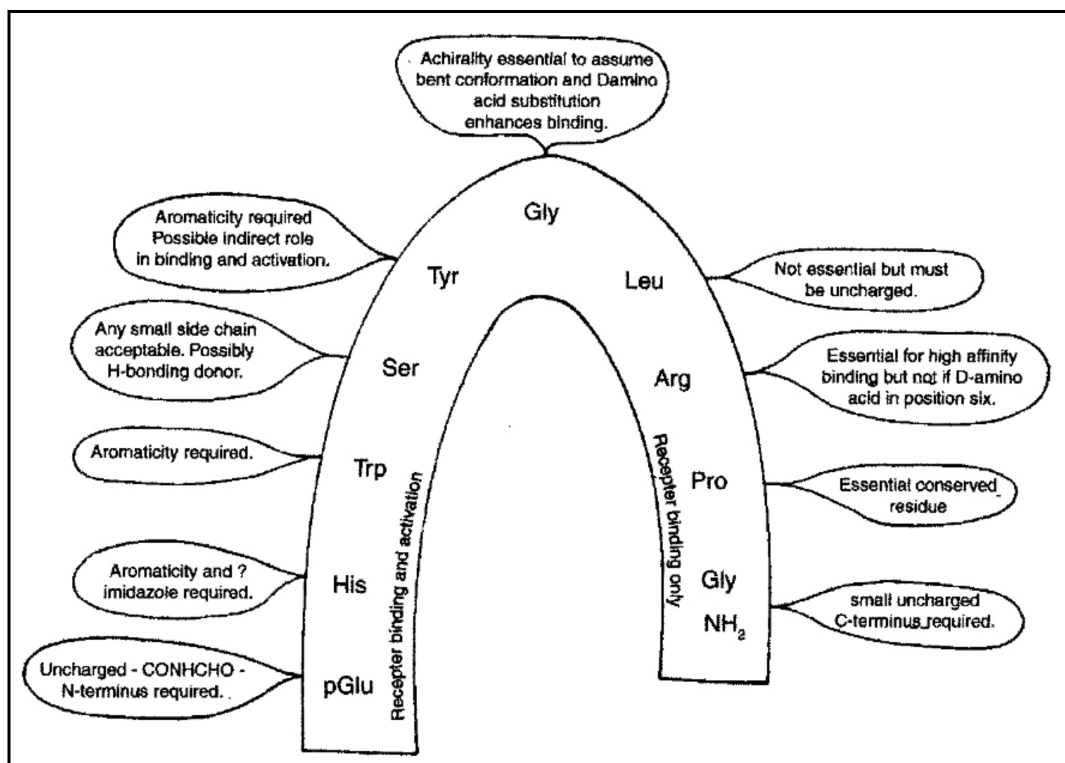

Figure 4: Representation of the GnRH peptide showing the structural and functional properties of individual amino acids (Sealfon *et al.,* 1997).

Upon administration, GnRH analogues initially stimulate the hypothalamic-pituitary axis by overriding endogenous GnRH levels, resulting in increased LH and FSH output, and subsequent increased testosterone or oestrogen production (D'Occhio *et al.,* 2000). Over time, usually one to two weeks, GnRH agonists result in decreased testosterone or oestrogen production through the negative feedback mechanism of the gondal-hypothalamic-pituitary axis (Hazum and Conn, 1988; Huckle and Conn, 1988). Continued long-term exposure to GnRH agonists results in GnRH receptor down-regulation, internalization, and signal uncoupling (Kutzler and Wood, 2006; Bertschinger *et al.,* 2006). GnRH agonists were incepted as fertility treatments prior to understanding that the feedback mechanism of the gondal-hypothalamic-pituitary axis would render the agonists contraceptives (Schriock, 1989). More recently, they were developed for contraception in the domestic dog and cat (Gobello, 2007; Trigg *et al.,* 2001; Munson *et al.,* 2001).

It is common for these agonists to induce ovulation and estrus in females shortly after administration (Munson, 2006; Pelican *et al.,* 2006; Trigg *et al.,* 2006; Herbert and Trigg, 2005; Bertschinger *et al.,* 2002; Bertschinger *et al.,* 2001; Munson *et al.,* 2001; Trigg *et al.,*

2001). Oestrus induced with a GnRH agonist may be suppressed with the administration of exogenous progestins (Wright *et al.,* 2001). Males treated with GnRH agonists will experience a short increase in systemic testosterone levels, followed by a drop of testosterone to baseline and, two to three months after administration, azoospermia (Trigg *et al*., 2001; Suprelorin® package insert, Peptech Animal Health Pty Limited, North Ryde, Australia). GnRH agonist formulations tested in domestic dogs include: leuprolide acetate, deslorelin, buserelin, lutrelin, histrelin and nafarelin, azagly nafarelin, with deslorelin and leuprolide acetate being the most commonly used (Concannon *et al.,* In press; Herbert and Trigg, 2005; Rubion *et al.,* 2003; Vickery *et al.,* 1989).

GnRH agonists are easy to administer, cost-efficient, biocompatible and, most importantly in the case of wild carnivores, allow long-term release of the contraception agent in a safe and reversible manner (Trigg *et al.,* 2001). In the case of deslorelin, contraception may be effective for 6 months to over 12 months depending on the formulation (Trigg *et al.,* 2006; Trigg *et al.,* 2001). Unlike the other classes of hormones mentioned above, GnRH agonists have had no untoward side effects reported (Trigg *et al.,* 2006). GnRH agonists are either formulated as implants or as microspheres of synthetic agonist suspended in a lipophylic vehicle (Herbert and Trigg, 2005). Most of the vehicle-based products were developed for use in humans and have a longevity of only one to three months. However, the implantable Suprelorin® is effective for six or more months (Herbert and Trigg, 2005; Trigg *et al.,* 2006). Originally developed for contraception of the domestic cat and dog, the safety, reversibility and long-acting release of GnRH agonists has made them attractive for use in zoo animals and free-ranging wildlife species.

GnRH agonists have been implemented successfully for control of fertility and aggression in a number of carnivores. Leuprolide acetate was one of the first GnRH agonists to be used as a contraceptive agent in dogs. In a study by Inaba *et al.,* (1996), reversible contraception with a long-acting (LA) leuprolide injection resulted in decreased testosterone and spermatogenesis in male dogs. After a single LA leuprolide injection, complete recovery of spermatogenesis occurred at 20 weeks and testosterone rose to pre-injection levels at 11 weeks (Inaba *et al.,* 1996). The mechanism by which pokeweed toxin works is quite different to that of GnRH agonists. The toxin is internalised within the GnRH molecule and destroys the pituitary cell. A GnRH agonist conjugated with pokeweed administered parenterally to domestic dogs as three injections over three days or as a single bolus injection resulted in decreased testis volume and decreased testosterone and LH levels for up to 20 weeks (Sabeur *et al.,* 2003).

Since its research in dogs, leuprolide acetate has been used in a number of zoo carnivores as well. Leuprolide acetate depot formulations administered at monthly intervals to male California sea otters resulted in decreased aggression, testicular volume, and serum testosterone. However, otters experienced anorexia, lethargy and injection site reactions to the hormone formulation (Calle *et al.,* 1998). More recently, a study by Pelican *et al.,* (2006) demonstrated that leuprolide acetate could be used to suppress oestrus and render the ovaries more sensitive to equine chorionic gonadotropin (eCG) and human chorionic gonadotropin (hCG) for subsequent artificial insemination in the clouded leopard. In addition to leuprolide, buserelin acetate has also been implemented in the contraception of wild carnivores. For example, buserelin has been used successfully in a multidose induction-phase and single annual dose maintenance-phase for fertility control in male harbour seals (Siebert *et al.,* in press). In many cases, these two injectable GnRH agonists have been replaced by deslorelin as the choice for contraception of zoo carnivores due its formulation and the longevity.

Deslorelin implants, which are 100 times more potent than GnRH (Padula, 2005), have been used successfully to control fertility and aggression in male red pandas (Bertschinger *et al.,* 2004b), male and female cheetah (Bertschinger *et al.,* 2006; Bertschinger *et al.,* 2001), female leopard, male and female wild dog, male and female lion, male sea otter, male and female red wolves, male and female grey wolves, male black-footed cat, female fennec fox and male bush dog (Bertschinger *et al.,* 2001). Six mg deslorelin implants were administered in all carnivore species mentioned with the exception of the lion, which received a 12 mg implant, and the fennec fox, which received a 3 mg implant (Bertschinger *et al.,* 2001). Deslorelin implant treatment may fail in seasonal breeders if it is administered too late or if too low a dose is used. Based on repeated trials, reduction of testosterone synthesis in wild dog and cheetah takes about four to six weeks, while cessation of spermatogenesis takes a bit longer (Bertschinger *et al.,* 2006; Bertschinger *et al.,* 2004a; Bertschinger *et al.,* 2001). Male cheetah experience no side effects from the deslorelin implant after repeated annual use for up to seven years, and it is a safe and reliable means of reversible male contraception in this species (Bertschinger *et al.,* 2006; Bertschinger *et al.,* 2004a). Deslorelin implants contracept lioness for 12-18 months, female cheetah and female leopard for a minimum of 12 months, male cheetah for 21 months, and male wild dogs for approximately 12 months (Bertschinger

Table 1: Commercially available contraceptives used in carnivores in the United States

Contraceptive	Brand Name	Manufacturer	Route of Delivery
Progestins			
Megestrol acetate	Ovaban®	Schering-Plough	PO
Megestrol acetate	Megace®	Bristol-Meyers Squibb	PO
Medroxyprogesterone acetate	Provera®	Pfizer Inc.	PO
Medroxyprogesterone acetate	Depo-provera®	Pfizer Inc.	IM
Melengestrol acetate	None	ZooPharm	Implant
Levonorgestrol	Norplant®	Wyeth-Ayerst	Implant
Antigestagens or Luteolytics			
Mifepristone	Mifeprex®	Danco Laboratories	PO
Cabergoline	Dostinex®	Pfizer Inc.	PO
Bromocriptine	Parlodel®	Novartis	PO
Dinoprost tromethamine	Lutalyse®	Pfizer Inc.	IM
Cloprostenol sodium	Estrumate®	Schering-Plough	IM
Oestrogens			
Oestrodiol cypionate	ECP®	Pfizer Inc.	IM
Diethylstilboestrol	Stiphostrol®	Bayer	PO
Androgens			
Mibolerone	Cheque® Drops	Pfizer Inc.	PO
Testosterone cypionate	Depotest 100®	Hyrex	IM
GnRH agonists			
Deslorelin	Suprelorin®	Peptech Animal Health	Implant
Leuprolide acetate	None	ZooPharm	IM depot
Leuprolide acetate	Lupron Depot®	TAP Pharmaceuticals	IM depot

et al., 2002). Female wild dogs do not respond consistently to the implant but tend to show signs of oestrous three to 21 months post implantation (Bertschinger *et al.,* 2002). It is advisable to limit male wild dog exposure to oestrous females within the first six weeks post deslorelin implantation, as this species continues to make viable sperm during this period (Bertschinger *et al.,* 2002; Bertschinger, personal communication).

2.3 Hormonal control of spermatogenesis, male reproductive characteristics and libido, and suppression of these functions and traits

2.3.1 Male hormonal feedback mechanisms

The testes perform two main functions in the dog both of which are regulated by gonadotropins: the production of testosterone by Leydig cells and spermatogenesis assisted by Sertoli cells (Pineda, 2003; Johnston *et al.,* 2001; Barth and Oko, 1989). Gonadotropins are synthesized and released from the adenohypophyseal cells of the hypothalamus and act on the anterior pituitary to stimulate the release of FSH and LH in a pulsatile manner. LH specifically stimulates secretion of testosterone by the Leydig cells (Pineda, 2003). High local concentrations of testosterone in the testes are necessary for spermatogenesis and may be maintained by transferral of testosterone from the pampiniform plexus to the testicular artery (Pineda, 2003). Whereas, FSH stimulates cell division of the germinal epithelium and uptake of Leydig-produced androgens by the Sertoli cells for conversion to estrogens. The testosterone produced by the Leydig cells is also taken into the systemic circulation and has global effects on: 1) the androgen-dependent organs in the body like the prostate and penis, 2) secondary sexual characteristics, and 3) libido and masculinization of the brain. Furthermore, testosterone in systemic circulation results in negative feedback on the hypothalamus and its production of GnRH, which subsequently results in decreased production and release of FSH and LH (Figure 5).

2.3.2 Spermatogenesis

Spermatogenesis occurs within the seminiferous tubules and consists of several steps through which spermatogonia become mature spermatozoa: 1) spermatogoniogenesis (mitosis), 2) spermatocytogenesis (meiosis), 3) spermiogenesis and maturation of spermatids, and 4)

spermiation (Holstein *et al.,* 2003). There are two main types of spermatogonia: A type and B type. A type spermatogonia belong to the germ cell pool, whereas, B type spermatogonia form the onset of germ cell development (Holstein *et al.,* 2003; Barth and Oko, 1989). Spermatogonia are located at the base of the seminiferous tubule and undergo a number of mitotic divisions to become spermatocytes located a layer higher in the tubule (Barth and Oko, 1989). These cell divisions are incomplete and the spermatocytes maintain a syncytial

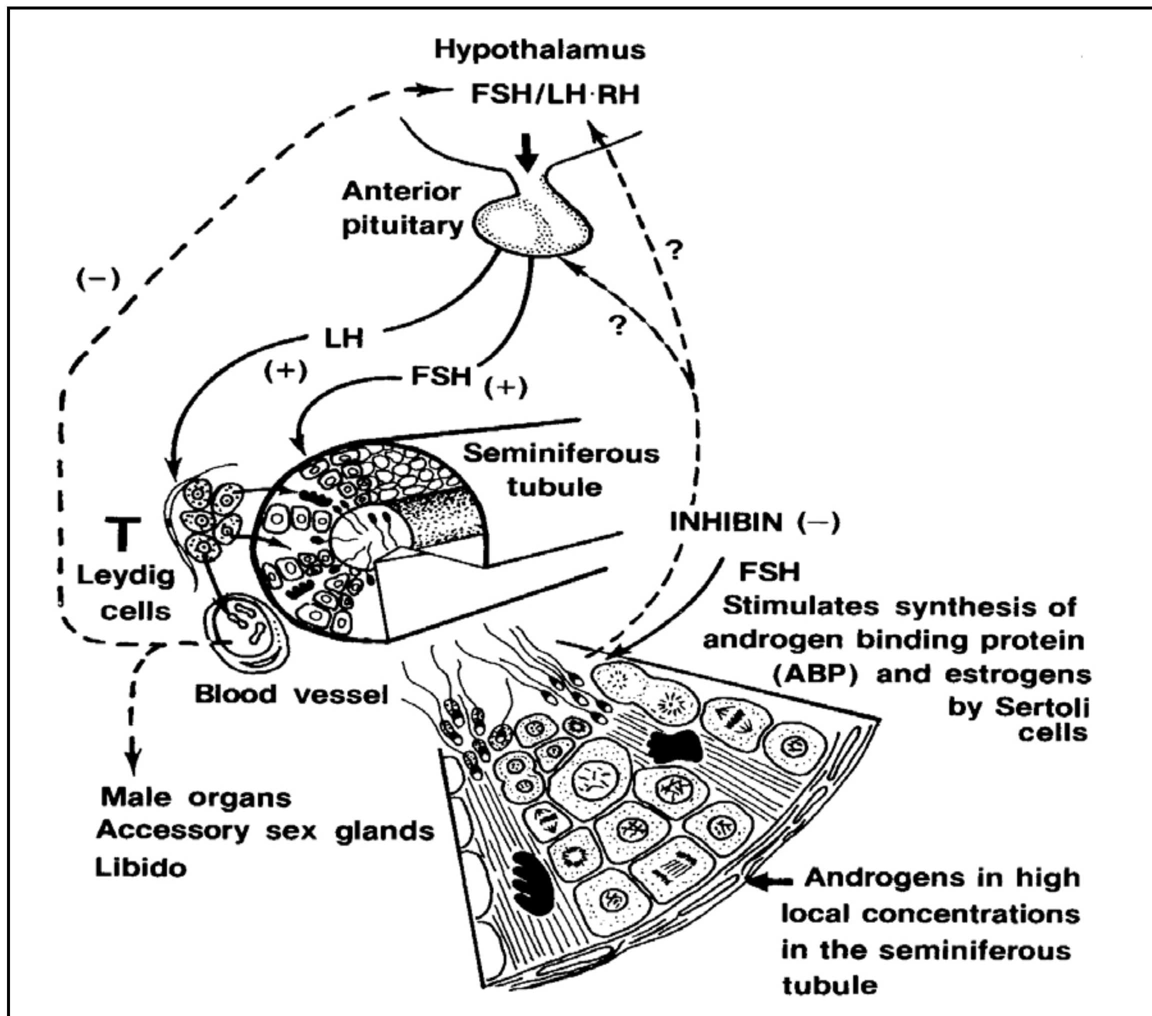

Figure 5: Relationship between hormonal control and testicular function (Pineda, 2003).

connection with each other. Additionally, not all spermatogonia divide to become spermatocytes; type A spermatogonia divide to continue the germ cell pool. The spermatocytes go through a number of meiotic divisions to become the haploid cells known as spermatids. Meiosis is a fragile process that is susceptible to defects and interruptions (Holstein *et al.,* 2003).

During stage 3, spermiogenesis, spermatids grow a tail, acquire a thickened midpiece where mitochondria will reside, form an axoneme, and their DNA becomes highly condensed. Similar to spermatocytogenesis, this process is frequently affected by noxious agents, which results in malformed acrosomes, absent acrosomes, malformed flagella, and poor nuclear condensation. The differentiation of the germ cells to spermatids is accompanied by upward mobility within the layers of the seminiferous tubule (Barth and Oko, 1989). Sertoli cells assist the mature spermatids to the lumen of the lumen of the seminiferous tubules. At the lumen, the spermatids close their syncytial cytoplasmic bridges and leave the germinal epithelium to become free cells called spermatozoa (Holstein *et al.,* 2003; Barth and Oko, 1989). Please refer to Figure 6.

2.3.3 Sertoli cells

Sertoli cells provide essential physical, nutritional and hormonal support to spermatogenesis (Barth and Oko, 1989). They change their shape in order to surround and move the developing germ cells through the seminiferous tubule cell layers; furthermore, the Sertoli cells, which can produce upwards of 60 different proteins, also change their metabolism based on the germ cell development level with which their assistance is needed. Sertoli cells phagocytose superfluous or defective germ cells and remove residual cytoplasm from spermatids prior to spermiation. Developing germ cells (haploid) are protected from the immune system of the body through maintenance of the blood-testis barrier by Sertoli cells' "tight junctions" (Holstein *et al.,* 2003; Johnston *et al.,* 2001). After spermatozoa are released into the lumen of the seminiferous tubules and travel toward the epididymis, Sertoli cells couple testosterone manufactured by the Leydig cells with androgen-binding protein (ABP) and secrete this complex into the lumen. The testosterone-ABP complex travels to the epididymis where the epididymal epithelium absorbs the complex to stimulate its own metabolism (Holstein *et al.,* 2003). In the epididymis testosterone is separated from ABP and is converted to dihydro-testosterone, which is actually responsible for the simulation of epididymal function (Pineda, 2003). Malfunction of Sertoli cells results in severe adverse effects on spermatozoa production.

The basal lamina is the location at which germ cell development begins and Sertoli cells await involvement in the process. In addition to forming a structural base for the seminiferous

tubules, the basal lamina promotes movement of lipid-soluble steroids through its layers to facilitate communication between the Sertoli cells and the pituitary and hypothalamus. Sertoli cells produce oestradiol from testosterone and secrete this to cause a negative feedback on GnRH and FSH secretion (Holstein *et al.,* 2003). Inhibin is secreted systemically by the

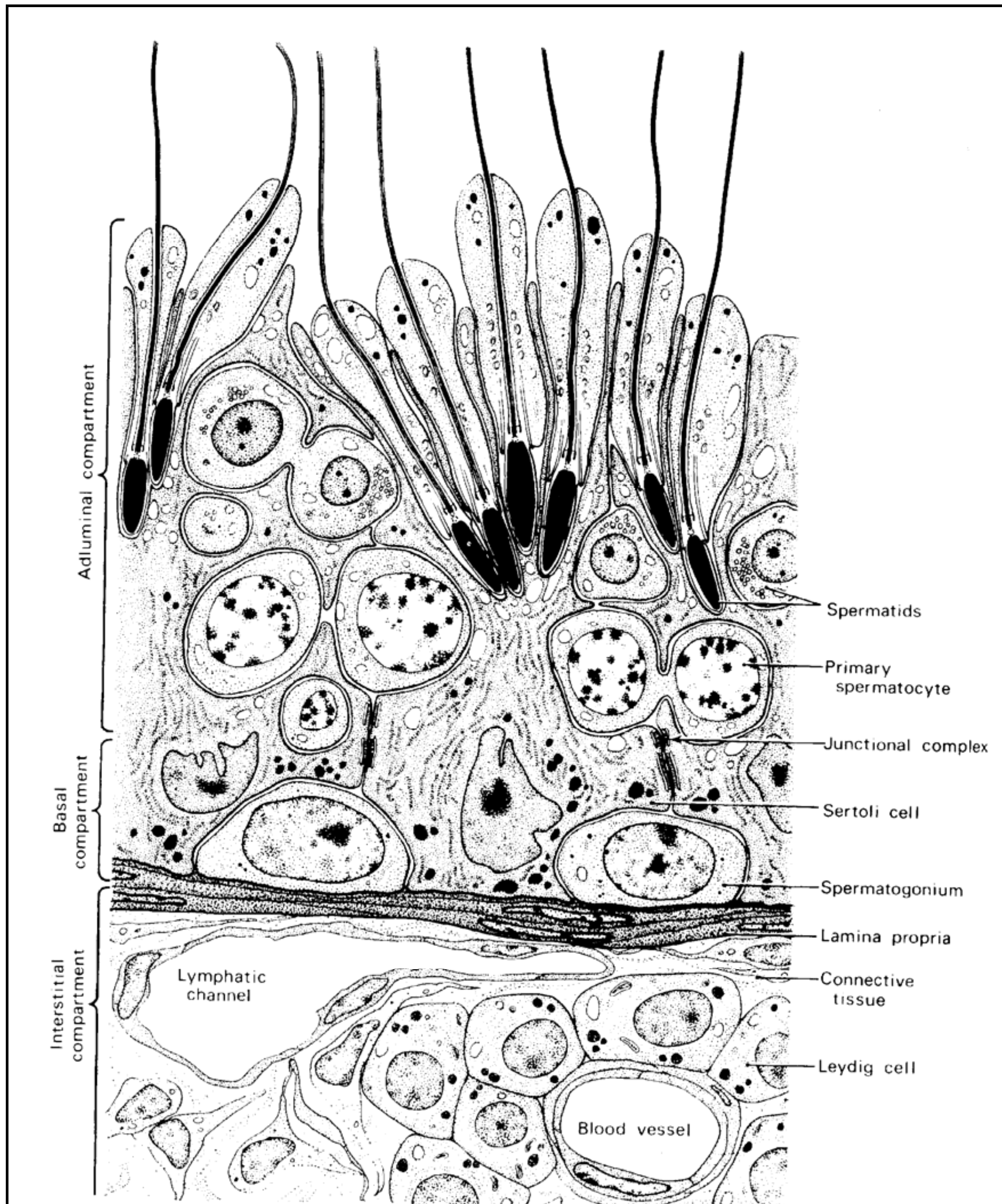

Figure 6: Seminiferous tubule and spermatogenesis (Johnston et al., 2001)

Sertoli cells to cause a negative feedback on FSH secretion only. Sertoli cells also direct the secretion of testosterone by the Leydig cells through the release of inhibin (stimulation) and

activin (suppression). FSH communicates directly with Sertoli cells to force an increase in their metabolic rate. LH indirectly affects spermatogenesis by instructing the Leydig cells to produce more testosterone (Holstein *et al.,* 2003). Prolactin released by the pituitary induces receptors for LH on the surface of Leydig cells and, therefore, also stimulates spermatogenesis.

2.3.4 Leydig cells

Leydig cells have recently been shown to have a neuroendocrine role, secreting serotonin, catecholamine synthesizing enzymes, neuropeptides and growth factors (Holstein *et al.,* 2003). Leydig cells are selectively stimulated to produce testosterone through local secretion of inhibin and activin by Sertoli cells. Thus, Sertoli cells are responsible for hypertrophy of Leydig cells during stages of spermatogenesis that require higher amounts of testosterone like stages VI and VII. Furthermore, the amount of testosterone produced in the testes is not related to the number of Leydig cells but the activity of the cells (Holstein *et al.,* 2003).

2.3.5 Suppression of androgen production and effects

Androgen production is necessary for spermatogenesis, maintenance of general sexual characteristics (eg, muscular and skeletal development), maintenance of special sexual characteristics (eg, antlers, manes and plumage), accessory organ size and function, libido and masculinization of the brain (Pineda, 2003). The germinal epithelium in the testis has a much higher requirement for androgens than other androgen-dependent tissues (Pineda, 2003). Suppression of androgen production through hormonal contraception like GnRH or through orchidectomy results in sharp decline in a plasma testosterone levels, atrophy of accessory organs and secondary sexual characteristics (Pineda, 2003; Martin and Lindsay, 1998). Peri-pubertal animals that experience suppression or removal of androgens demonstrate earlier epiphyseal closure and less developed skeletal structure (Pineda, 2003). Similarly, androgens have myotropic activity and their suppression results in atrophy of musculature (Pineda, 2003). Androgens also influence hair growth and pigmentation in mammals; the hair coat of masculine males tends to be darker and coarser than orchidectomized animals or females (Pineda, 2003). Orchidectomy of adult animals results in atrophy of accessory sexual organs (Pineda, 2003); however, function of accessory sexual organs appears to be a more accurate assessment of androgenic activity than size (Pineda, 2003). The continuous secretion of

androgens over the lifetime of a dog is responsible for enlargement of the prostate gland due to proliferation of glandular and stromal segments and the increase in the size of epithelial cells; similarly, orchidectomy or androgen suppression results in atrophy of the canine prostate gland (Lee, 1996; Zirkin and Strandberg, 1984; James and Heywood, 1979). Furthermore, androgens stimulate the growth of the penis and the preputial lamellae that form the preputial cavity (Pineda, 2003).

GnRH analogues have been shown to decrease testosterone levels in male dogs (*Ogawa et al.,* 1989). Inaba *et al.* (1996) demonstrated that leuprolide acetate (LA) does cause disturbances in spermatogenesis, including poorly motile spermatozoa and morphologically abnormal spermatozoa, through decreases in testosterone and LH that occur approximately one week after LA administration in the male dog. Production and secretion of GnRH is required for testicular development and function (Adams, 2005; Marshall *et al.,* 1983; Anderson, 1977).

In boars, long-acting GnRH agonists have been used to decrease systemic testosterone levels and, subsequently, to decrease the level of androstenone in the back fat and salivary glands and to decrease testicular size (Schneider *et al.,* 1998; Xue *et al.,* 1994). Androgen dependent behavioural traits are also affected by GnRH agonists (Adams, 2005). Additionally, GnRH antagonists and agonists will also decrease testicular size, libido and ejection fraction volumes in male dogs but may be unable to induce complete disruption of spermatogenesis as characterized by azoospermia (Valiente *et al.,* in press 2007; Paramo *et al.,* 1993). By contrast, GnRH agonists that will elevate testosterone for a period of time after their administration (as opposed to one week only) can result in increased libido and peripheral LH and testosterone levels in dogs (Purswell and Wilcke, 1993).

2.4 Use of vehicles in long-acting hormonal contraception

2.4.1 Lipid-based vehicles

Extended-release, parenteral administration of hormonal contraceptives requires the use of lipid-based vehicles. Lipid-based vehicles may retain the hormonal contraceptive within the aqueous environment of the muscle tissue, encapsulated in a lipid liposome (Uchegbu, 1999a, Uchegbu, 1999b). In an aqueous environment, phospholipid molecules arrange themselves in

a bilayer sphere with the lipophilic portion inside the sphere and the hydrophilic portion outside the sphere (Uchegbu, 1999a, Uchegbu, 1999b; Figure 7). Thus, hydrophobic synthetic hormones are encapsulated within the muscle in the centre of liposomes and are released slowly over time.

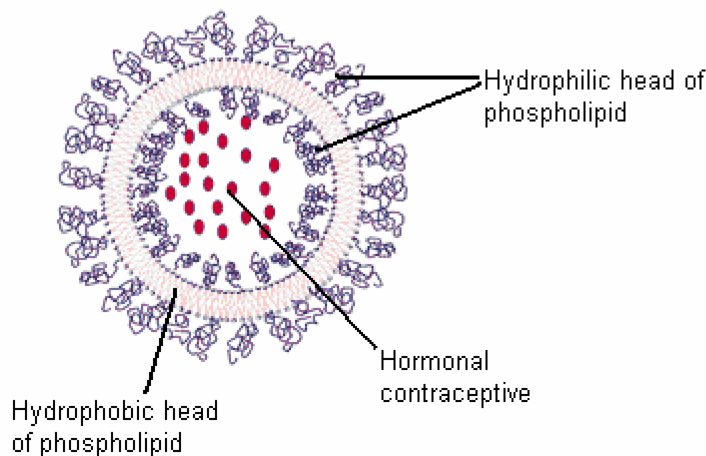

Figure 7: Liposomal encapsulation of hormonal contraceptive (adapted from Uchegbu, 1999a)

Lipid-based vehicles used previously for slow-release of GnRH agonists include: a 9:1 ratio of soybean L-α-lecithin: cholesterol (Mezei and Nugent, 1984), the ATRIGEL® drug delivery system (Atrix Laboratories, Inc., Ft. Collins, CO; Dunn *et al.,* 1994), and the polymer 85/15 poly (DL-lactide-co-glycolide) mixed 50:50 with N-methyl-2-pyrrolidone (Baker *et al.,* 2005).

2.4.2 Non-lipid-based vehicles

All classes of hormonal contraceptives have been administered in long-acting injectable formulations. Depo-provera® (Pfizer Inc, New York, NY), a synthetic progestin administered intramuscularly, is dispensed as a suspension comprised of polyethylene glycol, polysorbate 80, methylparaben, and propylparaben and sterile water (Depo-provera® contraceptive injection package insert, Pfizer Inc, New York, NY). Similarly, the GnRH agonist, leuprolide acetate or Lupron Depot® (TAP Pharmaceutical Products Inc, Lake Forest, IL), also contains polymers like: carboxymethylcellulose sodium, D-mannitol, D-lactic and glycolic acids copolymer, and polysorbate 80 (Lupron Depot® 3 month 11.25mg package insert, TAP Pharmaceutical Products Inc, Lake Forest, IL).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study site and husbandry of wild dogs

3.1.1 De Wildt Cheetah and Wildlife Center

The study site for this project, De Wildt Cheetah and Wildlife Center (De Wildt), was established in 1971 by Ann Van Dyke with the aim of breeding cheetah to support the long-term survival of this species outside protected areas in South Africa. De Wildt is 65 hectares in size and located at 25˚ 45' S latitude and 28˚ 14' E longitude in the Magaliesberg Mountains near Hartebeespoort Dam, South Africa.

In 1978, the wild dog breeding program at De Wildt began as a partnership with the National Zoo with a pack of 6 wild-caught dogs from Namibia. In the 1980's, offspring of this original group were released successfully into Hluhluwe Imfolozi Game Reserve in Kwa Zulu Natal. SASOL became a major partner with the wild dog breeding program in 1994, and their funding supported the construction of new enclosures designed to separate breeding lines, research, and releases into protected areas within South Africa. Successful releases of wild dog into Madikwe Reserve in 1995 and Venetia Game Reserve in 1999 were supported by SASOL. The De Wildt wild dog management program aims to ensure the long term survival of the free roaming wild dog and its ecosystem through the implementation of a management plan devised through integrated conservation plans, research, and education. Since the wild dog breeding program began, De Wildt has produced over 500 wild dog pups and releases of De Wildt wild dogs into the wild have been conducted in Hluhluwe Imfolozi Game Reserve, Etosha Game Reserve, Madikwe Reserve, Pilanesburg National Park, Karongwe Reserve, Marakele and Venetia Game Reserve.

3.1.2 African wild dog enclosures

During the study a total of 75 wild dogs, consisting of 26 females and 49 males, were housed at this facility. The 27 study animals were housed in 16 outdoor enclosures fenced with wire mesh and partially covered with indigenous bush. Ten of the enclosures were also fitted with a corner camp and an associated crush, which facilitated the capture of study animals for

immobilization and sample collection (Figure 8). All study dogs had olfactory and auditory contact with dogs in other enclosures. With the exception of two enclosures, containing two and four study dogs respectively, all study animals had visual contact with dogs in other enclosures. For an exact map of the dog enclosures refer to Appendix A.

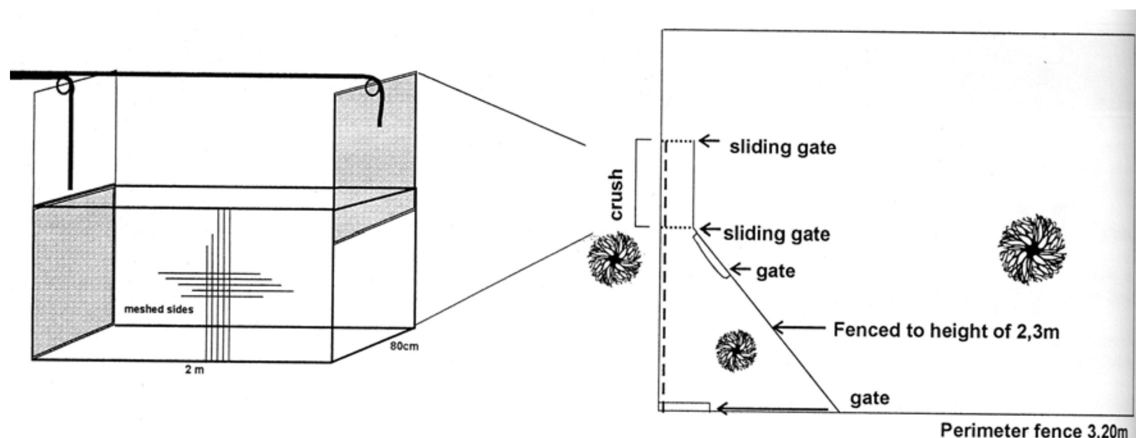

Figure 8. Design of enclosures with corner camps and crushes

3.1.3 Husbandry of African wild dogs

Each study dog was fed either a piece of raw horse meat (~0.5 kg), one whole chicken, or approximately 2 cups of pelleted Iams Adult Canine diet (The Iams Company, Dayton, Ohio, USA) once daily, six days a week. On the seventh day of the week dogs were starved for management purposes. Water was available *ad libitum.* Enclosures were cleaned of faecal matter and bones two times a week. Two times a year, each study dog was dewormed with pyrantel pamoate.

3.2 Study animals

3.2.1 Study animal past breeding success, contraception and current treatment type

Table 2 lists the male African wild dogs in the study, their age at the time of treatment, treatment administered, their previous breeding status, and their previous contraception status.

Table 2. Male African wild dogs (n=27) administered treatment (August and Sept. 2005)

			Previous breedings		Previous contraception	
Dog Id	Age (yr)[a]	Treatment[b]	Number of litters	Dates	Dose of deslorelin (mg)[c]	Date
M107	9.9	I	2	Nov. '02, May '05	Naïve	N/A
M130	9.1	J	2	May '04, May '05	4.7	Feb. '02
M173	7.25	J	2	May '01, May '02	Naïve	N/A
M176	7.25	I	1	Oct. '04	9.4	Sept. '04
M186	7.25	P	4	May '01, April '02, March '03, May '04	9.4	Dec. '04
M212	6.2	J	1	May '04	9.4	Dec. '04
M216	6.7	I	4	April '02, April '03, Nov. '03, April '05	9.4	Nov. '04
M255	4.4	I	0	N/A	9.4	Dec. '04
M256	4.4	J	2	May '04, May '04 (2 dams)	9.4	Dec. '04
M273	4.4	J	0	N/A	9.4	June '04
M287	4.2	I	0	N/A	9.4	Nov. '04
M301	4.2	J	0	N/A	9.4	Dec. '04
M325	3.4	J	0	N/A	4.7	Oct. '03
M333	3.3	I	0	N/A	9.4	Dec. '04
M334	3.3	P	0	N/A	Naïve	N/A
M341	3.3	J*	0	N/A	Naïve	N/A
M343	3.3	P	0	N/A	Naïve	N/A
M344	3.3	I	0	N/A	Naïve	N/A
M357	3.2	I	1	May '04	9.4	Dec. '04
M369	2.5	P	0	N/A	9.4	Dec. '04
M376	2.4	P	1	April '05	Naïve	N/A
M377	2.4	P	1	April '05	Naïve	N/A
M380	2.2	I	0	N/A	9.4	Dec. '04
M421	1.3	J	0	N/A	Naïve	N/A
M422	1.3	J	0	N/A	Naïve	N/A
M439	1.3	J	0	N/A	Naïve	N/A
M440	1.3	I	0	N/A	Naïve	N/A

[a]Age at treatment (figures to the right of the decimal indicate fractions of a year).
[b]Treatment administered for study: 4.7 mg deslorelin implant (I);~9.4 mg deslorelin injection (J); 2 ml vehicle as a placebo injection (P).
[c]Deslorelin contraception administered prior to the start of the current study.
*This dog received an additional 4.7 mg deslorelin implant in January 2006.

With the exception of two enclosures, containing two (M255 and M256) and four (M341, M343, M344, M334) study dogs respectively, all study animals had visual contact with dogs in other enclosures. At the start of the study, ten dogs were paired with female dogs, eight dogs were housed only with other male study dogs (two pairs and one group of four), one dog

was housed singly, and eight dogs were housed in mixed-sex groups of four or more dogs (Table 3). Also at the start of the study, two of the males paired with females (M107 and M130) where housed with their pups, which were sired in May 2005. Due to managerial needs, some dogs were moved during the course of the study. At the conclusion of the study, ten dogs were still paired with females (seven of which were paired with the same females they had been with during the entire study), all eight dogs housed only with other male study dogs were still housed as they had been at the beginning of the study, two dogs previously paired with females were housed singly, and six dogs were housed in mixed-sex groups (Table 3). Our study group consisted of the 13 male dogs contracepted in 2004, 12 naïve male dogs, and two male dogs respectively contracepted in 2002 and 2003. Males were randomly assigned to treatment groups without knowledge of their breeding or contraceptive history.

3.3 Treatments

3.3.1 Implant formulation

The deslorelin implant formulation used in this study had been used previously as a contraceptive for both female and male African wild dogs at De Wildt (Bertschinger et al 2001, Bertschinger et al. 2002), although the implants used previously each contained 6 mg of deslorelin, compared to 4.7 mg in the current study. The implant, Suprelorin (Peptech Animal Health Pty Limited, North Ryde, Australia), consists of a biocompatible, slow-release lipid-based implant diffusely impregnated with deslorelin acetate, which is a synthetic GnRH analogue. The active ingredient, deslorelin acetate, is [6-D-tryptophan-9-(N-ethyl-L-prolinamide)-10-deglycinamide]GnRH. The cylindrical implant comes in an implanter contained in a foil pouch. Just prior to administration, the Luer-lock cap is removed from the implanter in order to attach the implantation syringe. The implant is delivered through the combined apparatus with a 14-gauge needle. This product was developed for the reversible contraception of male domestic dogs. In male domestic dogs it is 100% effective and will maintain plasma testosterone levels below 1 ng/ml in 97.7 % of treated male domestic dogs for longer than 6 months (Suprelorin product insert). Furthermore, Suprelorin will decrease circulating testosterone levels below 1 ng/ml within eight to 14 days after implantation in male domestic dogs (Suprelorin product insert).

Table 3. Housing arrangements for study animals at the onset and end of study (numbers per enclosure)

Dog Id	At study onset				At study end			
	Enclosure name	No. of male dogs*	No. of female dogs	No. of pups	Enclosure name	No. of male dogs*	No. of female dogs	No. of pups
M107	Kennel 42	0	1	4	Kennel B	1	2	0
M130	Kennel B	0	1	3	OQ 29	0	0	0
M173	Kennel 45	0	1	0	Kennel 45	0	1	0
M176	Sasol F	0	1	0	Sasol F	0	1	0
M186[b]	JC	1	0	0	JC	1	0	0
M212	Kennel 46	0	1	0	Kennel 46	0	1	0
M216	Sasol B	0	1	0	OQ 31	0	0	0
M255[a]	Big Sasol	1	3	0	N/A#	N/A	N/A	N/A
M256[a]	Big Sasol	1	3	0	OQ30	0	1	0
M273	Sasol E	0	1	0	Sasol E	0	1	0
M287	Quar. A2	0	1	0	Quar. A2	0	1	0
M301	Kennel 43	0	1	0	Kennel 43	0	1	0
M325	Quar. A1	0	1	0	Quar. A1	0	1	0
M333[c]	Compound	3	3	0	Compound	2	3	0
M334	School	3	0	0	School	3	0	0
M341	School	3	0	0	School	3	0	0
M343	School	3	0	0	School	3	0	0
M344	School	3	0	0	School	3	0	0
M357[c]	Compound	3	3	0	Compound	2	3	0
M369[b]	JC	1	0	0	JC	1	0	0
M376[d]	Quar. B2	1	0	0	Quar. B2	1	0	0
M377[d]	Quar. B2	1	0	0	Quar. B2	1	0	0
M380	TC	0	0	0	Kennel A	0	1	0
M421[c]	Compound	3	3	0	Sasol B	0	1	0
M422[c]	Compound	3	3	0	Compound	2	3	0
M439[e]	Kennel 44	1	4	0	Kennel 44	1	4	0
M440[e]	Kennel 44	1	4	0	Kennel 44	1	4	0

* Number of male dogs in enclosure, excluding the study dog in question.
\# This animal died during the study.
[a] Letters indicate which study dogs were housed with one another.

3.3.2 Vehicle formulation for injection and placebo treatments

The experimental vehicle consisted of 1 methyl-2-pyrrolidone and the polymer, 85/15 poly (DL-lactide-co-gylcolide) (both from Sigma-Aldrich, Aston Manor, South Africa), mixed in a 3:1 ratio by weight. This lipid vehicle formulation was modified slightly from one used by Baker et al. (2005) in order to decrease its viscosity and ensure ease of delivery through an 18-gauge needle. Once the two vehicle components were combined, the mixture was placed in a 150 ml glass beaker in an ultrasonic cleaner (Transsonic T310, Labotec, Halfway House, South Africa) for four hours to insure adequate dissolution of the solid phase poly (DL-lactide-co-glycolide) into the liquid phase 1-methyl-2-pyrrolidone. Upon complete mixing of the two ingredients, ~10 ml of the resulting solution was transferred to 15 ml glass vials. The vials were capped with rubber stoppers and sealed with a metal ring top. The bottles were sterilized by γ-irradiation at a dose of approximately 25 kGy (Isotron South Africa (Pty) Ltd, Kempton Park, South Africa). The vials were then stored at room temperature (~20 ˚C) until use.

3.3.3 Preparation of the injectable formulation

Taking care to abide by sterile technique, the experimental injectable formulation was made by pulverizing ten 4.7 mg deslorelin implants and adding them to an uncapped 10 ml vial of the lipid-based vehicle. This created an injectable suspension containing approximately 4.7 mg of deslorelin per millilitre. The experimental injection was mixed thoroughly with an autoclave-sterilized metal rod before the bottle was recapped. All procedures were carried out under an ultraviolet hood (Gelaire VB85, Gelaire Pty Ltd, Sydney, Australia). The experimental injection was stored at room temperature (~20 ˚C) until use.

3.3.4 Treatment administration and dose

On Day 0, after baseline measurements of study parameters had been taken, each study animal received one of the following treatments: One 4.7 mg deslorelin implant placed subcutaneously in the lateral cervical region using an implanter as shown in Figure 7 (Group I); 2 ml of experimental injection containing ~9.4 mg of deslorelin, of which half was administered via an 18 gauge needle into the caudal muscles of each thigh (Group J); or 2 ml of experimental placebo vehicle, of which half was administered via an 18 gauge needle into the caudal muscles of each thigh(Group P). After administration of the vehicle and

experimental injection, pressure was applied to each injection site for two to three minutes to prevent subcutaneous leakage of the vehicle.

Figure 9:Administration of a subcutaneous implant in the lateral cervical region

3.4 Timeline and experimental procedures

3.4.1 Timeline

Treatment (implant, injection or placebo) was administered on Day 0, which was one of the following dates: August 23, 24, 25, 30, or September 1, 2005. On the same day, each animal was examined to obtain baseline data. Each dog was examined once at a specified interval after treatment in order to assess the effects of treatment. The examination dates were staggered within treatment groups on a random basis (Table 4). The scheduled dates for post-treatment examinations were November 28, 2005; January 16, February 23, April 3, May 24, August 29, and August 30, 2006. The exception to this statement was dogs assessed a year from the date of treatment (M173, M176, M186, M212, M216, M273, M301, M369, M376, M377, M380). This group of dogs was assessed at that time so haematology and blood biochemistry before and after treatment could be compared. Table 4 provides an overview of when and how each dog was treated and examined.

Table 4. Sample collection dates with dogs assessed in the different treatment groups by date

	Treatment type		
	Placebo	**Injection**	**Implant**
August 2005	M344, M343	M325, M341, M256, M422, M130, M173, M212, M301, M439, M273	M287, M344, M255, M357, M107, M440, M176, M216, M380
September 2005 (before treatment)	M186, M369, M376, M377	M421	
September 2005 (after treatment)	M343	M301, M256, M421, M301	
October 2005		M421	
November 2005	M344, M343 M186	M341	M344
December 2005	M377		
January 2006	M344, M343 M186	M325, M341 M421	M344, M287, M107 M216
February 2006	M334	M422 M341	M333, M257 M344
April 2006	M334, M343	M421, M439	M344, M440
May 2006		M130, M256	
June 2006			M380
August 2006	M186, M376, M377, M369	M273, M301, M173, M212 M256	M380, M176, M216

Table key: Red=serum testosterone measured only (opportunistic sampling); Yellow=serum testosterone and testes volume measured (opportunistic sampling); Green=all parameters measured (scheduled sampling); M186=all green parameters but semen quality were assessed.

For ethical reasons, most study animals were immobilized on two occasions only: the first for baseline procedures and treatment, and the second for post-treatment examination and sample collection. However, each of the dogs housed together in one group (M334, M341, M343 and M344) was immobilized and examined on three post-treatment sampling dates in order to better track potential reproductive system changes during the course of the study. This group consisted of two placebo dogs (Group P), one implant dog (Group I) and one injection dog (Group J). M341 was initially assigned to Group J but on February 1, 2006 he was also given an implant in an attempt to down-regulate his aggression toward the other dogs in the same enclosure. In addition to the above, opportunistic samples were collected from study dogs that were immobilized for reasons unrelated to the study, such as illness or fight wounds (Table 4).

3.4.2 Immobilization

Study dogs were immobilized by means of a dart gun or pole syringe with 70-110 mg Zoletil 100® (tiletamine, zolazepam combination, Virbac, France). One and a half ml Pneu-darts™ (Pneu-Dart, Inc. Williamsport, PA) were loaded with Zoletil 100® and fired from a Dan-inject dart gun (Dan-inject ApS, DK-7080 Børkop, Denmark) fitted with a modified barrel that accommodated Pneu-darts™. Dogs that could be chased into a crush were injected intramuscularly with Zoletil 100® by means of a pole syringe (Paxarms, Timaru, New Zealand). Once a dog was laterally recumbent with loss of voluntary movement, which occurred five to 10 minutes after drug administration, it was blind folded, loaded onto a truck, and taken to the hospital area for examination and sample collection. On arrival at the hospital, each dog was weighed and administered 0.1-0.6 mg medetomidine hydrochloride (Domitor, Pfizer Animal Health, Exton, PA) intramuscularly. On return to the camp, the effects of the medetomidine were reversed with 0.1-0.4 mg/kg atipamezole hydrochloride (Antisedan, Pfizer Animal Health, Exton, PA).

3.4.3 Procedures and sample collection

At each scheduled immobilization (before and after treatment), blood, urine and semen were collected. Additionally, measurements of the prostate testes and prepuce were conducted at each immobilization. The dimensions of the prostate and testes were used to calculate the approximate volumes of the respective organs. All anatomical measurements made on-site were recorded on the data sheet found in Appendix B.

3.4.3.1 Blood samples

Blood for the determination of the serum testosterone concentration and blood biochemistry (n=11) values was collected in red-topped tubes (BD vacutainer, Beliver Industrial Estate, Plymouth, UK) from either the cephalic or the lateral saphenous veins. Blood for haematology was collected in EDTA (BD vacutainer, Beliver Industrial Estate, Plymouth, UK) tubes (n=11). Blood collection occurred within 30 minutes of immobilization. Blood taken for serum samples was kept at room temperature until clotted and then stored at 4 ˚C for three to eight hours until processed. In the laboratory, serum was separated from the clot and blood cells by centrifugation (Beckman GS-6R Centrifuge, Beckman Coulter South Africa Pty Ltd, Halfway House, South Africa) for 15 minutes. The serum was decanted into plastic test tubes and frozen at –20 ˚C until analysed.

3.4.3.2 Urine samples

Urine was collected prior to electro-ejaculation by ultrasound-guided (Aloka 900) cystocentesis using a 5 ml syringe and a 20-gauge 1½″ needle. Upon collection, urine pH was measured with an indicator strip (Neutralit pH 5-10, Merck, Darmstadt, Germany). Following pH measurement, samples were immediately stored at 4 ˚C until analysed. In the laboratory, urine osmolality (mOsmol/kg) was assessed by placing 50 µl of urine in a 500 µl Eppendorf microcentrifgue tube (Eppendorf AG, Hamburg, Germany) and running the sample on an osmometer (Osmomat 030 cryoscopic osmometer, Gonotec GmbH, Berlin, Germany).

3.4.3.3 Semen samples

Semen samples were collected by means of electro-stimulation using a custom-built electro-ejaculator (Hans Kündig, Nürensdorf, Switzerland). This machine has variable settings for voltage output, Hz, wave pattern, and duration. The stimulus can either be automated or controlled manually. Using sterile plastic disposable gloves (Dispos-A-Glove, Johnson and Johnson, Midrand, South Africa) the penis was extruded manually from the preputial sheath, and was rinsed with a sterile physiological saline solution. The probe fitted with three ventral electrodes, which extended from a depth of 80 to 130 mm was placed into the rectum and over the internal genitalia. The position of the electrodes ensured that they were placed over the prostate gland once the probe was fully introduced to the level of the buttress**.** At this

stage a 15 or 50 ml plastic tube (Plastpro Scientific Pty Ltd, Edenvale, South Africa) was placed over the glans and electro-stimulation commenced. A 50 Hz sine wave was used and stimuli lasted 3 sec with a 3 sec rest period between each stimulation. The stimulation protocol is shown in Table 5. During the final two to three stimuli of each cycle from 2 volts and upwards the probe was slid out of the rectum while the stimulus continued and then switched off before introducing it once more. Once the dog started to ejaculate the amplitude of the stimulus was no longer increased.. Semen samples collected in a 15 or 50 ml plastic tube were maintained at room temperature until evaluation. Tubes were changed from time to time during collection from an individual dog to avoid possible contamination with urine which happens easily, especially when higher amplitudes are used. Upon collection, ejaculates were immediately assessed on-site for volume, colour, consistency, pH and motility. Semen smears were made and stored in order to assess sperm morphology at a later stage.

Table 5: Stimulation pattern used for electro-ejaculation of wild dogs

Cycle	Voltage	Number of stimuli
Cycle 1	< 1 volt (just sufficient amplitude required to produce anal contraction)	15
Cycle 2	< 1 volt (stronger anal contraction)	15
Cycle 3	1 volt	15
Cycle 4	1.5 volt	15
Cycle 5	2 volt	15*
Cycle 6	2.5 volt	15*
Cycle 7-10	3, 3.5, 4 and 5 volts	15 stimuli each*

* During the final two to three stimuli of each cycle the probe was slid out of the rectum while the stimulus continued

3.4.3.4 Volume of the prostate

The size of the prostate was measured by means of transcutaneous ultrasound. Prostate length and height were assessed on the longitudinal axis and width and height on the transverse axis. Height was taken as the average of the height values obtained in the longitudinal and transverse plains and the product of the length (L), width (W) and height (H) provided an estimation of the of the prostate. Due to the positioning and mobility of the prostate in the abdominal cavity, it was difficult to accurately measure each dimension of the organ; thus, the combined measurement value, volume, was implemented.

3.4.3.5 Testicular volume

Testicular volume (mL) was assessed by calliper measurement, in millimetres, of the length and width of each testis. The distance between the proximal and distal poles of a testis was taken as its width and the distance between its medial and lateral surfaces as its width. Testis volume was calculated by assuming that a testis is an ellipsoid and the formula for an ellipsoid is $\pi(LWH)/6$, where L, W and H are the length, width and height of the ellipsoid and, because W was assumed to be equal to H, the formula is equivalent to Volume = $\pi(LW^2)/6$ (Tapson, 1999).

3.4.3.6 Preputial measurements

The African wild dog has a number of sebaceous glands around its preputial orifice. These glands have been shown to shrink upon administration of stilboestrol, which may indicate that the size and activity of the glands are under hormonal control (Van Heerden, 1981). For this reason, the following measurements of the prepuce were made: 1) distance between the ventral aspect of the preputium to the abdominal wall (Figure 8), 2) preputial orifice diameter (Figure 9), 3) width of the right and left preputial flaps (Figure 10).

3.5 Sample Analysis

Semen evaluation, urine and hormone analyses were carried out at the Section of Reproduction while the Clinical Pathology Section performed the serum biochemistry and haematology. Both Sections belong to the Faculty of Veterinary Science.

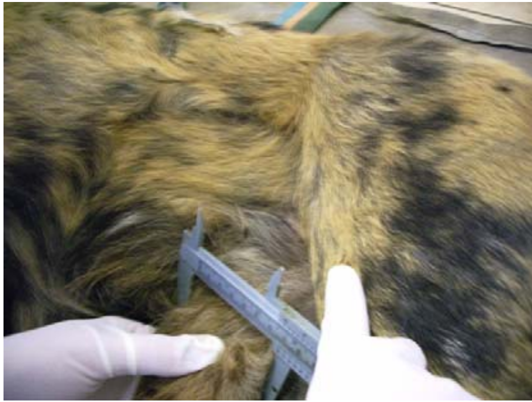

Figure 10. Distance between prepuce and the abdominal wall.

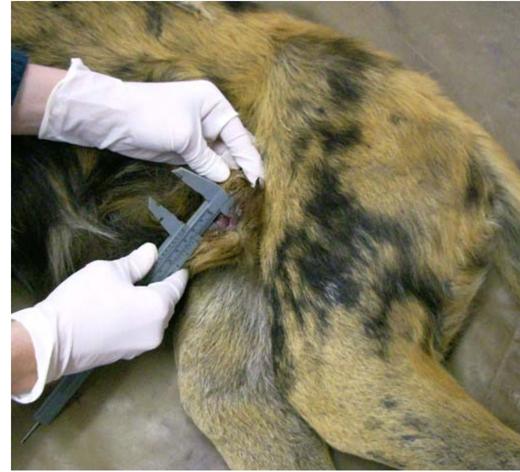

Figure 11. Diameter of preputial orifice.

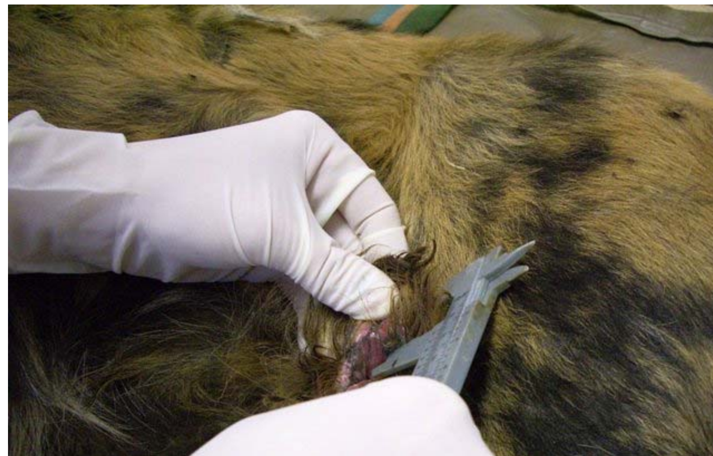

Figure 12. Measurement of preputial flaps

3.5.1 Serum testosterone assay

Serum testosterone was assessed using a double antibody DSL ^{125}testosterone radioimmunoassay (RIA) kit (Diagnostic Systems Laboratories, Inc, Webster, TX). This kit has been validated previously for measuring serum testosterone in male African wild dogs (Monfort *et al.,* 1997). The principle of this RIA is competition between testosterone (standard or sample) and radiolabelled testosterone for a fixed amount of antibody. The following aliquots are added to each reagent tube: the testosterone (standard or sample), the radiolabelled testosterone and the testosterone antibody. The testosterone (radiolabelled or sample/standard) binds with the antibody and then precipitates. Once the antibody-antigen precipitate is formed and centrifuged to the bottom of the test tube, the tubes are inverted and the liquid phase (unbound testosterone) is decanted. The radioactivity in the precipitate of

the tube is then counted using a gamma counter (Cobra® Series Auto-Gamma® Counting System, Packard Instrument Co., Meriden, USA). Each sample is run in duplicate and the mean concentration of testosterone per a sample is calculated using a standard curve.

3.5.2 Semen analysis

3.5.2.1 Initial collection parameters

Upon collection the following parameters were assessed immediately and recorded on the data sheet in Appendix C: volume (ml), colour, consistency, pH, and motility. Colour was assessed subjectively as one of the following: yellow, light yellow, dark yellow, white, grey, or clear. Consistency was termed subjectively: watery, mildly viscous or viscous. All consumables and stains used to process semen were pre-heated to 37 ˚C in order to protect spermatozoa from cold shock. pH was assessed using pH-indicator strips (Neutralit®, Merck, Darmstadt, Germany) with a range of 5.0 to 10.0 and a resolution of 0.5 units. Approximately10 µl of semen was added with a Pasteur pipette to each strip before comparing the colour changes to a colour chart. The preparation used for the assessment of motility consisted of about 10 µl of semen that was flattened under a coverslip on a microscope slide placed on the heated stage (37 °C) of an Olympus phase-contrast microscope (Olympus Optical Co., Ltd, Tokyo, Japan). Using X 200 magnification, motility was assessed as the percentages of spermatozoa that were progressively motile, aberrantly motile, or immotile. If the motility was very high (> 85% progressive), mass motility was determined by assessment of a hanging-drop of about 25 µl of undiluted semen. Mass motility was rated on a scale of one to five as follows: 0=no motility other than Brownian movement; 1=slight movement of individual spermatozoa, but no waves; 2=outspoken movement of individual sperm movement, but no waves; 3=weak waves; 4=strong waves but without whiplash movement; 5=strong waves forming whiplashes near the edge of the droplet is seen. If the subjective assessment revealed that a semen sample had a sufficiently high concentration of spermatozoa, a morphology smear was made from the tube with the highest concentration and best motility of spermatozoa from the particular animal. A Pasteur pipette was used to mix approximately 10 µl of semen with several drops of Eosin-nigrosin stain on a warm coverslip, subsequent to which the mixture was incubated on a warm stage at 37 °C for two minutes to allow spermatozoa with dysfunctional membranes to take up stain. Approximately 10 µl of the mixture was then placed on a clean slide and a smear was made

for the assessment of sperm morphology. Morphology smears were stored in slide boxes until assessment in the laboratory.

3.5.2.2 Count of spermatozoa per an ejaculate and separation of the seminal plasma

All tubes with semen collected from each study animal were transported to the laboratory where they were centrifuged at 300g for 15 minutes. If several tubes from the same study animal contained a watery, yellowish fluid, the contents of the tubes were combined. Then, the tubes were centrifuged only once prior to removal and storage of the supernatant at 4 ˚C and resuspension of the pellet in water. If several tubes from the same study animal contained viscous, whitish-grey fluid, the tubes were centrifuged twice. For these tubes, after the first centrifugation period, the supernatant, or seminal plasma, was removed from each tube by means of a Pasteur pipette, placed in a 2 ml tube, and stored at 4 ˚C until assessment of osmolality. The pellet in each tube was resuspended in a small amount of water, drawn up in a Pasteur pipette and combined with pellets from other tubes from the same study animal in a tube, which was again centrifuged at 300g for 15 minutes. After the second centrifugation, the supernatant was removed and discarded, and the pellet was resuspended in a graduated cylinder in one to 64 ml of water, depending on the concentration of spermatozoa in the sample. The resuspended sample was mixed thoroughly several times by inverting the graduated cylinder. Then some of the sample was placed in one half of a haemocytometer (Improved Neubauer, Boeco, Germany) to perform a count. The haemocytometer was examined at x200 using phase contrast. The number of spermatozoa seen above 20 large squares on the haemocytometers was tabulated. Spermatozoa were counted if their head touched one of the 20 squares, even if the entire spermatozoon was not in the square. The number of spermatozoa per ejaculate, expressed in millions of spermatozoa (which amounts to the number of spermatozoa rendered on the day (N) may be calculated with Equation 1:

Equation 1

$$N = \frac{Vr}{8ml} \times 0.1 \times n_{20sq}$$

where the meanings of the symbols in Equation 1 are as follows:

N is the number of spermatozoa rendered on the day, expressed in millions,

v_r is the volume to which the spermatozoal sediment was resuspended in millilitre and

n_{20sq} is the number of spermatozoa counted above 20 counting squares of the haemocytometer.

If the sediment was resuspended to 8 ml, Equation 1 would simplify to $N = 0.1 \times n_{20sq}$.

If the sediment was resuspended to a volume below 8 ml, say 4 ml, Equation 1 would simplify to $N = \frac{4ml}{8ml} \times 0.1 \times n_{20sq}$, which is equivalent to $N = 0.5 \times 0.1 \times n_{20sq}$.

If the sediment was resuspended to a volume larger than 8 ml, say 16 ml, Equation 1 would simplify to $N = \frac{16ml}{8ml} \times 0.1 \times n_{20sq}$, which is equivalent to $N = 2 \times 0.1 \times n_{20sq}$.

Once a count was tabulated for a given semen sample, the sample was discarded.

3.5.2.3 Sperm morphology and percent live

Once completely dry, each eosin-nigrosin smear (Section 3.4.2.1) was mounted under a coverslip by means of a mounting medium (Entellan®, Merck Darmstadt, Germany). Subsequent to mounting, smears were allowed to dry for at least one hour before they were evaluated. Each morphology smear was assessed under oil at x1000 magnification, using phase contrast and the morphology of 100 neighbouring spermatozoa was recorded. Twenty-eight different morphological defects, as they occur in the spermatozoa of bulls and domestic dogs (Barth and Oko, 1989), were defined and were assumed to be applicable to the wild dog (Appendix D). Defects one to 20 were considered to be of testicular origin, whereas defects 22 to 28 may be from epididymal origin or may have arisen as artefacts. If defect 21 occurred mainly with Defects one to 20 it was assumed to indicate testicular malfunction, whereas if it occurred together with Defects 22 to 28, without many defects of Types one to 20, it was assumed to indicate epididymal malfunction. All defects occurring in a spermatozoon were recorded but accounted for in such a way (see Appendix D) that the percentages of spermatozoa with nuclear defects, flagellar defects, and morphologically normal spermatozoa could be derived accurately. The number of spermatozoa among the 100 that were evaluated that were affected by a particular defect was the percentage of that defect per sample. During the assessment of morphology, the live status of the spermatozoa was also determined. White spermatozoa with no stain uptake were considered live, while spermatozoa that stained pink or bright red due to stain uptake were considered dead. The number of live spermatozoa among the 100 that were evaluated was taken as the percentage live spermatozoa in the sample.

3.5.2.4 Seminal plasma osmolality and pH

The osmolality (osmol/kg) of the seminal plasma was assessed by placing 50 µl of seminal plasma in a 500 µl Eppendorf microcentrifuge tube (Eppendorf AG, Hamburg, Germany) and running the sample on an osmometer (Osmomat 030 cryoscopic osmometer, Gonotec GmbH, Berlin, Germany). In the case of multiple seminal fluid samples from a single dog that appeared yellow and watery, urine contamination was suspected. In this case, the tubes of seminal fluid were combined and a single osmolality was taken. If multiple seminal fluid samples from a single dog appeared white and viscous, then the seminal fluid was assumed to be of a high quality and each sample tube had its osmolality assessed individually. Samples were discarded after their osmolalities were measured. Seminal plasma was also assessed for pH. These values were compared with values obtained for urine samples via cystocentesis prior to electro-ejaculation.

3.6 Data analysis

The data collected during the study period was grouped into two categories: before treatment and after treatment. Data were analysed by means of a Repeated Measures ANOVA (Bogartz, 1994). Dogs were the subjects, Treatment was the between subjects source of variation, and Time was the within subjects source of variation. Dog was nested in Treatment. Treatment had three levels, namely Implant, Injection and Placebo. Time had three levels: before treatment (Aug 23, 2005 to September 1, 2005), the whole time frame after treatment (September 2, 2005 to August 30, 2006), and subsections of the time frame after treatment (1: September 2, 2005 to November 28, 2005, 2: September 2, 2005 to January 30, 2006, 3: September 2, 2005 to February 23, 2005, 4: September 2, 2005 to April 3, 2006, 5: September 2. 2005 to May 24, 2006, 6: September 2, 2005 to August 30, 2006). The subsections-of-time analysis was implemented to elucidate whether season had an effect on post-treatment parameters. Prior to analysis, and arcsine transformation was done on variables measured as percentages, namely the percentages live, morphologically defective, morphologically normal and progressively motile spermatozoa. The arcsine transformation was done after values of zero or 100 were modified according to Bartlett's method (Steel and Torrie, 1980).

The potential confounders of: age (subadult, adult or geriatric), body mass (underweight or normal), access to female dogs (all access, no access or only visual and olfactory access), season of the year (summer, characterized by rain, warm temperatures and long photoperiods-

October through April or winter, characterized by arid conditions, cool temperatures and short photoperiods-May through September) and previous contraception status (naïve or not naïve) were assessed with respect to each reproductive parameter using Repeated Measures ANOVA. Dogs were the subjects and Confounder (ie, age, weight, access to females, season and previous contraception) was the between subject source of variation. A separate Repeated Measures ANOVA was done for each confounder. Multivariate correlations were run between urine pH, semen pH, urine osmolality, semen osmolality, urine sperm count, semen sperm count.

The α-level of significance was set at < 0.05 for all tests. Statistical analyses and graphs were performed using NCSS Statistical Software 2004 (©2004) and JMP 6 by SAS (©2005). . Although the statistical analyses on some data were done after transformation, untransformed data are presented for ease of interpretation. Variation is indicated as mean ± SEM, unless otherwise stated. The standard errors of the means reported are not the same as those that would be obtained when calculated separately for each group, but the ones derived by means of the Repeated Measures ANOVA procedure in NCSS. These standard errors are appropriate to use for the multiple comparisons and each is calculated as the square root of the mean square of the particular error term, divided by the count.

CHAPTER 4

RESULTS

4.1 Testicular volume

a) The main effect of Treatment on testicular volume

Treatment had no effect on combined testicular volume (the volumes of the left and the right testis were added for all analysis) at any time period after treatment (DF=2, $F \leq 2.64$, $p > 0.1$; Table 6). The combined testicular volume of all dogs was significantly lower before treatment than at any time after treatment except for the three-month post-treatment window (ending November 2005), as shown in Table 7.

When all months were pooled for analysis, there was no significant difference between the combined testicular volume before treatment compared with after treatment for any treatment group (DF=5, F=0.75, $p=0.59$, 21132 ± 3812.8 mm^3 (n=6) for the implant group, 27128 ± 2953.3 mm^3 (n=10) for the injection group and 27910 ± 3301.9 mm^3 (n=8) for the placebo group).

Table 6. Mean (±SEM) combined testicular volume (mm^3) for each treatment group measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

		Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
Treatment	Immediately prior to treatment	Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Implant	21024±2146.8 (n=10)	31715±2375.6 (n=1)	24960±2432.7 (n=5)	22843 ±1236.8 (n=8)	22769±916.1 (n=10)	21769±2238.5 (n=10)
Injection	23126±2717.7 (n=11)	31035±2375.6 (n=1)	41400±2719.8 (n=4)	41793±1564.5 (n=5)	41514±1094.9 (n=7)	39502±2359.6 (n=9)
Placebo	23014±1798.8 (n=6)	28325±1371.5 (n=3)	28835±2220.7 (n=6)	30060±1322.2 (n=7)	30373±965.6 (n=9)	30738±2359.6 (n=9)

Table 7. Mean (±SEM) combined testicular volume (mm^3) before treatment and measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
Stage of measurement	Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Before treatment	21002 ± 0.0 (n=6)	22715 ± 1813.2 (n=9)[a]	22542 ± 1009.9 (n=12)[a]	21230 ± 1769.7 (n=14)[a]	22240 ± 1660.6 (n=16)[a]
After treatment	30359 ± 0.0 (n=5)	31732 ± 1404.5 (n=15)[b]	31566 ± 782.2 (n=20)[b]	31340 ± 1337.8 (n=26)[b]	30670 ± 1271.4 (n=28)[b]

[a, b] Within columns, means with different superscripts differ ($p \leq 0.003$)

b) The effect of confounding variables on testicular volume

Dogs had significantly higher combined testicular volume during the summer, October to April (DF=1, F=3.76, p=0.05, 27458 ± 2289.1 mm^3 (n=30) for the summer and 21773 ± 1828.9 mm^3 (n=47) for the winter). See Figure 11 for the testicular volume ranges during the summer and winter.

Age did not affect testicular volume (DF=2, F=0.25, p=0.78, 24001 ± 1754.3 mm^3 (n=54) for adults, 25489 ± 3445.4 mm^3 (n=14) for geriatric dogs and 21579 ± 4297.2 mm^3 (n=9) for sub-adults).

Underweight dogs tended to have lower combined testicular volumes than normal-weight dogs, although this finding was not statistically significant (DF=1, F=3.0, p=0.09, 25253 ± 1613.6 mm^3 (n=61) for normal-weight dogs, 19167 ± 3150.7 mm^3 (n=16) for underweight dogs).

Previous contraception history did not affect testicular volume (DF=1, F=1.07, p=0.30, 25433 ± 2017.3 mm^3 (n=40) for naïve dogs and 22426 ± 2097.5 mm^3 (n=37) for dogs that had received contraceptive treatment before).

Proximity to female dogs did not affect testicular volume (DF=2, F=1.29, p=0.28, 22114 ± 1874.9 mm^3 (n=46) for dogs housed with females, 27286 ± 2843.4 mm^3 (n=20) for dogs not housed with females and 25825 ± 3824.1 mm^3 (n=11) for dogs with olfactory contact with females only).

Very few dogs were azoospermic during the study: Five dogs were azoospermic prior to treatment, of which one was also azoospermic five months after treatment and the remaining four had spermatozoa in their ejaculates after treatment. Seven other dogs had spermatozoa in their ejaculates prior to treatment but were azoospermic when re-examined after treatment. Twenty-two ejaculates from 22 dogs contained spermatozoa prior to treatment compared with 25 ejaculates from 18 dogs, which contained spermatozoa after treatment. Azoospermic dogs had significantly lower combined testicular volume than non-azoospermic dogs (DF=1, F=14.8, p=0.0003, 18635 ± 2496.1 mm^3 (n=13 measurements in 12 dogs) for azoospermic dogs and 29499 ± 1312.8 mm^3 (n=47 measurements in 25 dogs) for non-azoospermic dogs.

Figure 12 shows the variability in the combined testicular volume for azoospermic and non-azoospermic dogs.

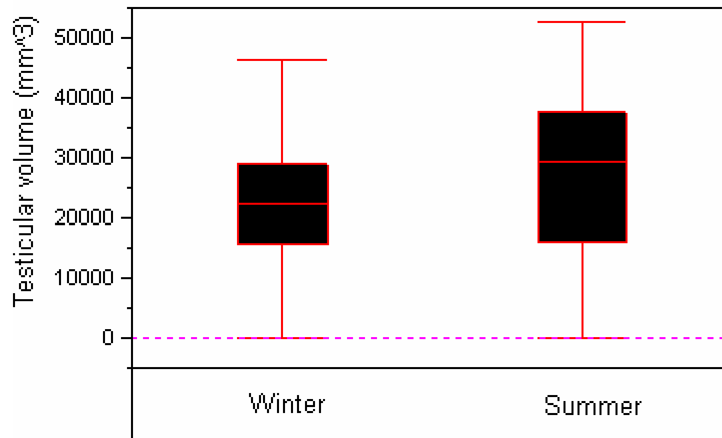

Figure 13. Median, interquartile range and range of testicular volume (mm^3) measurements for winter (n=47) and summer (n=30).

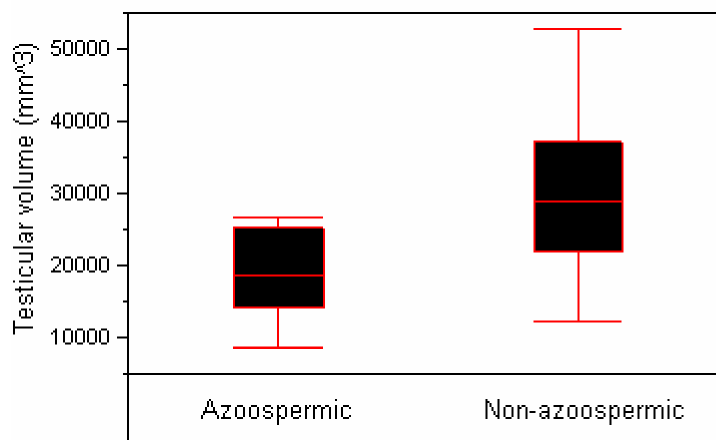

Figure 14. Median, interquartile range and range of testicular volume (mm^3) for azoospermic (n=13 readings from 12 dogs) and non-azoospermic (n=47 readings from 25 dogs) dog samples.

4.2 Semen quality

4.2.1 Semen volume and colour

a) The main effect of Treatment on semen volume and colour

There was no significant difference in the semen volume between different treatment groups (DF=2, F=2.02, *p*=0.14, 2.9 ± 0.9 ml (n=21) for the implant group, 5.5 ± 0.9 ml (n=23) for the injection group and 4.1 ± 1.0 ml (n=16) for the placebo group). Small volumes of semen were usually white or grey in colour, whereas larger volumes of semen were often yellow in colour due to urine contamination (see Figure 13).

b) The effects of confounding variables on semen volume

A significantly larger volume of semen was collected from dogs during winter, May to September, as compared with summer, October to April (DF=1, F=4.88, *p*=0.03, 5.1 ± 0.7 ml (n=40) for the winter and 2.5 ± 0.9 ml (n=20) for the summer).

Age did not affect semen volume (DF=2, F=0.19, *p*=0.83, 4.1 ± 0.7 ml (n=42) for adults, 4.0 ± 1.3 ml (n=12) for geriatric dogs and 5.2 ± 1.8 ml (n=6) for sub-adult dogs).

Body mass did not effect semen volume (DF=1, F=0.86, *p*=0.36, 4.5 ± 0.6 ml (n=46) for normal-weight dogs, 3.3 ± 1.2 ml (n=14) for underweight dogs).

Previous contraception history did not affect semen volume (DF=1, F=0.67, *p*=0.42, 4.7 ± 0.8 (n=31) for naïve dogs and 3.7 ± 0.8 (n=29) for dogs that had received contraceptive before).

Proximity to female dogs did not affect semen volume (DF=2, F=0.56, *p*=0.58, 3.8 ± 0.7 ml (n=37) for dogs housed with females, 5.2 ± 1.1 ml (n=15) for dogs housed without females, and 4.1 ± 1.5 ml (n=8) for dogs with olfactory contact with females only).

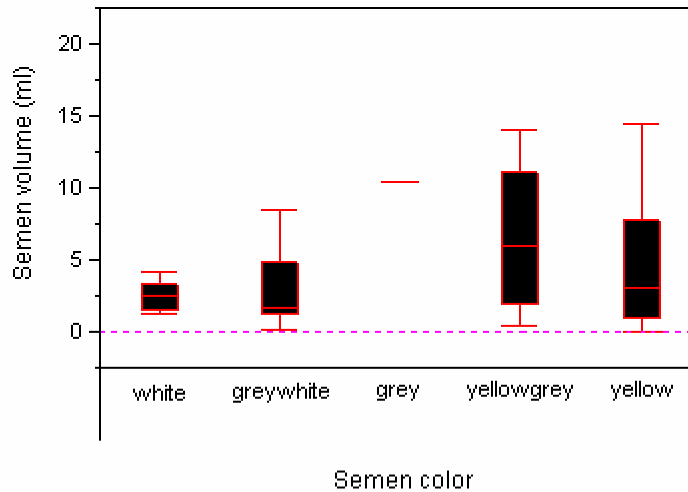

Figure 15. Median, interquartile range and range of semen volume (ml) in relation to semen color (white, n=6; greywhite, n=8; grey, n=3; yellowgrey, n=5;yellow, n=21).

4.2.2 Sperm motility

a) The main effect of Treatment on motility

Figure 14 demonstrates the variability of progressive sperm motility before and after treatment. With all post-treatment data pooled to give a twelve-month post-treatment window of observation, there was no significant difference in progressive motility before as opposed to after treatment, regardless of treatment type administered (DF=5, F=1.13, *p*=0.36, 15% ± 11.7% (n=8) for the implant group before treatment; 23% ± 14.8% (n=6) for the implant group after treatment, 10% ± 11.0% (n=9) for the injection group before treatment, 36% ± 10.0% (n=11) for the injection group after treatment, 0.0% ± 0.0% (n=6) for the placebo group before treatment, and 23% ± 11.0% (n=9) for the placebo group after treatment). The aberrant motility of the injection group after treatment (17% ± 4.4% (n=11)) was significantly greater than the pre-treatment aberrant motility of the injection (DF=5, F=2.01, *p*=0.01, 0.0% ± 0.0% (n=9) for the injection group before treatment), implant (DF=5, F=2.01, *p*=0.02, 0.0% ± 0.0% (n=8) for the implant group before treatment) and placebo (DF=5, F=2.01, *p*=0.04, 0.0% ± 0.0% (n=6) for the placebo group before treatment) groups.

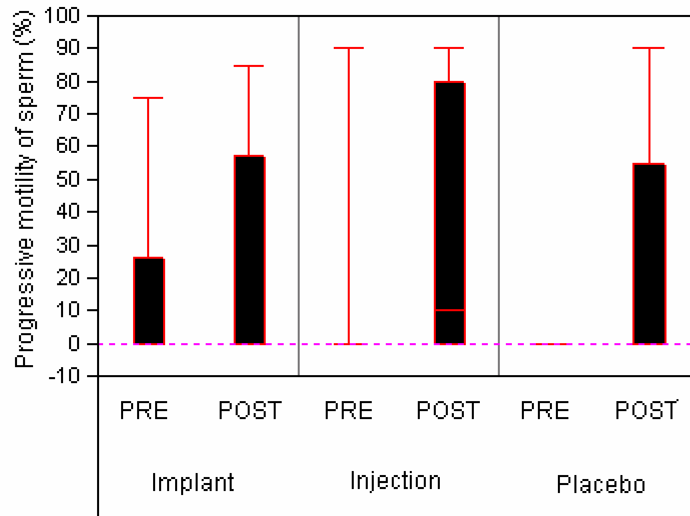

Figure 16. Median, interquartile range and range of progressive motility (%) before and after treatment by group (Implant pre n=8, post n=6; Injection pre n=9, post n=11; Placebo pre n=6, post n=9).

For the treatment groups combined, the mean percentage of progressively motile spermatozoa was significantly higher after treatment than before treatment for the six-month post-treatment window (ending February 2006, DF=1, F=14.63, p=0.02) as well as the nine-month post-treatment window (ending May 2006, DF=1, F=9.06, p=0.02; Table 8).

Table 8. Mean (±SEM) percentage progressively motile spermatozoa before treatment and measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
Stage of measurement	Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 April 2006 (7 months)	Until 24 May 2006 (9 months)
Before treatment	0.0 ± 0.0 (n=4)	0.0 ± 9.4 (n=5)	0.0 ± 6.7 (n=7)[a]	0.0 ± 11.7 (n=7)	0.0 ± 9.5 (n=9)[a]
After treatment	10 ± 0.0 (n=4)	33.2 ± 7.0 (n=9)	33.4 ± 5.4 (n=11)[b]	35.5 ± 7.7 (n=16)	37.7 ± 6.7 (n=18)[b]

[a,b] Within a column, means with different superscripts differ ($p \leq 0.07$)

b) The effect of confounding variables on motility

During summer, which lasts from October to April, the mean percentage of progressively motile spermatozoa was 36 ± 7.9 (n=16), which was higher than the 11 ± 5.6 (n=31) during the winter (DF=1, F=6.7, *p*=0.01). Figure 15 demonstrates the difference in the progressive motility in the summer, as opposed to the winter. Season had no effect on the percentage of aberrantly motile spermatozoa (DF=1, F=0.96, *p*=0.33, 4 ± 2.8 (n=31) for winter and 8 ± 3.8 (n=16) for summer).

Age had no effect on the percentage progressively motile spermatozoa (DF=2, F=0.27, *p*=0.76, 21 ± 5.9 (n=33) for adults, 14 ± 10.2 (n=11) for geriatric dogs and 27 ± 19.5 (n=6) for sub-adults). Age also had no effect on the percentage aberrantly motile spermatozoa (DF=2, F=0.86, *p*=0.43, 4 ± 2.7 (n=33) for adults, 11 ± 4.7 (n=11) for geriatric dogs, 3 ± 8.9 (n=6) for sub-adults).

Body mass had no effect on the percentage progressively motile spermatozoa (DF=1, F=0.07, *p*=0.80, 20 ± 5.5 (n=38) for normal-weight dogs and 17 ± 11.2 (n=9) for underweight dogs) nor on the percentage of aberrantly motile spermatozoa (DF=1, F=0.08, *p*=0.78, 5 ± 2.5 (n=38) for normal-weight dogs and 7 ± 5.2 (n=9) for underweight dogs).

Previous contraception history had no effect on the percentage progressively motile spermatozoa (DF=1, F=0.003, *p*=0.96, 20 ± 6.7 (n=25) for naïve dogs and 20 ± 7.2 (n=22) for previously contracepted dogs) nor on the percentage of aberrantly motile spermatozoa (DF=1, F=0.01, *p*=0.92, 5 ± 3.1 (n=25) for naïve dogs and 6 ± 3.3 (n=22) for previously contracepted dogs).

Proximity to females had no effect on the percentage progressively motile spermatozoa (DF=2, F=1.23, *p*=0.30, 22 ± 6.4 (n=27) for dogs housed with females, 23 ± 8.9 (n=14) for dogs housed without females and 0.0 ± 0.0 (n=6) for dogs with olfactory contact with females). Proximity to females also had no effect on the percentage aberrantly motile spermatozoa (DF=2, F=0.42, *p*=0.66, 6 ± 3.0 (n=27) for dogs housed with females, 6 ± 4.2 (n=14) for dogs housed without females and 0.0 ± 0.0 (n=6) for dogs with olfactory contact with females only).

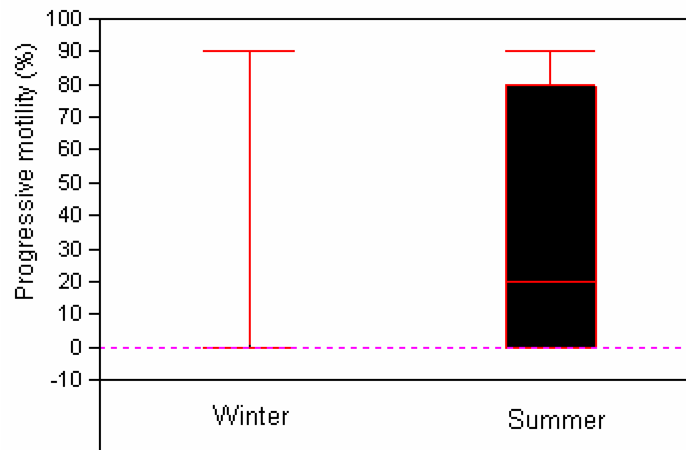

Figure 17. Median, interquartile range and range of progressive motility (%) of samples for winter (n=31) and summer (n=16).

4.2.3 Prevalence of live spermatozoa

a) The main effect of Treatment on the percentage live spermatozoa

When the percentage of live spermatozoa before treatment was compared with that after treatment, with post-treatment data pooled into observational windows of different durations after treatment, neither Treatment (DF=2, F≤0.22, *p*≥0.79) nor Stage (before or after treatment; DF=1, F≤0.93, *p*≥0.45) had an effect on the percentage live spermatozoa. See Tables 9 and 10 for comparisons of the mean percentages of live spermatozoa by treatment type and stage relative to treatment. When all post-treatment data were pooled, Stage had no effect on the percentage live spermatozoa in any of the treatment groups (DF=5, F=0.57, *p*=0.72, 51.8 ± 16.1 (n=6) for the implant group before treatment, 37.0 ± 18.0 (n=6) for the implant group after treatment, 26.5 ± 12.8 (n=8) for the injection group before treatment, 42.0 ± 10.9 (n=11) for the injection group after treatment, 29.3 ± 20.82 (n=6) for the placebo group before treatment and 51.2 ± 12.0 (n=9) for the placebo group after treatment). Figure 16 demonstrates the lack of variability in the percentage of live spermatozoa between treatment groups, both before and after the treatment was applied.

Table 9. Mean (±SEM) percentage of live spermatozoa for each treatment group measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

Treatment	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
	Immediately prior to treatment	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Implant	51.8 ± 21.3 (n=5)	49.0 ± 0.0 (n=2)	32.7 ± 36.5 (n=3)	37.0 ± 35.3 (n=4)	37.0 ± 25.9 (n=4)
Injection	26.5 ± 11.3 (n=8)	62.0 ± 0.0 (n=3)	46.5 ± 31.6 (n=4)	36.3 ± 28.8 (n=6)	43.1 ± 18.3 (n=8)
Placebo	29.3 ± 14.7 (n=3)	58.3 ± 0.0 (n=4)	58.3 ± 31.6 (n=4)	61.2 ± 28.8 (n=6)	61.2 ± 21.2 (n=6)

Table 10. Mean (±SEM) percentage of live spermatozoa before and measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

Stage of measurement	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)			
	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Before treatment	50.9 ± 0.0 (n=3)	0.0 ±31.6 (n=4)	12.8 ± 35.3 (n=4)	17.5 ± 23.2 (n=5)
After treatment	56.4 ± 0.0 (n=9)	45.8 ± 19.0 (n=11)	44.8 ± 17.6 (n=16)	47.1 ± 12.2 (n=18)

b) The effect of confounders on the percentage of live spermatozoa

Season had no effect on the percentage of live spermatozoa (DF=1-2, F≤2.61, $p \geq 0.09$, 37.4 ± 7.2 (n=24) for the winter and 45.8 ± 8.8 (n=16) for the summer).

Age had no effect on the percentage of live spermatozoa (DF=1-2, F≤2.61, $p \geq 0.09$, 42.0 ± 6.3 (n=29) for adults, 51.4 ± 11.9 (n=8) for geriatric dogs and 0.0 ± 0.0 (n=6) for sub-adults).

Body mass had no effect on the percentage of live spermatozoa (DF=1-2, F≤2.61, $p \geq 0.09$, 38.5 ± 6.1 (n=33) for normal-weight dogs and 51.3 ± 13.3 (n=7) for underweight dogs).

Previous contraception history had no effect on the percentage of live spermatozoa (DF=1-2, F≤2.61, $p \geq 0.09$, 36.5 ± 7.7 (n=21) for naïve dogs and 45.5 ± 8.0 (n=19) for previously contracepted dogs).

Proximity to females had no effect on the percentage of live spermatozoa (DF=1-2, F≤2.61, $p \geq 0.09$, 38.4 ± 7.5 (n=23) for dogs housed with females, 47.1 ± 10.3 (n=12) for dogs housed without females and 36.4 ± 16.0 (n=6) for dogs with olfactory contact with females only).

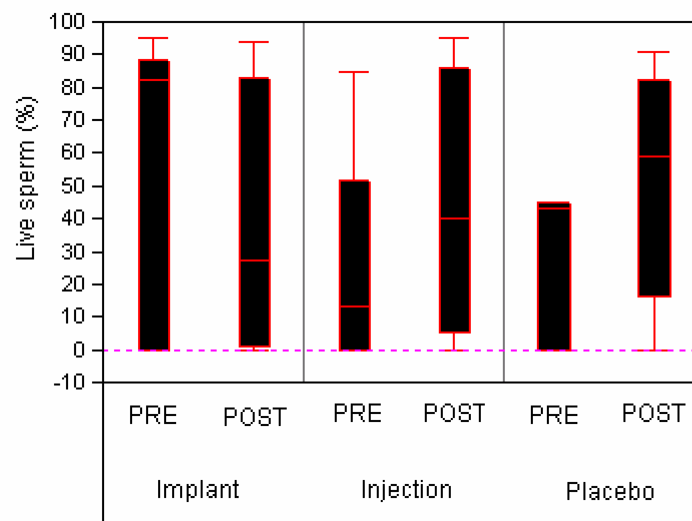

Figure 18. Median, interquartile range and range of live spermatozoa (%) before and after treatment by group (Implant pre n=6, post n=6; Injection pre n=8, post n=11; Placebo pre n=6, post n=9).

4.2.4 Sperm Morphology

Table 11 summarises the effects of treatment and time on sperm morphology.

Table 11. Mean (±SEM) percentage of sperm defect types before and measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment				
Morphology type and treatment category	Immediately prior to treatment	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Normal spermatozoa					
Implant	25.6 ± 9.9 (n=5)	27.5 ± 0.0 (n=2)[a]	27.3 ± 4.9 (n=3)[a]	36.5 ± 17.9 (n=4)	36.5 ± 12.9 (n=4)
Injection	38.1 ± 5.2 (n=8)	69.0 ± 0.0 (n=3)[b]	65.5 ± 4.3 (n=4)[b]	48.3 ± 14.6 (n=6)	54.6 ± 9.1 (n=8)
Placebo	22.3 ± 11.3 (n=3)	46.3 ± 0.0 (n=4)[b]	46.3 ± 4.3 (n=4)[b]	40.2 ± 14.6 (n=6)	40.2 ± 10.5 (n=6)
Defects of testicular origin					
Implant	5.8 ± 1.4 (n=5)	2.5 ± 0.0 (n=2)	19.3 ± 3.7 (n=3)	21.3 ± 5.1 (n=4)	21.3 ± 5.6 (n=4)
Injection	9.4 ± 3.7 (n=8)	1.0 ± 0.0 (n=3)	2.0 ± 3.2 (n=4)	4.7 ± 4.1 (n=6)	5.8 ± 4.0 (n=8)
Placebo	8.7 ± 3.2 (n=3)	15.5 ± 0.0 (n=4)	15.5 ± 3.2 (n=4)	15.5 ± 4.1 (n=6)	15.5 ± 4.6 (n=6)
Defects forming in the epididymis or as artefacts					
Implant	68.0 ± 9.2 (n=5)	NA	54.7 ± 8.0 (n=3)	43.5 ± 14.9 (n=4)	43.5 ± 11.6 (n=4)
Injection	45.9 ± 6.8 (n=8)	NA	33.8 ± 6.9 (n=4)	45.7 ± 12.2 (n=6)	37.8 ± 8.2 (n=8)
Placebo	66.7 ± 4.8 (n=3)	NA	32.0 ± 6.9 (n=4)	42.5 ± 12.2 (n=6)	42.5 ± 9.5 (n=6)
Nuclear defects					
Implant	1.2 ± 0.6 (n=5)	0.5 ± 0.0 (n=2)	7.7 ± 0.9 (n=3)	12.3 ± 0.7 (n=4)	12.3 ± 1.5 (n=4)
Injection	3.4 ± 1.7 (n=8)	0.3 ± 0.0 (n=3)	1.0 ± 0.8 (n=4)	3.7 ± 0.6 (n=6)	3.9 ± 1.1 (n=8)
Placebo	2.0 ± 1.2 (n=3)	6.5 ± 0.0 (n=4)	6.5 ± 0.8 (n=4)	7.2 ± 0.6 (n=6)	7.2 ± 1.2 (n=6)
Bent tails					
Implant	59.2 ± 13.1 (n=5)	52.5 ± 0.0 (n=2)	44.3 ± 0.0 (n=3)	35.0 ± 15.9 (n=4)	35.0 ± 8.9 (n=4)
Injection	41.4 ± 8.3 (n=8)	19.0 ± 0.0 (n=3)	22.0 ± 0.0 (n=4)	24.5 ± 13.0 (n=6)	21.8 ± 6.3 (n=8)
Placebo	57.0 ± 6.0 (n=3)	35.5 ± 0.0 (n=4)	35.5 ± 0.0 (n=4)	41.8 ± 13.0 (n=6)	41.8 ± 7.2 (n=6)
Retained proximal droplets					
Implant	4.6 ± 2.5 (n=5)	8.0 ± 0.0 (n=2)	10.0 ± 0.0 (n=3)	7.5 ± 0.0 (n=4)	7.5 ± 0.1 (n=4)
Injection	10.0 ± 6.3 (n=8)	1.3 ± 0.0 (n=3)	1.3 ± 0.0 (n=4)	4.5 ± 0.0 (n=6)	5.6 ± 0.0 (n=8)
Placebo	9.3 ± 6.8 (n=3)	11.5 ± 0.0 (n=11)	11.5 ± 0.0 (n=4)	8.0 ± 0.0 (n=6)	8.0 ± 0.0 (n=6)

[a,b] Within a column means with different superscripts differ (DF=2, F=22.00, p≤0.008)

4.2.4.1 Normal spermatozoa

a) The main effect of Treatment on the percentage normal spermatozoa

The percentage of normal spermatozoa of the implant group differed from that of the injection group and the placebo group, respectively, over a five-, as well as a six-month observation period after treatment (DF=2, F=22.00, $p \leq 0.008$, Table 11).

Regardless of the treatment group and the duration of the window of observation after treatment, there was no significant difference in the mean percentage of normal spermatozoa before as opposed to after treatment (DF=1, F≤1.01, p>0.41). The means for the seven-month post-treatment window of observation (until the end of April 2006) were 44.4% ± 17.9% (n=4) before treatment and 41.7% ± 8.9% (n=16) after treatment. For the nine-month post-treatment period (ending May 2006) the means were 29.6% ± 11.5% (n=5) before treatment and 43.8% ± 6.1% (n=18) after treatment.

b) The effect of confounders on the percentage of normal spermatozoa

Season had no effect on the percentage of normal spermatozoa (DF=1, F=1.43, p=0.24, 33.4 ± 4.7 (n=24) for winter and 42.3 ± 5.8 (n=16) for summer.

Age had no effect on the percentage of normal spermatozoa (DF=2, F=0.24, p=0.79, 38.5 ± 4.4 (n=29) for adults, 33.4 ± 8.4 (n=8) for geriatric dogs and 31.3 ± 13.7 (n=6) for sub-adults).

Body mass had no effect on the percentage of normal spermatozoa (DF=1, F=0.78, p=0.38, 38.4 ± 4.1 (n=33) for normal-weight dogs and 29.9 ± 8.8 (n=7) for underweight dogs.

Previous contraception history had no effect on the percentage of normal spermatozoa (DF=1, F=0.04, p=0.85, 36.3 ± 5.1 (n=21) for naïve dogs and 37.7 ± 5.4 (n=19) for dogs that had received contraceptive treatment previously.

Proximity to female dogs had no effect on the percentage of normal spermatozoa (DF=2, F=2.09, p=0.14, 38.0 ± 4.7 (n=23) housed with females, 42.8 ± 6.5 (n=12) housed without females and 18.4 ± 10.1 (n=6) for dogs with olfactory contact with females only).

4.2.4.2 Percentage of spermatozoa with defects of testicular origin

a) The main effect of Treatment on the percentage of spermatozoa with defects of testicular origin

Regardless of the treatment group and the duration of the window of observation after treatment, there was no significant difference in the percentage of spermatozoa with defects of testicular origin before as opposed to after treatment (DF=2, F≤1.34, p≥0.31; See Table 11). Similarly, when data of treatment groups were pooled and the percentage of spermatozoa with defects of testicular origin prior to treatment was compared with that after treatment (post-treatment data were pooled into observational windows of different durations after treatment), there was no significant difference in the percentage of defects of testicular origin before as opposed to after treatment (DF=1, F≤4.93, p≥0.26, 23.6 ± 3.2 (n=4) for the before treatment values of the six month post-treatment window, 12.3 ± 2.0 (n=11) for the after treatment values of the six month post-treatment window, 4.9 ± 5.1 (n=4) for the before treatment values of the seven month post-treatment window, 13.8 ± 2.5 (n=16) for the after treatment values of the seven month post-treatment window, 18.9 ± 5.0 (n=5) for the before treatment values of the nine month post-treatment window, 14.2 ± 2.7 (n=18) for the after treatment values of the nine month post-treatment window. Dog had a significant effect on the percentage of spermatozoa with defects of testicular origin (DF=2-9, F≤37.11, 0.003≤p). Table 12 shows that Dogs M173, M343, M357, M376, M377 and M440 each had more than 20% of spermatozoa with defects of testicular origin, which was a distinctly higher percentage than those of the other dogs. Overall, the mean prevalence of defects of testicular origin was 13%.

Table 12. Percentage (Mean ± SEM) of spermatozoa with defects of testicular origin for individual dogs

Dog	Percentage testicular origin defects
M130	5.0 ± 9.4 (n=2)
M173	39.5 ± 6.6 (n=2)
M176	5.0 ± 9.4 (n=2)
M186	13.5 ± 6.6 (n=2)
M212	7.0 ± 6.6 (n=2)
M216	7.0 ± 9.4 (n=2)
M255	1.0 ± 9.4 (n=1)
M256	7.5 ± 6.6 (n=2)
M273	12.5 ± 6.6 (n=2)
M301	4.0 ± 9.4 (n=2)
M325	2.5 ± 6.6 (n=2)
M333	9.0 ± 9.4 (n=2)
M334	8.3 ± 5.4 (n=4)
M341	2.7 ± 5.4 (n=4)
M343	21.7 ± 5.4 (n=4)
M344	2.5 ± 6.6 (n=3)
M357	53.0 ± 9.4 (n=2)
M369	6.0 ± 9.4 (n=2)
M376	24.0 ± 9.4 (n=2)
M377	39.0 ± 9.4 (n=2)
M380	7.0 ± 9.4 (n=2)
M421	4.0 ± 9.4 (n=2)
M422	17.5 ± 6.6 (n=2)
M439	16.0 ± 9.4 (n=2)
M440	27.0 ± 9.4 (n=2)

b) The effect of confounders on the percentage of spermatozoa with testicular origin defects

Season had no effect on the percentage of spermatozoa with testicular origin defects (DF=1, F=0.00, p=0.98, 13.0 ± 2.8 (n=24) for winter and 12.9 ± 3.4 (n=16) for summer).

Age had no effect on the percentage of spermatozoa with testicular origin defects (DF=2, F=0.29, p=0.75, 12.0 ± 2.6 (n=29) for adults, 16.3 ± 4.9 (n=8) for geriatric dogs and 13.0 ± 8.0 (n=6) for sub-adults).

Body mass had no effect on the percentage of spermatozoa with testicular origin defects (DF=1, F=2.38, p=0.13, 11.5 ± 2.3 (n=33) for normal-weight dogs and 20.0 ± 5.0 (n=7) for underweight dogs.

Previous contraception history had no effect on the percentage of spermatozoa with testicular origin defects (DF=1, F=2.25, p=0.14, 16.0 ±2.9 (n=21) for naïve dogs and 9.6 ± 3.0 (n=19) for previously contracepted dogs).

Proximity to female dogs had no effect on the percentage of spermatozoa with testicular origin defects (DF=2, F=1.00, p=0.38, 13.5 ± 2.8 (n=23) for dogs housed with females, 9.3 ± 3.9 (n=12) for dogs housed without females and 19.2 ± 6.1 (n=6) for dogs with olfactory contact with females).

4.2.4.3 Percentage of spermatozoa with defects that originate in the epididymis or as artefacts

a) The main effect of Treatment on the percentage of spermatozoa with defects that originate in the epididymis or as artefacts

Neither Treatment nor Stage of treatment (before or after treatment) affected the percentage of spermatozoa with defects that were of epididymal origin or that arose as artefacts (DF=2, F≤0.42, p≥0.68; Table 11). Similarly, when data of different treatment groups were pooled and the percentage of spermatozoa with defects that were of epididymal origin or arose as artefacts before treatment was compared to the percentage after treatment (post-treatment data pooled into observational windows of different durations after treatment), there was no

significant difference (DF=1, F≤2.45, p≥0.34, 58.5% ± 6.9% (n=4) for the before treatment values of the six month post-treatment window, 40.1% ± 4.2% (n=11) for the after treatment values of the six month post-treatment window, 54.6% ± 14.9% (n=4) for the before treatment values of the seven month post-treatment window, 43.9% ± 7.4% (n=16) for the after treatment values of the seven month post-treatment window, 49.8% ± 10.4% (n=5) for the before treatment values of the nine month post-treatment window and 41.3% ± 5.5% (n=18) for the after treatment values of the nine month post-treatment window).

b) The effect of confounders on the percentage of spermatozoa with defects that originate in the epididymis or as artefacts

Season had no effect on the percentage of spermatozoa with defects of epididymal origin or as artefacts (DF=1, F=1.51, p=0.23, 53.5 ± 5.0 (n=24) for winter and 43.9 ± 6.1 (n=16) for summer).

Age had no effect on the percentage of spermatozoa with defects of epididymal origin or as artefacts (DF=2, F=0.16, p=0.86, 49.5 ± 4.6 (n=29) for adults, 47.6 ± 8.8 (n=8) for geriatric dogs and 57.0 ± 14.4 (n=6) for sub-adults).

Body mass had no effect on the percentage of spermatozoa with defects of epididymal origin or as artefacts (DF=1, F=0.31, p=0.58, 50.7 ± 4.3 (n=33) for normal-weight dogs and 45.0 ± 9.3 (n=7) for underweight dogs).

Previous contraception history had no effect on the percentage of spermatozoa with defects of epididymal origin or as artefacts (DF=1, F=0.36, p=0.55, 47.5 ± 5.4 (n=21) for naïve dogs and 52.2 ± 5.6 (n=19) for previously contracepted dogs).

Proximity to female dogs had no effect on the percentage of spermatozoa with defects of epididymal origin or as artefacts (DF=2, F=1.23, p=0.31, 47.4 ± 5.1 (n=23) for dogs housed with females, 47.5 ± 7.0 (n=12) for dogs housed without females, 65.6 ± 10.9 (n=6) for dogs with olfactory contact with females only).

4.2.4.4 Percentage of spermatozoa with nuclear defects

a) The main effect of Treatment on the percentage of spermatozoa with nuclear defects

Regardless of Treatment group or Stage (before or after treatment) there was no significant difference in the percentage of spermatozoa with nuclear defects (DF=2, F≤1.40, p≥0.42; See Table 11). When the percentage of spermatozoa with nuclear defects before treatment was compared with that after treatment (post-treatment data pooled into observational windows of different durations after treatment), there was no significant difference (DF=1, F≤4.48, p≥0.29, 7.7 ± 0.8 (n=4) for before treatment values for the six month post-treatment window, 5.1 ± 0.5 (n=11) for after treatment values for the six month post-treatment window, 0.0 ± 0.7 (n=4) for before treatment values for the seven month post-treatment window, 7.7 ± 0.4 (n=16) for after treatment values for the seven month post-treatment window, 6.9 ± 1.3 (n=5) for before treatment values for the nine month post-treatment window and 7.8 ± 0.7 (n=18) for after treatment values for the nine month post-treatment window).

The percentage of spermatozoa with nuclear defects differed between individual dogs for a six (DF=4, F=13.8, p=0.01), seven (DF=7, F=12.6, p=0.003) and nine (DF=9, F=9.76, p=0.006) month post-treatment period. Refer to Table 13 for the percentage of spermatozoa with nuclear defects for each dog. The statistical difference between the percentage of spermatozoa with nuclear defects of individual dogs can be attributed to M173, M357, M376, M377, M339 and M340, which all had percentages of spermatozoa with nuclear defects greater than 14. The mean nuclear defect percentage for the whole study population was 7.3%.

Table 13. Percentage (Mean ± SEM) of spermatozoa with nuclear defects for individual dogs

Dog	Percentage of nuclear defects
M130	2.0 ± 8.4 (n=2)
M173	32.0 ± 5.9 (n=2)
M176	0.0 ± 0.0 (n=2)
M186	4.5 ± 5.9 (n=2)
M212	3.0 ± 5.9 (n=2)
M216	1.0 ± 5.9 (n=2)
M255	2.0 ± 8.4 (n=1)
M256	3.0 ± 5.9 (n=2)
M273	8.0 ± 5.9 (n=2)
M301	2.0 ± 8.4 (n=2)
M325	0.0 ± 0.0 (n=2)
M333	0.0 ± 0.0 (n=2)
M334	4.0 ± 4.8 (n=4)
M341	0.7 ± 4.8 (n=4)
M343	9.0 ± 4.8 (n=4)
M344	0.5 ± 5.9 (n=4)
M357	22.0 ± 8.4 (n=2)
M369	5.0 ± 8.4 (n=2)
M376	17.0 ± 8.4 (n=2)
M377	32.0 ± 8.4 (n=2)
M380	3.0 ± 8.4 (n=2)
M421	4.0 ± 8.4 (n=2)
M422	6.5 ± 5.9 (n=2)
M439	14.0 ± 8.4 (n=2)
M440	26.0 ± 8.4 (n=2)

b) The effect of confounders on the percentage of spermatozoa with nuclear defects

Season had no effect on the percentage of spermatozoa with nuclear defects (DF=1, F=0.005, p=0.94, 7.4 ± 2.2 (n=24) for winter and 7.1 ± 2.7 (n=16) for summer.

Age had no effect on the percentage of spermatozoa with nuclear defects (DF=2, F=0.37, p=0.70, 6.7 ± 2.0 (n=29) for adults, 10.1 ± 4.0 (n=8) for geriatric dogs and 5.7 ± 6.2 (n=6) for sub-adults).

Underweight dogs had 15.4% ± 3.8% (n=7) spermatozoa with nuclear defects, which was significantly higher than the 5.5% ± 1.7% (n=33) for normal-weight dogs (DF=1, F=5.72, p=0.02).

Previously contracepted dogs had 3.9% ± 2.3% (n=19) spermatozoa with nuclear defects, which was significantly lower than the 10.3% ± 2.2% (n=21) for naïve dogs (DF=1, F=3.89, p=0.05).

Proximity to female dogs had no effect on the percentage of spermatozoa with nuclear defects (DF=2, F=1.31, p=0.28, 8.0% ± 2.2% (n=23) for dogs housed with females, 3.8% ± 3.0% (n=12) for dogs housed without females and 12.4% ± 4.7% (n=6) for dogs with olfactory contact with females only).

4.2.4.5 Percentage of spermatozoa with bent tails

a) The main effect of Treatment on the percentage of spermatozoa with bent tails

Regardless of the post-treatment window, Treatment did not affect the percentage of spermatozoa with bent tails (DF=2, F≤2.79, p≥0.17). When the percentage of spermatozoa with bent tails before treatment was compared with that after treatment (post-treatment data pooled into observational windows of different durations after treatment), there was no significant difference (DF=1, F≤4.02, p≥0.18, 48.3% ± 14.3% (n=5) for before treatment for the seven month post-treatment window, 33.8% ± 8.0 (n=16) for after treatment for the seven month post-treatment window, 54.6% ± 8.0% (n=5) for before treatment for the nine month

post-treatment window and 32.9% ± 4.2% (n=18) for after treatment for the nine month post-treatment window).

b) The effect of confounders on the percentage of spermatozoa with bent tails

Although there was not a significant difference in the percentage of spermatozoa with bent tails by season, there was a trend for there to be more bent tail defects during the winter than the summer (DF=1, F=3.32, p=0.06, 46.6% ± 4.5% (n=24) for winter and 33.6% ± 5.5% (n=16) for summer).

Age had no effect on the percentage of spermatozoa with bent tails (DF=2, F=0.19, p=0.82, 41.4 ± 4.3 (n=29) for adults, 44.1 ± 8.2 (n=8) for geriatric dogs and 34.3 ± 13.4 (n=6) for sub-adults).

Body mass had no effect on the percentage of spermatozoa with bent tails (DF=1, F=0.04 p=0.86, 41.1 ± 4.0 (n=33) for normal-weight dogs and 42.8 ± 8.7 (n=7 for underweight dogs).

Previous contraception history had no effect on the percentage of spermatozoa with bent tails (DF=1, F=0.81, p=0.37, 38.3 ± 5.0 (n=21) for naïve dogs and 44.8 ± 5.2 (n=19) for previously contracepted dogs).

Proximity to female dogs had no effect on the percentage of spermatozoa with bent tails (DF=2, F=0.94, p=0.40, 38.6 ± 4.8 (n=23) for dogs housed with females, 41.7 ± 6.6 (n=12) for dogs housed without females and 54.0 ± 10.2 (n=6) for dogs with olfactory contact with females only).

4.2.4.6 Percentage of spermatozoa with proximal cytoplasmic droplets

a) The main effect of Treatment on the percentage of spermatozoa with proximal cytoplasmic droplets

When the percentage of spermatozoa with proximal cytoplasmic droplets before treatment was compared with that after treatment, with post-treatment data pooled into observational windows of different durations after treatment, there was no significant difference in the

percentage of proximal cytoplasmic droplets between treatment groups (DF=2, F≤3.63, p≥0.13; See Table 11). Treatment and Time interacted in their effect on the percentage of spermatozoa with proximal cytoplasmic droplets (Figure 17). Prior to treatment, but not thereafter, the implant group had significantly more proximal droplet defects than the placebo group (DF=2, F=221.25, p=0.004).

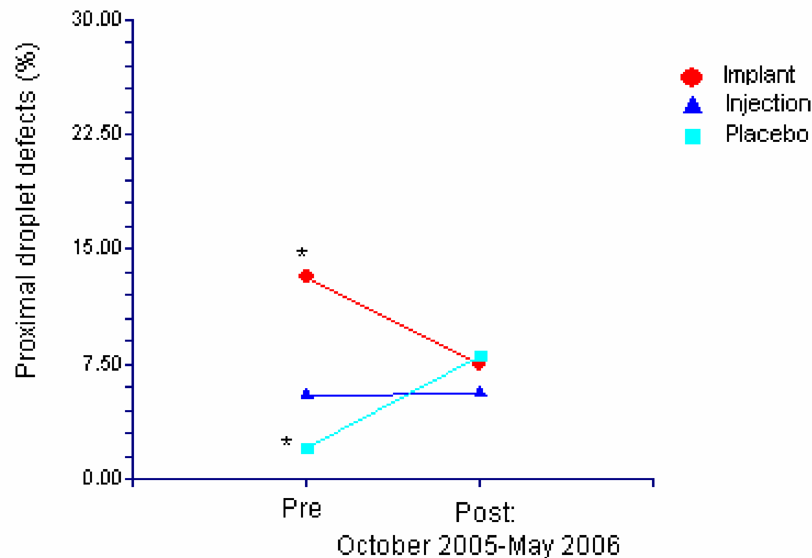

Figure 19. The interaction between treatment and time relative to treatment with respect to the prevalence of proximal cytoplasmic droplets (* indicate that means differ (P<0.05))

b) The effect of confounders on the percentage of spermatozoa with proximal cytoplasmic droplets

Season had no effect on the percentage of spermatozoa with proximal cytoplasmic droplets (DF=1, F=0.02 p=0.88, 8.9 ± 2.4 (n=21) for winter and 9.5 ± 3.4 (n=11) for summer).

Age had no effect on the percentage of spermatozoa with proximal cytoplasmic droplets (DF=2, F=0.71, p=0.50, 9.0 ± 2.3 (n=24) for adults, 12.2 ± 4.5 (n=6) for geriatric dogs and 1.5 ± 7.8 (n=6) for sub-adults).

Normal-weight dogs had 17.0% ± 4.2% (n=26) spermatozoa with proximal cytoplasmic droplets which was significantly greater than the 7.3% ± 2.0% (n=6) spermatozoa with proximal cytoplasmic droplets for underweight dogs (DF=1, F=4.24, p=0.05)

Previous contraception history had no effects on the percentage of spermatozoa with proximal cytoplasmic droplets (DF=1, F=1.54, p=0.22, 11.5 ± 2.7 (n=16) for naïve dogs and 6.8 ± 2.7 (n=16) for previously contracepted dogs).

Proximity to female dogs had no effects on the percentage of spermatozoa with proximal cytoplasmic droplets (DF=2, F=0.07, p=0.93, 9.4 ± 2.7 (n=18) for dogs housed with females, 8.0 ± 3.8 (n=9) for dogs housed without females and 10.2 ± 5.0 (n=6) for dogs with olfactory contact with females only).

4.2.4.7 Detailed distribution of defects

Table 14 shows that neither Treatment nor Stage (before or after treatment) had an effect on the percentage of individual sperm defects (p>0.05). Pre-treatment sperm morphology assessments were made in the months of August and September, the end of winter. During these months many semen samples were contaminated with urine as well. Midpiece reflex (34) and distal droplet (14) were the two most common defects seen before treatment. Defects seen after treatment were seen with similar frequency in each treatment group.

4.2.5 Azoospermia

a) The main effect of Treatment on azoospermia

Table 15 demonstrates the distribution of azoospermic dogs between the treatment groups before and after treatment. Very few dogs were azoospermic during the study: five at the time of initial treatment and eight thereafter. Of the dogs receiving implants that were azoospermic after treatment, immediately prior to the administration of treatment one was azoospermic, three oligospermic (M107 had 10,000, M333 had 20 million and M344 had one and a half million spermatozoa per ejaculate), and two normospermic (M380 had 94 million and M216 had 376 million spermatozoa per ejaculate). The latter two implant dogs were housed with a female at the start of the study, but were housed without a female dog during some later period of the study. The placebo dog that was azoospermic after treatment was housed with a male dog only but had olfactory contact with nearby female dogs. The injection dog that was azoospermic after treatment was housed with a female dog only.

Table 14. Mean (±SEM) percentage of different spermatozoa defects for each treatment group before treatment and after treatment

	Immediately prior to treatment (23 Aug. 2005 to 1 Sept. 2005)	After treatment (2 Sept. 2005 to 30 Aug. 2006)		
	All groups (n=27)	Implant (n=10)	Injection (n=11)	Placebo (n=6)
Teratoid	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Double	1.2 ± 0.4	1.0 ± 0.5	1.5 ± 0.6	2.0 ± 0.8
Macrocephalic	0.0 ± 0.0	11.0 ± 0.8	1.8 ± 0.4	1.5 ± 0.4
Microcephalic	0.0 ± 0.0	1.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0
Rolled head	1.0 ± 0.6	2.5 ± 0.8	2.0 ± 0.6	1.3 ± 0.6
Pyriform	1.0 ± 0.5	2.0 ± 0.4	1.0 ± 0.2	1.7 ± 0.2
Tapered head	2.5 ± 3.4	3.0 ± 6.6	10.0 ± 4.2	5.6 ± 4.2
Diadem	4.0 ± 2.0	3.5 ± 2.2	5.0 ± 1.6	3.8 ± 1.5
Narrow base	0.0 ± 0.0	2.0 ± 2.7	0.0 ± 0.0	3.0 ± 1.5
Abnormal base	1.0 ± 1.1	5.0 ± 0.9	1.7 ± 0.7	2.5 ± 0.5
Other abnormal head shapes	1.0 ± 0.5	1.0 ± 0.7	1.3 ± 0.4	3.5 ± 0.5
Abnormal loose heads	0.0 ± 0.0	0.0 ± 0.0	1.5 ± 0.5	1.0 ± 0.7
Knobbed acrosome	0.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Stump tail	0.0 ± 0.0	2.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pseudodroplet	0.0 ± 0.0	5.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0
Aplasia of mitochandria	1.0 ± 2.7	8.0 ± 2.7	2.8 ± 1.6	2.8 ± 1.9
Cork screw	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.0 ± 0.0
Dag	2.5 ± 0.7	1.0 ± 2.2	1.5 ± 1.1	1.5 ± 1.6
Other midpiece defects	1.0 ± 2.1	0.0 ± 0.0	0.0 ± 0.0	2.5 ± 1.5
Coiled principle piece	4.4 ± 1.2	5.0 ± 3.3	1.3 ± 1.9	5.8 ± 1.6
Proximal droplet	9.4 ± 3.0	10.0 ± 6.8	7.3 ± 4.1	10.4 ± 4.4
Midpiece reflex	34.2 ± 4.6	18.5 ± 9.3	15.5 ± 5.6	15.4 ± 6.2
Normally shaped loose heads	5.4 ± 4.9	5.3 ± 8.3	11.0 ± 5.8	9.5 ± 8.3
Fractured flagellum	1.7 ± 0.5	3.7 ± 1.0	2.0 ± 0.8	1.3 ± 0.9
Distal droplet	14.4 ± 3.2	12.0 ± 9.2	6.5 ± 3.3	3.0 ± 4.1
Degenerate acrosome	2.5 ± 3.6	1.0 ± 8.9	7.0 ± 6.3	9.0 ± 4.4
Bent midpiece	9.5 ± 4.5	3.0 ± 7.5	0.0 ± 0.0	8.0 ± 3.8
Bent principle piece	11.9 ± 3.4	13.0 ± 5.6	12.0 ± 3.3	25.0 ± 4.2

Table 15. The number of azoospermic dogs in the treatment groups before and after treatment (Number of azoospermic/total number in group)

	Placebo	Injection	Implant
Before treatment	1/6	2/11	2/10
After treatment	1/6	1/11	6/10
Before and after treatment	0/6	0/11	1/10

b) The effect of confounders on sperm count and the relationship between other reproductive parameters and sperm count

Body mass did not effect the sperm count per ejaculate (DF=1, F=0.03, *p*=0.85, 89.8 ± 16.7 (n=46) for normal-weight dogs and 35.7 ± 0.3 (n=14) for underweight dogs).

Age did not effect the sperm count per ejaculate (DF=1, F=0.43, *p*=0.51, 88.06 ± 17.80 (n=42) for adults, 57.2 ± 33.3 (n=12) for geriatric dogs and 41.2 ± 7.1 (n=6) for sub-adults).

Although not a statistically significant finding, dogs with greater semen osmolality (DF=1, F=1.89, *p*=0.19, 1.45 ± 0.27 osmol (n=6) for azoospermic dogs and 1.02 ± 0.16 osmol (n=14) for non-azoospermic dogs) and lower semen pH (DF=1, F=1.84, *p*=0.18, 6.3 ± 0.2 (n=11) for azoospermic dogs and 6.5 ± 0.1 (n=46) for non-azoospermic dogs) were more likely to be azoospermic. Azoospermic dogs had significantly lower serum testosterone concentrations compared with non-azoospermic dogs (DF=1, F=9.2, *p*=0.004, 0.11 ± 0.10 ng/ml (n=13) for azoospermic dogs and 0.45 ± 0.05 ng/ml (n=47) for non-azoospermic dogs).

4.3 Urine and semen pH, osmolality, and sperm count

4.3.1 Urine pH , osmolality and sperm count

a) The main effect of Treatment on urine pH, osmolality and sperm count

Dogs commonly started ejaculating when the stimulus reached an amplitude of one and a half volts and seldom required greater amplitudes than three volts. Regardless of Treatment, there was no significant difference in urine pH (DF=5, F=0.16, *p*=0.85, 6.2 ± 0.1 (n=7) for the implant group, 6.2 ± 0.1 (n=7) for the injection group and 6.1 ± 0.1 (n=8) for the placebo group). The implant group had a significantly lower urine osmolality than the injection group (DF=5, F=2.70, *p*=0.05, 1.72 ± 0.21 osmol (n=7) for the implant group and 2.35 ± 0.21 osmol (n=7) for the injection group). The sperm count (in millions) per ml of urine for each treatment group varied little. Urine pH, osmolality and sperm counts by treatment group for the months of January to August 2006 are shown in Figures 18, 19, and 20.

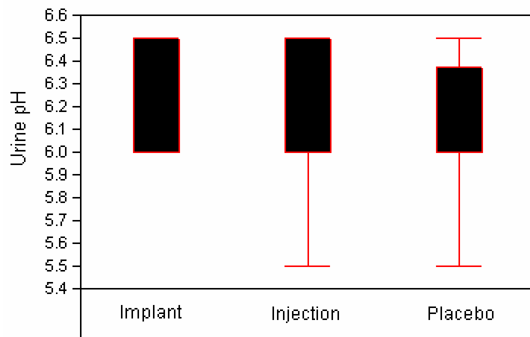

Figure 20. Median, interquartile range and range of urine pH for each treatment group (Implant, n=7; Injection, n=7; Placebo, n=8).

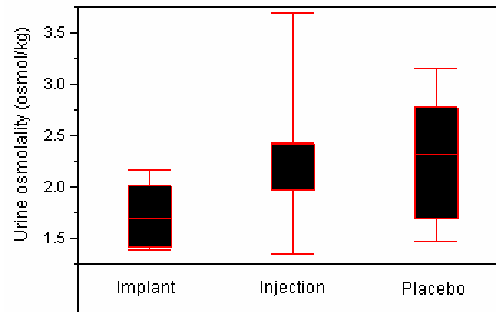

Figure 21. Median, interquartile range and range of urine osmolality for each treatment group (Implant, n=7; Injection, n=7; Placebo, n=8).

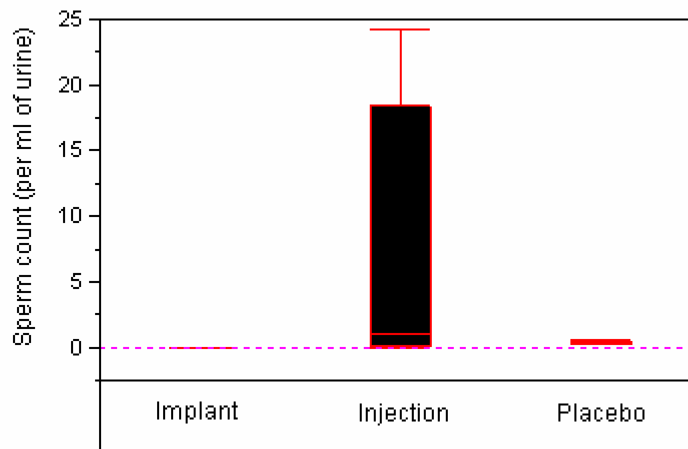

Figure 22. Median, interquartile range and range of sperm in millions count per ml of urine by treatment group (Implant, n=2; Injection, n=4; Placebo, n=3).

4.3.2 Semen pH, osmolality, and sperm count

Figures 21 and 22 demonstrate the variability of semen osmolality and pH between treatment groups.

a) The main effect of Treatment on semen pH, osmolality and sperm count

Semen pH was higher after treatment than before treatment for the injection group (DF=5, F=2.16, p=0.01, 6.3 ± 0.2 (n=10) for the injection group before treatment, 6.8 ± 0.1 (n=13) for the injection group after treatment). Regardless of treatment group, the semen pH was inversely correlated with the semen osmolality (Corr=-0.7337, p=0.002; Semen pH: n=58, Mean=6.5, SD=0.54; Semen osmolality: n=20, Mean=1.09, SD=0.62). The remaining urine

and semen variables (pH, osmolality and sperm count) were not significantly correlated with one another (Corr=-0.3177 to 0.4142, $p \geq 0.13$; Semen pH: n=19, Mean=6.7, SEM=0.58; Semen osmolality: n=19, Mean=1.05, SEM=0.61; Semen sperm count: n=60, Mean=77.2, SEM=111.67; Urine pH: n=19, Mean=6.1, SEM=0.32; Urine osmolality: n=19, Mean=2.14, SEM=0.64; Urine sperm count: n=9, Mean=3.0, SEM=7.94). Semen osmolality was measured for samples collected from January to August 2006 only.

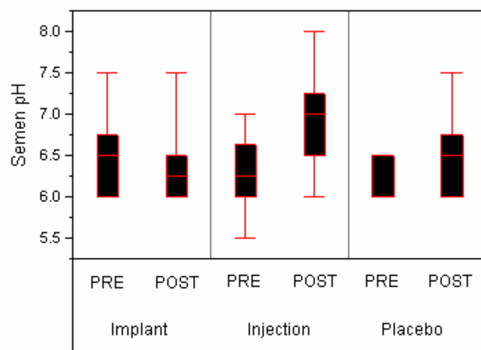

Figure 23. Median, interquartile range and range of semen pH for each treatment group before and after treatment (Implant, n=19; Injection, n=23; Placebo, n=16).

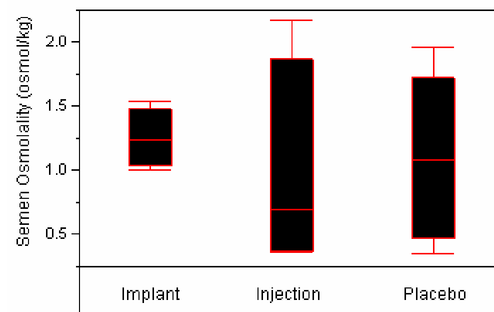

Figure 24. Median, interquartile range and range of semen osmolality for each treatment group (Implant, n=4; Injection, n=8; Placebo, n=8).

The placebo group had a higher sperm count (in millions) per ejaculate than the implant group during the six months immediately post-treatment and higher than the implant and injection groups during the nine month post-treatment window. During the seven-month post-treatment window, the sperm counts of all three groups were different (Table 16). Only those dogs stimulated to ejaculate during a given month after treatments were assessed for sperm counts before and after treatment; therefore the number of dogs included in each month subset varied.

Figure 23 demonstrates the variability in the sperm counts before and after treatment. Assessment of the effects of Treatment, with post-treatment data pooled into one continuous 12 month group, showed that the sperm count (in millions) per ejaculate of the placebo group after treatment was significantly higher than the sperm count (in millions) of the implant group after treatment (DF=5, F=3.01, p=0.02, 55.9 ± 43.4 (n=6) for the placebo group before treatment, 175.2 ± 33.6 (n=10) for the placebo group after treatment, 61.3 ± 33.6 (n=10) for the implant group before treatment, and 21.5 ± 32.0 (n=11) for the implant group after

Table 16. Mean (±SEM) sperm counts (million/ejaculate) for each treatment group measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

Treatment	Immediately prior to treatment	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
		Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Implant	61.3 ± 37.0 (n=10)	161.0 ± 9.4 (n=1)	85.6 ± 45.3 (n=2)	29.6 ± 17.4 (n=6)[a]	29.6 ± 21.1 (n=8)[a]	29.6 ± 22.7 (n=8)[a]
Injection	35.3 ± 14.0 (n=11)	42.2 ± 9.4 (n=1)	110.4 ± 37.0 (n=3)	95.7 ± 21.4 (n=4)	145.5 ± 24.3 (n=6)[b]	112.7 ± 22.7 (n=8)[a]
Placebo	55.9 ± 31.6 (n=6)	110.1 ± 6.6 (n=2)	162.6 ± 32.0 (n=4)	162.6 ± 21.4 (n=4)[b]	258.0 ± 24.3 (n=6)[c]	258.0 ± 26.3 (n=6)[b]

[a,b,c] Within a column, means with different superscripts differ ($p \leq 0.01$)

treatment). When assessing the Stage of treatment, the placebo group had a significantly higher sperm count (in millions) per ejaculate after treatment compared with before treatment (DF=5, F=2.96, p=0.03, 55.9 ± 43.4 (n=6) for the placebo group before treatment and 175.2 ± 33.6 (n=10) for the placebo group after treatment). Furthermore, the sperm count (in millions) per ejaculate of the placebo group after treatment was significantly higher than the sperm count (in millions) per ejaculate of the implant group before treatment (DF=5, F=2.96, p=0.02, 175.2 ± 33.6 (n=10) for the placebo group after treatment and 61.3 ± 33.6 (n=10) for the implant group before treatment) and sperm count of the injection group before treatment (DF=5, F=2.96, p=0.004, 35.3 ± 32.0 (n=11) for the injection group before treatment). Additionally, the sperm count (in millions) per ejaculate of the placebo group after treatment was also significantly higher than the sperm count (in millions) of the implant group after treatment (DF=5, F=2.96, p=0.002, 175.2 ± 33.6 (n=10) for the placebo group after treatment and 21.5 ± 32.0 (n=11) for the implant group after treatment). Furthermore, the pre-treatment sperm count (in millions) per ejaculate of the placebo group was higher than the sperm count (in millions) of the implant group before treatment (DF=5, F=2.96, p=0.02, 55.9 ± 43.4 (n=6) for the placebo group before treatment and 61.3 ± 33.6 (n=10) for the implant group before treatment) and injection group before treatment (DF=5, F=2.96, p=0.004, 35.3 ± 32.0 (n=11) for the injection group before treatment), respectively. The sperm count (in millions) per ejaculate of the placebo group before treatment was also higher than the sperm count (in millions) of the implant group after treatment (DF=5, F=2.96, p=0.002, 21.5 ± 32.0 (n=11) for the implant group after treatment).

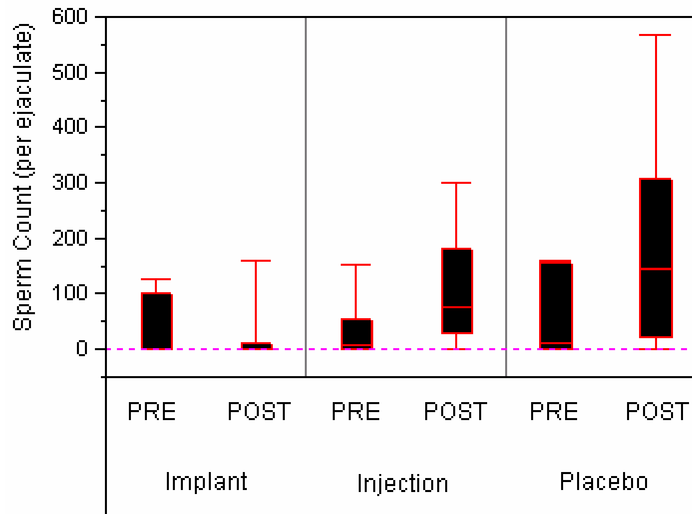

Figure 25. Median, interquartile range and range of sperm count (in millions) per ejaculate before and after treatment for each group (Implant, n=21; Injection, n=23; Placebo, n=16).

When the sperm count (in millions) before treatment was compared with that after treatment, with post-treatment data pooled into observational windows of different durations after treatment, the sperm count before treatment was significantly lower than the sperm count after treatment for the six month (DF=1, F=8.23, p=0.01), seven month (DF=1, F=21.68, p=0.001), and nine month (DF=1, F=16.05, p=0.002; Table 17) post-treatment windows.

Table 17. Mean (±SEM) sperm counts (million/ejaculate) before treatment and measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

Stage of measurement	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
	Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Before treatment	45.5 ± 4.7 (n=4)	45.7 ± 28.7 (n=5)	41.9 ± 14.2 (n=9)^{a}	36.5 ± 14.2 (n=12)^{a}	37.0 ± 17.2 (n=14)^{a}
After treatment	104.4 ± 4.7 (n=4)	119.5 ± 21.4 (n=9)	96.0 ± 11.4 (n=14)^{b}	144.4 ± 11.4 (n=20)^{b}	133.4 ± 13.7 (n=22)^{b}

a,b=Within a column, means with different superscripts differ (p≤0.01)

b) The effect of confounders on sperm count

Dogs had significantly higher sperm counts per ejaculate during summer, October to April, as opposed to winter (DF=1, F=7.90, *p*=0.007, 49.4 ± 17.2 (n=40) for summer and 132.9 ± 24.3 (n=20) for winter).

Age had no effect on sperm count per ejaculate (DF=2, F=0.66, *p*=0.52, 88.1 ± 17.8 (n=42) for adults, 57.2 ± 33.3 (n=12) for geriatric dogs and 41.2 ± 47.1 (n=12) for sub-adults).

Body mass had no effect on sperm count per ejaculate (DF=1, F=2.44, *p*=0.12, 89.8 ± 16.7 (n=46) for normal-weight dogs and 35.7 ± 30.3 (n=14) for underweight dogs).

Previous contraception history had no effect on sperm count per ejaculate (DF=1, F=1.19, *p*=0.28, 92.8 ± 20.6 (n=31) for naïve dogs and 60.5 ± 21.3 (n=29) for previously contracepted dogs).

Throughout the study, dogs housed with females had significantly lower sperm counts per ejaculate than dogs housed without females (DF=2, F=3.53, *p*=0.01, 57.4 ± 18.1 (n=37) for dogs housed with females and 142.3 ± 28.4 (n=15) for dogs housed without females).

4.4 Serum testosterone

a) The main effect of Treatment on serum testosterone

Stage (before or after treatment) had a significant effect on the concentration of testosterone in serum, regardless of the type of treatment, for the post-treatment windows spanning six months post-treatment (DF=1, F=9.82, *p*=0.01), seven months post-treatment (DF=1, F=13.25, *p*=0.003), and ten months post-treatment (DF=1, F=8.06, *p*=0.01; see Table 18). Only dogs bled during a given post-treatment month window (ie, 3,6,7,9,10 months post-treatment) were assessed for serum testosterone concentration before and after treatment; therefore the number of dogs included in each month subset varied, as seen in Table 18.

Table 18. Mean (±SEM) concentrations of testosterone in serum (ng/ml) before treatment and measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

Stage of measurement	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)					
	Until 28 Nov. 2005 (3 months	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)	Until 30 Aug. 2006 (12 months)
Before treatment	0.02 ± 0.10 (n=4)	0.11 ± 0.14 (n=5)	0.20 ± 0.11 (n=13)[a]	0.20 ± 0.08 (n=15)[a]	0.26 ± 0.08 (n=18)[a]	0.46 ± 0.10 (n=12)
After treatment	0.52 ± 0.10 (n=4)	0.46 ± 0.11 (n=9)	0.62 ± 0.08 (n=23)[b]	0.56 ± 0.06 (n=29)[b]	0.55 ± 0.06 (n=32)[b]	0.27 ± 0.10 (n=12)

[a,b] Within a column means with different superscripts differ ($p \leq 0.01$)

Figure 24 shows the concentrations of serum testosterone before and after treatment for the three treatment groups assessed in the study.

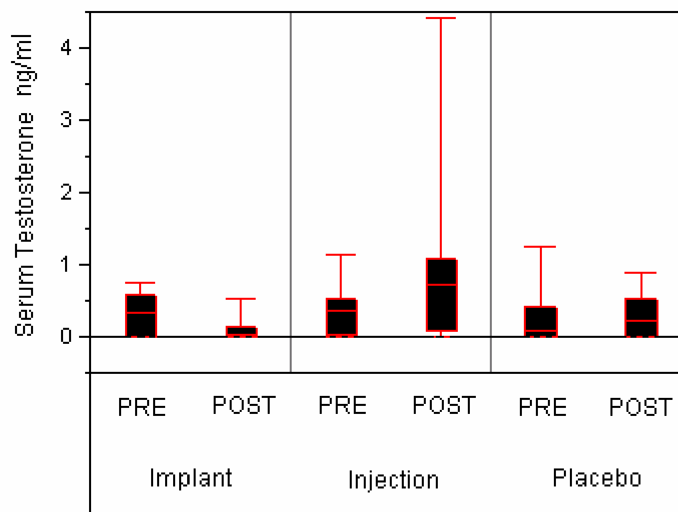

Figure 26. Median, interquartile range and range of concentration of testosterone (ng/ml) in serum before and after treatment for each treatment group (Implant: n=19; Injection: n=17; Placebo: n=14).

After treatment, the injection group had significantly higher serum testosterone concentration levels compared with the other two treatment groups for the 10-month post-treatment window (DF=1, F=3.60, p=0.05; see Table 19).

Table 19. Mean (±SEM) serum testosterone (ng/ml) concentrations for each treatment group measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)					
Treatment	Immediately prior to treatment	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)	Until 30 Aug. 2006 (12 months)
Implant	0.34 ± 0.09 (n=10)	0.52 ± 0.26 (n=1)	0.26 ± 0.22 (n=2)	0.16 ± 0.13 (n=9)	0.13 ± 0.00 (n=11)[a]	0.12 ± 0.00 (n=12)[a]
Injection	0.40 ± 0.11 (n=11)	0.47 ± 0.26 (n=1)	0.63 ± 0.18 (n=3)	1.26 ± 0.16 (n=6)	1.11 ± 0.11 (n=8)[b]	1.11 ± 0.11 (n=10)[b]
Placebo	0.26 ± 0.20 (n=6)	0.58 ± 0.18 (n=2)	0.48 ± 0.16 (n=4)	0.44 ± 0.14 (n=8)	0.43 ± 0.00 (n=10)[a]	0.43 ± 0.11 (n=10)[a]

[a,b]Within a column means with different superscripts differ (p=0.05)

b) The effect of confounders on serum testosterone

During winter, which lasted from May to September, the concentration of testosterone in serum was 0.33 ± 0.09 ng/ml (n=47), which was similar to the 0.55 ± 0.11 ng/ml (n=30) during the summer, which lasted from October to April (DF=1, F=2.39, p=0.13).

Age had an effect on serum testosterone concentrations of sub-adult dogs whose testosterone was 0.85 ng/ml (n=9 samples from 4 dogs) and adult dogs whose serum testosterone was 0.33 ng/ml (n=54 samples from 18 dogs; DF=2, F3.00, p=0.02, 0.33 ± 0.40 ng/ml (n=54) for sub-adults, 0.47 ± 0.41 ng/ml (n=14) for geriatric dogs and 0.85 ± 1.41 ng/ml (n=9) for adults). However, there was no significant difference between the serum testosterone concentrations of geriatric dogs whose testosterone was 0.47 ng/ml (n=14 samples from 5 dogs) and either sub-adult dogs (DF=2, F=3.00, p=0.15) or adult dogs (DF=2, F=3.00, p=0.41).

Body mass had no effect on serum testosterone concentrations (DF=1, F=0.40, p=0.53, 0.44 ± 0.08 ng/ml (n=61) for normal-weight dogs and 0.33 ± 0.15 ng/ml (n=16) for underweight dogs).

Previous contraception history had no effect on serum testosterone concentration (DF=1, F=0.37, p=0.54, 0.45 ± 0.10 ng/ml (n=40) for naïve dogs and 0.37 ± 0.10 ng/ml (n=37) for previously contracepted dogs).

Proximity to female dogs had no effect on the serum testosterone concentration (DF=2, F=1.27, p=0.29, 0.50 ± 0.09 ng/ml (n=46) for dogs housed with females, 0.33 ± 0.14 ng/ml (n=20) for dogs housed without females and 0.21 ± 0.18 ng/ml (n=11) for dogs with olfactory contact with females only).

4.5 Prostate volume and preputial sheath size

4.5.1 Prostate volume

a) The main effect of Treatment on prostate volume

When the prostate volume before treatment was compared with that after treatment, with post-treatment data pooled into observational windows of different durations after treatment, the following results were found. After treatment, the prostate volume of the injection group was significantly greater than the prostate volume of the implant and placebo groups, respectively, during the three month post-treatment window (DF=2, F=253.3, p=0.04) and significantly greater than the prostate volume of implant group, during the nine month post-treatment window (DF=2, F=3.9, p=0.05; Table 20).

Table 20. Mean (±SEM) prostate volume (mm^3) for each treatment group measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)					
Treatment	Immediately prior to treatment	Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Implant	5705 ± 1008.3 (n=10)	9240 ± 418.0 (n=1)^a	9404 ± 1082.8 (n=2)	7747 ± 1339.6 (n=6)	7964 ± 1684.0 (n=8)	7964 ± 1878.8 (n=8)^a
Injection	7283 ± 1416.6 (n=10)	13120 ± 418.0 (n=1)^b	13215 ± 884.1 (n=3)	14366 ± 1640.6 (n=4)	13831 ± 1944.5 (n=6)	15515 ± 1878.8 (n=8)^b
Placebo	5534 ± 1331.5 (n=6)	5568 ± 595.6 (n=2)^a	8247 ± 765.6 (n=4)	9546 ± 1467.4 (n=5)	10890 ± 1800.2 (n=7)	10890 ± 2008.6 (n=7)

^{a,b}Within a column means with different superscripts differ (p≤0.05)

When the data of treatment groups were combined and post-treatment data grouped into observation periods of different duration, every post-treatment observation window had a significantly higher mean prostate volume than the respective means before treatment (see Table 21). In all cases, the prostate volume after treatment was about two times as high as it was before treatment.

Table 21. Mean (±SEM) prostate volume (mm^3) for each treatment group measured over different time spans after treatment, which occurred between 23 Aug. 2005 and 1 Sept. 2005

Stage of measurement	Time span (from 28 Nov. 2005) over which post-treatment values were pooled for analysis (and the approximate window period in months after treatment)				
	Until 28 Nov. 2005 (3 months)	Until 30 Jan. 2006 (5 months)	Until 23 Feb. 2006 (6 months)	Until 3 Apr. 2006 (7 months)	Until 24 May 2006 (9 months)
Before treatment	4008 ± 209.0 (n=4)^a	4345 ± 684.8 (n=5)^a	5559 ± 1037.6 (n=10)^a	5003 ± 1375.0 (n=12)^a	5606 ± 1420.2 (n=14)^a
After treatment	9309 ± 209.0 (n=4)^b	10289 ± 510.4 (n=9)^b	10553 ± 847.2 (n=15)^b	10895 ± 1039.4 (n=21)^b	11456 ± 1108.1 (n=23)^b

^{a,b}Within a column means with different superscripts differ ($p \leq 0.04$).

When all post-treatment data of a treatment group were pooled into one continuous 12 month observational window, Stage (before or after treatment) had no effect on the prostate volume, regardless of treatment group (DF=5, F=0.24, p=0.94, 4466 ± 2376.6 mm^3 (n=5) for the implant group before treatment, 7693 ± 1878.8 mm^3 (n=8) for the implant group after treatment, 6292 ± 2169.5 mm^3 (n=6) for the injection group before treatment, 15514 ± 1878.8 mm^3 (n=8) for the injection group after treatment, 6059 ± 3068.1 mm^3 (n=3) for the placebo group before treatment and 10890 ± 2008.6 mm^3 (n=7) for the placebo group after treatment).

b) The effect of confounders on prostate volume

Season had no effect on prostate volume (DF=1, F=0.32, p=0.57, 6854 ± 743.1 mm^3 (n=39) for winter and 10744 ± 1028.5 mm^3 (n=22) for summer).

Geriatric dogs (> 7 years old) had significantly larger prostate volumes than adults and sub-adults combined (DF=2, F=3.3, p=0.04, 4873 ± 544.3 mm^3 (n=54) for sub-adults, 7627 ± 1069.0 mm^3 (n=14) for geriatric dogs and 3831 ± 1333.3 mm^3 (n=9) for adults).

Body mass had no effect on prostate volume (DF=1, F=0.24, *p*=0.63, 5134 ± 529.9 mm^3 (n=61) for normal-weight dogs and 5702 ± 1034.7 mm^3 (n=16) for underweight dogs).

Previous contraception history had no effect on prostate volume (DF=1, F=0.48, *p*=0.49, 4937 ± 653.4 mm^3 (n=40) for naïve dogs and 5592 ± 679.3 mm^3 (n=37) for previously contracepted dogs),

Proximity to female dogs had no effect on prostate volume (DF=2, F=0.36, *p*=0.70, 5542 ± 612.4 mm^3 (n=46) for dogs housed with females, 5043 ± 928.7 mm^3 (n=20) for dogs housed without females and 4418 ± 1252.2 mm^3 (n=11) for dogs with olfactory contact with females only).

If dogs were azoospermic at the time of measurement they also had significantly smaller prostate volumes than those that were not azoospermic (DF=1, F=4.6, *p*=0.04, 4795 ± 945.8 mm^3 (n=13 measurements in 12 dogs) for azoospermic dogs and 7098 ± 497.4 mm^3 (n=47 measurements in 25 dogs) for non-azoospermic dogs). Figure 25 shows the variability of prostate volume with respect to azoospermia.

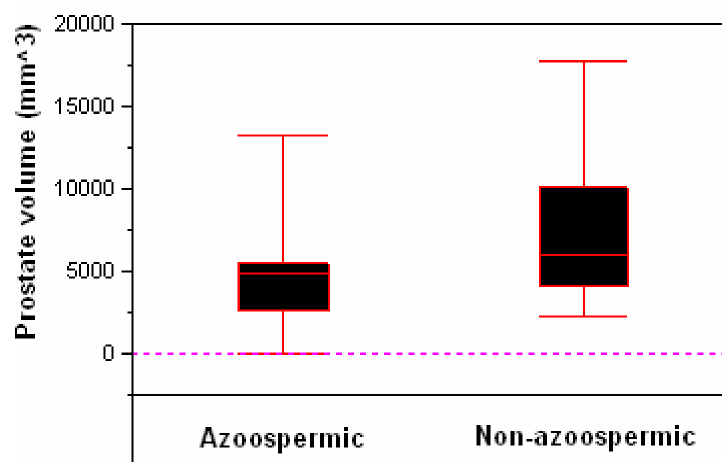

Figure 27. Median, interquartile range and range of prostate volume (mm^3) for azoospermic (n=13) and non-azoospermic dogs (n=47).

4.5.2 Preputial measurements

4.5.2.1 Preputial flap gland width

a) The main effect of Treatment on preputial gland width

When all post-treatment data were pooled into one continuous 12 month observational window, the flap gland width of the implant group before treatment was larger than that of the placebo group before treatment and was also larger than the width of the implant and placebo groups, respectively, after treatment (DF=5, F=1.9, P<0.05, 21 ± 0.9 mm (n=10) for the implant group before treatment, 18 ± 0.9 mm (n=11) for the implant group after treatment, 8 ± 1.2 mm (n=6) for the placebo group before treatment and 18 ± 0.9 mm (n=11) for the placebo group after treatment).

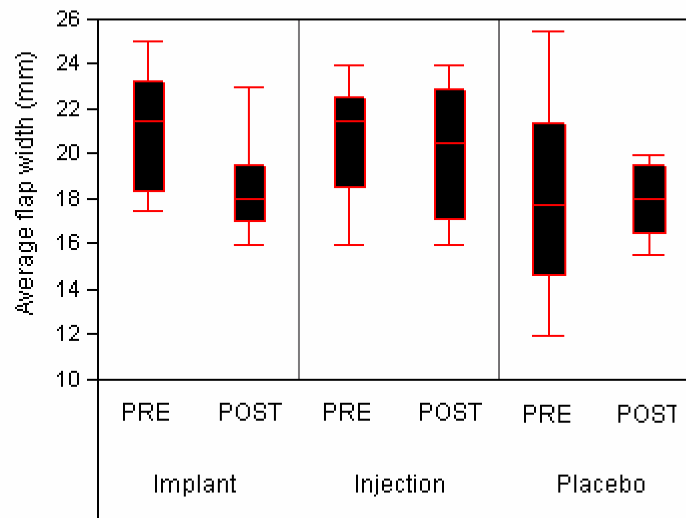

Figure 28. Median, interquartile range and range of preputial flap width (mm) before and after treatment by treatment group (Implant, pre: n=10; Implant, post: n=11; Injection, pre: n=11; Injection, post: n=12; Placebo, pre: n=6; Placebo, post: n=11)

b) The effect of confounders on preputial gland width

Flap gland widths were significantly larger during winter as opposed to summer (DF=1, F=8.38, *p*=0.005, 20 ± 0.4 mm (n=40) for winter and 18 ± 0.6 mm (n=21) for summer).

Geriatric dogs had significantly larger flap gland widths than either adults (DF=2, F=7.44, *p*=0.0003, 22 ±0.7 mm (n=13) for geriatric dogs and 19 ± 0.4 mm (n=42) for adults) or sub-adults (DF=2, F=7.44, *p*=0.03l, 20 ± 1.0 mm (n=6) for sub-adults).

Body mass did not significantly affect flap gland width (DF=1, F=1.31, *p*=0.26, 19 ± 0.4 mm (n=47) for normal-weight dogs and 20 ± 0.8 mm (n=14) for underweight dogs).

Naïve dogs had significantly smaller flap gland widths than previously contracepted dogs (DF=1, F=7.4, *p*=0.009, 18 ± 0.5 (n=31) for naïve dogs and 20 ± 0.5 (n=30) for previously contracepted dogs).

Dogs housed with females had significantly larger flap gland widths than dogs housed without females (DF=3, F=2.07, *p*=0.05, 20 ± 0.4 mm (n=37) for dogs housed with females and 17 ± 0.7 mm (n=15) for dogs housed without females and 19 ± 0.9 mm (n=9) for dogs with olfactory contact with females only).

4.5.2.2 Preputial orifice diameter

a) The main effect of Treatment on preputial orifice diameter

For each treatment the preputial orifice diameter enlarged after treatment (Placebo group: DF=5, F=5.14, *p*=0.003, 52 ± 3.4 mm (n=6) for before treatment and 65 ± 2.5 mm (n=11) for after treatment; Injection group: DF=5, F=5.14, *p*=0.01, 59 ± 2.5 mm (n=11) for before treatment and 68 ± 2.43 mm (n=12) for after treatment; Implant group: DF=5, F=5.14, *p*=0.006, 56 ± 2.7 mm (n=11) for before treatment and 67 ± 2.5 mm (n=12) for after treatment). Figure 27 demonstrates the variability between groups before and after treatment with respect to their preputial orifice diameter.

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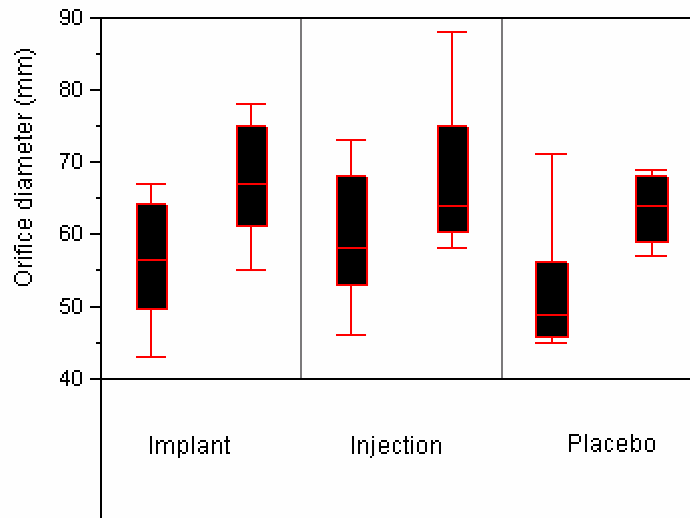

Figure 29. Median, interquartile range and range of preputial orifice diameter (mm) before and after treatment by treatment group (Implant, pre: n=10; Implant, post: n=11; Injection, pre: n=11; Injection, post: n=12; Placebo, pre: n=6; Placebo, post: n=11)

b) The effect of confounders on preputial gland width

Preputial orifice diameter was significantly larger during summer than winter (DF=1, F=4.33, *p*=0.04, 60 ± 1.50 mm (n=40) for winter and 66 ± 2.1 mm (n=21) for summer).

Geriatric dogs had significantly larger preputial orifice diameters than adults (DF=2, F=7.44, *p*=0.0003, 71 ± 2.5 mm (n=13) for geriatric dogs, 60 ± 1.36 mm (n=42) for adults) and sub-adults (DF=2, F=7.44, 0.03, 61 ± 3.6 mm (n=12) for sub-adults).

Body mass had no effect on preputial orifice diameter (DF=2, F=0.08, *p*=0.78, 62 ± 1.4 mm (n=47) for normal-weight dogs and 63 ± 2.6 mm (n=14) for underweight dogs).

Previous contraception history had no effect on preputial orifice diameter (DF=1, F=2.97, *p*=0.09, 60 ± 1.7 mm (n=31) for naïve dogs and 64 ± 1.8 mm (n=30) for previously contracepted dogs).

Dogs housed with females had significantly larger preputial diameters than dogs housed without females (DF=1, F=2.07, p=0.05, 64 ± 1.6 mm (n=37) for dogs housed with females and 58 ± 2.5 mm (n=15) for dogs housed without females and 62 ± 3.2 mm (n=9) for dogs with olfactory contact with females only).

4.6 Haematology, serum chemistry and body mass

4.6.1 Haematology

Table 22 summarizes the mean haematology and serum chemistry values before and after treatment, irrespective of treatment type administered.

The concentration of haemoglobin in the blood of the placebo group before treatment was significantly greater than after treatment (DF=5, F=9.56, p=0.005, 172.25 ± 0.94 g/l (n=4) for the placebo group before treatment and 186.00 ± 2.94 (n=4) g/l for the placebo group after treatment). Additionally, the haemoglobin of the injection group before treatment was significantly greater than after treatment (DF=5, F=9.56, p=0.006, 171.50 ± 2.94 g/l (n=4) for the injection group before treatment and 158.25 ± 2.94 g/l (n=4) for the injection group after treatment).

The mean red blood cell count of the injection group was significantly higher after treatment than before treatment (DF=5, F=2.04, p=0.04, 8.65 ± 0.25 (n=4) for the injection group before treatment and 9.44 ± 0.25 (n=4) for the injection group after treatment). Additionally, the mean haematocrit of the injection group was higher after treatment than before treatment (DF=5, F=7.97, p=0.01, 0.45 ± 0.008 (n=4) for the injection group before treatment and 0.48 ± 0.008 (n=4) for the injection group after treatment). The MCHC of the placebo group was significantly higher after treatment than before treatment (DF=5, F=5.93, p=0.0002, 34.90 ± 0.18 g/dl (n=4) for the placebo group before treatment and 36.15 ± 0.18 g/dl (n=4) for the placebo group after treatment).

4.6.2 Serum chemistry

The serum albumin of the placebo group was significantly higher before treatment as opposed to after treatment (DF=5, F=3.29, p=0.02, 28.70 ± 0.84 g/l (n=4) for the placebo group before

treatment and 25.72 ± 0.84 g/l (n=4) for the placebo group after treatment). The serum albumin of the injection group was also higher before treatment than after treatment (DF=5, F=3.29, p=0.05, 27.63 ± 0.84 g/l (n=4) for the injection group before treatment and 25.13 ± 0.84 g/l (n=4) for the injection group after treatment). Table 22 shows the serum chemistry information of the treatment groups combined before and after treatment.

4.6.3 Body mass

Body mass did not differ significantly before treatment as opposed to after treatment for any of the treatment groups (DF=5, F=5.87, $p \geq 0.59$, 24.9 ± 0.8 kg (n=10) for the implant group before treatment, 26.3 ± 0.8 kg (n=11) for the implant group after treatment, 24.7 ± 0.8 kg (n=11) for the injection group before treatment, 25.9 ± 0.7 kg (n=11) for the injection group after treatment, 25.1 ± 1.1 kg (n=6) for the placebo group before treatment and 26.2 ± 0.8 kg (n=6) for the placebo group after treatment). Figure 28 shows the variability in body mass between the three treatment groups before and after treatment.

Table22. Mean (±SD) haematology and serum chemistry values before and after treatment (data of treatment groups were combined)

Haematology Values	Before treatment	After treatment
Hb (g/l)	166.64 ± 7.50	175.09 ± 11.19
RCC (x10^12/lx10^9/l)	9.04 ± 0.48	9.51 ± 0.54
Hct	0.48 ± 0.02	0.49 ± 0.03
MCV (fl)	52.64 ± 1.79	51.52 ± 2.38
MCHC (g/dl)	35.08 ± 0.35	35.76 ± 0.47
RDW (%)	20.62 ± 1.37	20.94 ± 1.35
WCC (x10^9/l)	9.53 ± 1.80	8.72 ± 3.65
Mature Neutrophils (x10^9/l)	7.01 ± 1.61	6.82 ± 2.99
Bands (x10^9/l)	0.04 ± 0.08	0.06 ± 0.08
Lymphocytes (x10^9/l)	1.41 ± 0.56	0.97 ± 0.54
Monocytes (x10^9/l)	0.46 ± 0.30	0.41 ± 0.24
Eosinophils (x10^9/l)	0.58 ± 0.25	0.45 ± 0.25
Basophils (x10^9/l)	0.03 ± 0.04	0.02 ± 0.04
Thrombin C (x10^9/l)	387.00 ± 60.83	393.09 ± 78.43
Chemistry Values		
TSP (g/l)	61.58 ± 4.34	58.17 ± 4.22
Alb (g/l)	28.40 ± 2.03	25.70 ± 1.14
Glob (g/l)	33.18 ± 3.94	32.47 ± 3.88
A/G ratio	0.87 ± 0.12	0.80 ± 0.09
ALT (U/l)	105.09 ± 169.49	61.45 ± 37.61
ALP (U/l)	32.45 ±34.08	26.18 ± 17.53
Urea (mmol/l)	10.20 ± 2.00	9.23 ± 3.33
Creatinine (µmol/l)	114.09 ± 13.92	109.18 ± 17.73

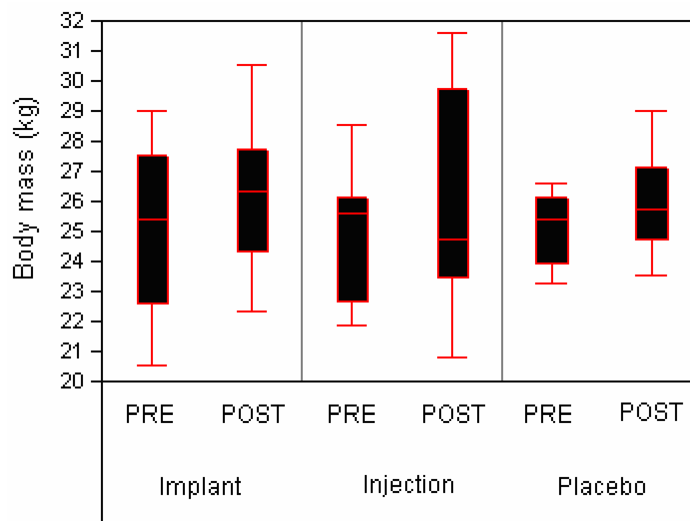

Figure 30. Median, interquartile range and range of body mass (kg) for each treatment group before and after treatment (Implant, pre: n=3; Implant, post: n=3; Injection, pre: n=4; Injection, post: n=4; Placebo, pre: n=4, Placebo, post: n=4).

4.7 Side-effects of treatment administration

4.7.1 Implant administration

Prior to this study, the 4.7 mg deslorelin implant had been administered in the cervical area of numerous wild dogs (male and female) without incident. The same findings held true in this study. The subcutaneous implant was administered with ease, and during the two weeks after implantation no adverse side effects were noticed at the site of the implant or systemically in any of the implanted dogs.

4.7.2 Experimental injection administration

The viscosity of the experimental injection did not impede its intramuscular administration via 18 gauge needle. However, some of the crushed deslorelin implant suspended in the vehicle had a larger diameter than the lumen of the administration needle. Such particles blocked the needle on several occasions, necessitating the use of a new needle in order to

complete the administration. During the observation period after administration (two weeks), no adverse side effects at the site of the injection were noted in any of the injected dogs. One injected dog, M255, developed multi-focal, raised, ulcerated cutaneous lesions on the flank, hind legs, scrotum and testes about two weeks after injection. Although a relationship to the injection could not be ruled out completely, none of the lesions were located at the injection site and most of the lesions appeared to be the result of mechanical trauma, like biting (Figure 29). Additionally, bite wounds commonly occur on the hind legs and testes of wild dog.

Figure 31. Scrotal and testicular lesion in M255 two weeks after injection

4.7.3 Placebo injection

The placebo injection was easily administered intramuscularly through an 18-gauge needle. No adverse systemic side effects were noted in any placebo dogs after the injection. However, one of the six placebo dogs, M343, did have an injection-site reaction on the medial aspect of the left hind leg about one week after injection (Figure 30). The area was diffusely alopecic, focally ulcerated in the centre, and granulated around the ulcerated area. The affected dog was immobilized and treated with 2.5 ml of prednisolone acetate (Pfizer Animal Health, Sandton 2146, South Africa) and 1.5 ml of amoxicillin with clavulanic acid (Synulox®, Pfizer Animal Health, Sandton 2146, South Africa) given intramuscularly; the lesion fully resolved within two weeks after treatment was administered. Due to the location and appearance of this lesion, it was assumed the lesion was a hypersensitivity reaction to some component of the vehicle that was inadvertently deposited subcutaneously.

Figure 32. Focal injection-site lesion in M343 two weeks after injection

CHAPTER 5

DISCUSSION

5.1 Effect of deslorelin on reproductive parameters and testosterone

This project aimed to develop a deslorelin formulation that could be delivered by remote delivery or pole-syringe injection. Such a contraceptive formulation would obviate the need to immobilise wild dogs for administration of contraception. The 4.7 mg deslorelin implant (Suprelorin®, Peptech Animal Health, Sydney) was used as the gold standard in this study, because it had been used successfully in wild dogs and cheetahs previously (Bertschinger *et al.,* 2001; Bertschinger *et al*., 2002; Bertschinger *et al.,* 2004a; Bertschinger *et al.,* 2006) . The injectable formulation consisted of DL-lactide-co-glycolide and 1-methyl-2-pyrrolidone polymers mixed with pulverized deslorelin implants. These polymers were mixed in a 3:1 ratio, which differed slightly from the ratio used by Baker *et al.* (2005). Each dog in the injection treatment group was administered 2 ml of the formulation, which contained approximately 9.4 mg of active deslorelin product. A control group of dogs was administered 2 ml of the polymer vehicle only.

It was expected that the 4.7 mg deslorelin implant would down-regulate serum testosterone to baseline levels, decrease testicular and prostatic volumes, and render treated dogs azoospermic. In previous studies of deslorelin administration in male cheetah and African wild dog, serum testosterone went to baseline within six weeks after treatment and study animals were rendered oligo- or azoospermic within three months after treatment (Bertschinger *et al.,* 2001; Bertschinger *et al*., 2002; Bertschinger *et al.,* 2004a; Bertschinger *et al.,* 2006). Based on the performance of a vehicle formulation used by Baker *et al.* (2005) and the use of pulverized 4.7 mg deslorelin implants, the long-acting contraception injection was expected to decrease serum testosterone to baseline and decrease testicular volume with or without azoospermia for a period of three to six months. The placebo dogs were expected to show no response to vehicle administration and to show reproductive trait seasonality from February through May (Bertschinger, personal communication; Nöthling *et al.,* 2002). Confounders thought to potentially affect reproductive trait changes, regardless of treatment administered, included: season, age, body mass, previous contraception history, and contact with female dogs.

In dogs that were evaluated from November to May, a period spanning up to nine months after treatment, the implant group had a smaller combined testicular volume than the two other groups. The differences in combined testicular volume, however, were not statistically significant. Based on this finding, it appears that the implant may have had some partial contraceptive effects; however, 50% of the implant dogs, despite having smaller testes during the breeding season, were still producing spermatozoa. This finding is in direct contrast to previously published reports (Bertschinger *et al.,* 2001, Bertschinger *et al.,* 2002) that supported the efficacy of this contraceptive in male African Wild dogs and other wild canid species. In one report, the authors warned against administration of the contraceptive to male red and grey wolves too close to the onset of the breeding season (Bertschinger *et al.,* 2001). Previous studies have reported that wild dogs are rendered oligo- or azoospermic three months after treatment with deslorelin (Bertschinger *et al.,* 2001; Bertschinger *et al*., 2002; Bertschinger *et al.,* 2004a; Bertschinger *et al.,* 2006). Although oligospermia does not imply infertility, one may infer that it may take as long as three months to render some wild dogs infertile. If it can take this long to render wild dogs infertile and if this scenario can be extrapolated to grey wolves, grey wolves that were treated shortly before their short breeding season may have reproduced successfully throughout the short season, and only been rendered infertile post-breeding season. It would be risky to administer contraception shortly before the breeding season in grey wolves. In domestic dogs, deslorelin has been shown to initially stimulate LH release and, subsequently, testosterone production, a process which usually does not last longer than five days and eventually results in a dramatic feedback decrease in these hormones by no later than 25 days post contraception (Junaidi *et al.,* 2007).

As was suggested for grey wolves, it is possible that it takes longer than six weeks for deslorelin to significantly suppress reproductive parameters in African wild dog, possibly up to 24 weeks based on our data. Based on previous studies, it seems impossible that the implant could take so long to down-regulate LH and testosterone concentrations and spermatogenesis, however, we do not know the details of the implant formulation (Junaidi *et al.,* 2007). Furthermore, this implant dose did effectively contracept male African wild dogs in previous studies where it was administered as close to the assumed breeding season as November and December. Perhaps, the failure of the implant in this study is as simple as a flaw or change in the formulation of the implant (ie, lower than stated dose or a change in the deslorelin release characteristics of the implant). It may be that the payout properties of the 4.7 mg implants were altered specifically to suit the requirements of the domestic dog. This

may have altered the time taken to reach threshold levels for effective suppression or the actual concentrations reached were just too low. Finally, some female African wild dogs did appear to experience a secondary oestrus around July, which may have been correlated with a mild improvement in reproductive parameters of male African wild dogs around the same time period (personal observation, De Wildt). If male African wild dogs do experience an improvement in reproductive parameters starting in July, when females enter a secondary oestrus, it is possible that the administration of the implant in August/September was so close to this period that it may have prevented the implant from adequately dampening hormone feedback mechanisms and spermatogenesis.

As the implant was not effective for contraception in male African wild dogs, it is not surprising that the injection was completely ineffective as well. The injection formulation relied on the efficacy of the deslorelin chemical and formulation in the implant, because it was composed of pulverized implant particles and a polymer formulation, albeit at double the dose that the implant was administered. The injection was expected to be short-lived in the body because Baker *et al*. (2005) demonstrated that a similar vehicle yielded contraception for a period of 215 days post-treatment. But this study showed that the injection was ineffective for short- or long-term contraception. Although it is possible that dogs in the injection group happened to have superior reproductive variables compared to dogs in the two other groups it is unlikely, because the dogs were assigned to treatment groups randomly. Ultimately, the amount of deslorelin released from the injection product could have been below what is required to successfully down-regulate the pituitary-gonadal axis.

The placebo group had higher sperm counts than the implant group for the time period of November to February, a period spanning six months after treatment, and higher sperm counts than the implant and injection groups for the time periods of November to April, a period spanning eight months after treatment, and November to May, a period spanning nine months after treatment. Furthermore, even when treatment groups were analysed together over time, the average sperm count for all dogs was higher during those same periods. Analysis of the season confounder supports the finding that season had a significant effect on sperm count. Interestingly, dogs housed without females had higher sperm counts than those housed with females. The main group of dogs housed without females was comprised of individual dogs with very high sperm counts, which may have had a large effect on the overall sperm counts of the group of dogs housed without females.

Semen volume was significantly greater during winter. During winter, most semen samples were contaminated with urine, which was accompanied by increased semen volumes. These findings mirror those of Nöthling *et al.* (2002). Dogs with urine contamination had acidic semen pH values and high semen osmolalities. Dogs with lower semen osmolality and high semen pH tended to have better sperm motility. Seminal pH is routinely measured as a part of semen quality assessment and is directly related to the amount of prostatic fluid or urine (samples obtained by means of electro-stimulation) contained in a sample. Domestic canine semen pH ranges from 6.3 to 7.0 and prostatic fluid pH ranges between 6.0 and 7.4 with a mean of 6.8 (Feldman and Nelson, 2004b).

There were few differences in sperm motility with respect to time or treatment. However, the confounder analysis did show that peak motility occurred during summer, as opposed to winter. Decreased sperm motility was more likely found during the winter months as a result of urine contamination of semen samples (Feldman and Nelson, 2004b), which occurred more commonly in the winter. During summer it was much easier to obtain semen and the voltage required for successful electro-ejaculation was lower. We suspect that the higher voltages used for electro-ejaculation increase the likelihood of obtaining a urine-contaminated sample

There were no statistically significant effects of treatment or time of year on the percentage live sperm per a semen sample. Although in Table 10, many post-treatment percentages of live spermatozoa appear to be higher than pre-treatment percentages, it is impossible to state that because the sample sizes are too small (n values between three and 18) and the variability within sample sizes too large.

Sperm morphology reflects both testicular and epididymal function (Nöthling, personal communication; Barth and Oko, 1989). A number of extra-testicular factors may influence interpretation of sperm morphology results. Spermatozoa should be evaluated for motility and preserved for the assessment of morphology as soon after collection as possible; motility decreases over time and also decreases in response to storage media, and loose acrosomes may result from prolonged incubation post-collection (Saacke and Almquist, 1964 cited by Salisbury *et al.,* 1978). Hot pipettes or extenders will degrade spermatozoa quickly. In contrast, cold shock may cause poor motility, bent mid-pieces, and bent principle pieces (Barth and Oko, 1989). Hypo-osmotic shock as a result of contamination with water during

semen collection or the use of hypotonic stains and extenders will cause sperm to swell and tails to bend or coil and resemble mid-piece reflex defects (Saacke and Almquist, 1964 cited by Salisbury *et al.,* 1978; Barth and Oko, 1989). Depending on the concentration, urine may have a hyper or hypotonic effect on semen sample samples, both of which could affect individual motility due to morphological changes to the tail (Olson, 1984; Feldman and Nelson, 2004b). It is important to take all of these factors into consideration in the evaluation of morphologic data. Apart from urine contamination, the other factors were controlled and should not have affected the semen quality of the wild dog samples.

There were no significant differences in the prevalence of spermatozoa with normal morphology between the treatment groups for the duration of the study as a whole. However, significant improvements in the prevalence of spermatozoa with normal morphology were seen for the months of January and February. The improvement in the prevalence of spermatozoa with normal morphology supports optimisation of semen quality in preparation for the breeding season.

There were no significant differences in the prevalence of testicular-origin defects by treatment or time period during the study. Dogs M173, M343, M357, M376, M377 and M440 each had more than 20% of spermatozoa with defects of testicular origin, which was a distinctly higher percentage than the mean prevalence of defects of testicular origin (13%). It is unclear what commonality existed between these dogs with higher prevalences of defects of testicular origin. Neither treatment nor the duration of the post-treatment observation period affected the prevalence of defects of epididymal origin.

When different types of morphological defects were analysed, a few trends and differences appeared. Although there were no significant differences between treatment group or time period with respect to the prevalence of nuclear defects, dogs M357 and M440 (implant dogs) and M377 (placebo dog) had higher prevalences (22%, 26% and 32% respectively as compared with a population mean of 7.3%) of nuclear defects than the other dogs in the study. Treatment does not appear to be related to the prevalence of nuclear defects, because these dogs were administered different treatments. All three dogs were younger than three years of age, suggesting that age probably did not affect spermiogenesis either. M357 had been contracepted previously but the two other males were naïve. The cause of inadequate spermiogenesis in these three males is unknown. Naïve dogs and underweight dogs both had

higher prevalences of nuclear defects than previously contracepted dogs or dogs with adequate body condition. Although one may expect previously contracepted dogs to have a higher prevalence of defects, it is important to note that contraception from the previous year (2004) was not completely effective, as demonstrated by the three litters which were sired by males contracepted in 2004 (Bertschinger, personal communication). Although underweight dogs may lack sufficient body fat stores, nutritional balance has to be highly disturbed before it will affect semen quality (Bertschinger, personal communication). Many underweight dogs were also older and may have been approaching reproductive senescence. Lower body weight due to senile atrophy of muscles and other tissues occurs simultaneously with senile testicular degeneration, resulting in decreased fertility. Senescence is an age-related decrease in an organism's survivorship or fecundity and is associated with decreased physiologic function (Adams, 1985). Reproductive senescence has been documented in many long-lived mammals, including non-human primates (Paul *et al.,* 1993, Johnson and Kapsalis 1995), ungulates and carnivores (Eberhardt 1985, Fisher *et al.,* 1996, Packer *et al.,* 1998).

There were no significant differences in the prevalence of bent-tail defects between treatment groups, but there was a trend for a higher prevalence of bent-tails during the winter than the summer. During the winter the temperatures were cooler and the samples tended to be contaminated with urine. It is possible that urine contamination may have induced the secondary defect of bent-tails (Johnston, 1991). It is not likely that the cooler winter temperatures resulted in an increased prevalence of bent-tail defects, because the sample handling methods used prevented cold shock. However, the increased prevalence of bent-tail defects also could have been the effect of long-term epididymal storage (Barth and Oko, 1989).

There also were no significant differences in the prevalence of proximal droplet defects over time. Prior to treatment, the implant group had a higher prevalence of proximal droplets than the placebo group, which was probably a coincidental finding. Underweight dogs also had a higher prevalence of proximal droplets than dogs with normal body masses. Generally, mid-piece reflex and proximal or distal droplet defects were seen with high prevalence before treatment, during the non-breeding season. During the non-breeding season it is likely that spermatozoa had a higher prevalence of proximally retained droplets, because the sperm that migrated through the epididymis or the function of the epididymis or both were abnormal, such that the droplets failed to migrate from their proximal to their distal position. The

control of spermiogenesis is complex and highly influenced by photoperiod. With the wild dog, it is possible that decreasing daylight length stimulates the neuro-endocrine system to optimise spermatogenesis and maturation of sperm. Conversely as daylight increases, the neuro-endocrine system would be less stimulated or, even, inhibited, perhaps resulting in increases in proximal droplets and other defects.

Azoospermia was not significantly related to treatment group in this study. Furthermore, although oligospermia was seen during the non-breeding season, rarely did dogs with sperm counts greater than five million per ejaculate prior to treatment administration become completely azoospermic during that time of the year. Out of a total of 10 implant dogs, five were azoospermic after treatment, although three of these five dogs had been azoospermic prior to treatment. The remaining two dogs were housed with a female at the start of the study and, due to circumstances beyond our control, were moved to singular enclosures later in the study. With the number of confounding factors involved, it is difficult to determine the success rate of the implants. A more controlled study would be necessary to make that conclusion. As one may expect, azoospermic dogs had significantly lower serum testosterone concentrations compared with non-azoospermic dogs. Testosterone, synthesized by the Leydig cells in the testis, is essential for spermiogenesis. Although blood concentrations of testosterone are only an indirect measure of local testicular concentrations, they are the means of evaluation used for routine purposes. Testicular testosterone concentrations are usually 50-100 times as high as serum testosterone concentrations. Therefore, serum testosterone concentrations may not accurately reflect alterations in testicular testosterone or be useful for assessment of spermatogenesis (Olson, 1984).

In the current study, azoospermic dogs tended to have acidic semen pH and higher semen osmolality than non-azoospermic dogs. Furthermore, the ejaculates of azoospermic dogs often were clearly contaminated with urine, which could explain their low pH and high osmolality. It is also possible that in addition to being more prone to urine contamination, the ejaculates of azoospermic dogs contained little prostatic fluid. An ejaculate lacking alkaline prostatic fluid would certainly be more likely to have an acidic pH. It is important to note that dogs with which we had to increase the electroejaculation voltage also were more prone to have urine contamination of their ejaculates. This finding relates to ease or difficulty of obtaining a semen sample. Progressively higher stimuli are required as it becomes more difficult to obtain a semen sample and, furthermore, the chances of urine contamination also

increase with higher stimuli. Azoospermic dogs probably had lower serum testosterone concentrations and decreased function of the prostate gland, the source for seminal fluid, resulting in stimulation of the bladder, rather than the prostate, during electro-ejaculation. In support of this theory, azoospermic dogs had significantly smaller prostate volumes than dogs with spermatozoa.

For the implant and placebo groups, the semen pH did not vary significantly throughout the course of the study, despite prevalent urine contamination during the collection periods before treatment (winter 2005). The injection group had a higher semen pH after treatment than before treatment, which is not easily explained, but follows a trend of improved reproductive parameters after treatment for the injection group.

Regardless of treatment type, for the seven month (2 Sept 2005 to 3 April 2006) and nine month (2 Sept 2005 to 24 May 2006) post-treatment windows, testes size was significantly larger after treatment than before treatment. This finding supports seasonal breeding in the male African wild dog. Confounder analysis also showed that dogs had larger testicular volumes during the summer months, which include the months of April and May. This is the first study to show that male African wild dogs definitely experience an improvement in reproductive parameters during certain times of year, similar to wolf species (Asa *et al.,* 2006; Asa and Valdespino, 1998; Asa *et al.,* 1996). Seasonally breeding species often have increases in body mass, testicular volume and spermatogenesis following increases in testosterone concentrations in the blood (Newell-Fugate *et al.,* 2007; Goodman, 1999). In this study, wild dogs experienced increases in sperm count per ejaculate, testicular volume, prostatic volume and serum testosterone which corresponded with the breeding season (summer).

Over the course of the study there were no significant differences in testicular volume between treatment groups. Azoospermic dogs and underweight dogs had smaller testicular volumes than dogs with spermatozoa and dogs with normal body masses. It is obvious that azoospermic dogs would experience decreased fertility. Underweight dogs, which have smaller testicular volumes, may or may not experience decreased fertility. The wild dog breeding season occurs only once to twice a year; thus, dogs with smaller testicular volumes might have adequate fertility in response to the infrequent demands of mating. However, it is important to note that dogs with small testicular volumes are also more likely to have

hypoplastic tubules, defective parenchyma or other problems with their testicular tissue which could render them azoospermic or oligospermic (Feldman and Nelson, 2004b).

There was no difference in the serum testosterone before as opposed to after treatment for the entire data set. However, the serum testosterone of the injection group was significantly greater than that of the implant group during the 10 months post-treatment of November through June. There were also differences in the serum testosterone of the two groups during the after-treatment month subsets of November to February, a period spanning six months post-treatment, and November to April, a period spanning eight months post-treatment, but these were not large enough to be statistically significant. When data were analyzed in subsets, irrespective of treatment group, serum testosterone after treatment was significantly higher than before treatment for the post-treatment month windows of November to February (six months), November to April (seven months), and November to June (nine months). Although there was a seasonal increase in systemic testosterone concentrations in male African wild dogs for the post-treatment month windows of February through June (six to nine months post-treatment), the seasonal confounder analysis did not support this trend. Increases in serum testosterone during February to June were very slight and did not impact the data set when it was analyzed as a whole. Therefore, the methodology of the seasonal confounder analysis, wherein the data set was analyzed in its entirety and divided into the months of May to Sept (winter) and Oct to April (summer), did not permit the increase in serum testosterone from February to June to be detected. Furthermore, the division of the year into summer and winter for the purposes of the seasonal confounder analysis did not mirror the potential breeding season of the African wild dog as demonstrated by the other reproductive data in this study. This study also showed that sub-adult dogs had higher circulating testosterone levels than adult dogs, possibly due to the growth demands on sub-adults as they mature muscularly and sexually (Feldman and Nelson, 2004b).

The prostate volume, regardless of treatment group, was larger after treatment than before treatment for all post-treatment month windows analysed. This finding was likely an effect of season as all measurements taken before treatment were conducted during the non-breeding season and many of the measurements after treatment were taken during the breeding season. Increased testosterone during the breeding season likely positively affected prostate volumes. By treatment group, the injection group had a significantly larger mean prostate volume than the two other groups in November, and a significantly larger prostate volume than the implant

group in May, the tail end of the breeding season. Geriatric dogs (dogs older than 8 years of age) had significantly larger prostate volumes than younger dogs possibly due to the incidence of benign prostatic hyperplasia in intact older male dogs (Johnston *et al*., 2001; Feldman and Nelson, 1996; Krawiec and Heflin, 1992).

Preputial glands are unique to the African wild dog and may be controlled by circulating androgen concentrations. This study showed that preputial flap width decreased in the implant group after treatment, supporting the theory that these glands are hormonally controlled. Similarly, geriatric dogs (dogs older than 8 years of age) and dogs housed with females had larger preputial flap widths than other dogs. Age and proximity to females may be causes of increased testosterone production in African wild dogs. Perhaps like benign prostatic hyperplasia, preputial flaps become larger with age. Female pheromones, acting as chemical signals, may stimulate increased activity and size of these glands. This physiologic process would be mediated through the neuro-endocrine system. Naïve dogs were found to have smaller preputial flap measurements than previously contracepted males. However, naïve dogs were also younger and, therefore, usually had less flap development. Dogs were found to have larger flap widths during the winter season compared with the summer season. If these glands were used in conjunction with breeding only, this finding would be nonsensical. Perhaps the glands are used more for territory marking and during the dry winter, when the scent may linger longer on plants. Preputial orifice diameter was significantly larger for each group after treatment. The study design lends itself to measurement bias due to inexperience with measurement techniques early in the study. It is likely that the technique used to measure the orifice may have changed over time during the course of this study, thus accounting for the change in orifice diameter. Age, proximity to females, and the summer all correlated with larger preputial orifice diameters. Despite the measurement bias, it is possible that older dogs, dogs housed with females, and dogs assessed during the breeding season all experienced increased coital activity, resulting in increased preputial orifice diameters.

5.2 Side-effects of treatment

Similar to previous studies in African wild dogs (Bertschinger *et al.,* 2001; Bertschinger *et al.,* 2002), no side effects could be related to deslorelin implant treatment. Administration of the vehicle or the vehicle with deslorelin resulted in side effects in only 10% of cases. All side

effects were localized to the skin. Although the rate of side-effects seems quite low, it is based on administration in 17 dogs only, suggesting that it is advisable to monitor the injection site for at least one week after injection to ensure that a subcutaneous reaction to the vehicle does not arise. Baker *et al.* (2004, 2005) used an analogous vehicle formulation (1:1 ratio) in leuprolide contraception studies of female deer (n=13) and elk (n=15) without any adverse side effects. Contrary to Baker *et al.* (2005), this study group elected to decrease the viscosity of the vehicle formulation by increasing the solvent ratio from 1:1 to 3:1 in order to improve delivery via an 18 gauge needle. Administration of the placebo was easily accomplished; however, there was difficulty with delivery of the experimental deslorelin injection. It is recommended that future long-acting deslorelin injections be composed of vehicle and powdered deslorelin, rather than pulverized implant-deslorelin. This should facilitate homogenisation of the product throughout the vehicle and increased ease of administration through an 18 gauge needle. Additionally, scientific grade powdered deslorelin could be more effective at contraception than the pulverized implants used in this study.

As there were no adverse changes in haematology or serum chemistry values (n=11) after treatment and no difference in body weight (n=27) after treatment, the deslorelin implant and injection appear to be safe products for use in African wild dog males. Continued long-term studies of the individual dogs used in this study would be required in order to definitively establish the safety of this product in male African wild dogs.

Conclusions

Our data demonstrate that:

- The deslorelin long-acting injection is an ineffective contraceptive in male African wild dogs based on assessment of reproductive parameters (testicular volume, presence or absence of semen, semen quality, serum testosterone and prostate volume).
- The deslorelin implant may be an effective contraceptive in male African wild dogs based on assessment of reproductive parameters, however, its efficacy appears to vary among dogs.
- Both the deslorelin injection and implant appear to be safe for use in male African wild dogs, as judged by haematology, serum chemistry and body weight.
- Male African wild dogs experience improvement in reproductive parameters (increased testicular volume, less urine contamination of semen, increased sperm count per ejaculate, lower prevalence of sperm bent tail defects) during summer, lasting from October to April.
- Based on significant increases in testicular volume, sperm progressive motility, sperm count per ejaculate, serum testosterone and prostate volume during the months of January to May, we suspect that male African wild dog experience a breeding season during these months.

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APPENDIX A: August 2005 map of African Wild Dog enclosures and dogs in this study

NEW QUARANTINE
A
FB409 : M287
F293 ; M325
B
M376 ; M377
F374 ; M375 ; M378

OLD QUARANTINE
31
M358
30
M327 : M326
F359 : F360
29

KENNELS
A
B
F126 : M130
?475 ?477
?476
42
F166 : M107
?480 ?481
?482 ?483
43
F323
M301
44A
M484
M485
M486
M487
44B
F180
F370 ; F371
F438 ;
M439
M440
45
F324
M173
46
F320
M212

NEW SASOL CAMP
C
F286 ; M213
F403 ; M405
M406 ; M407
M408
D
F242 ; M214
M401
M402
B
F215
M216
E
F211
M273
A
F364 : F365
M366 ; M367
M368
F
F241
M176

SCHOOL
M341
M343
M344
M334

SASOL
F291
F302
F303
M255
M256

SHINGWEDZI
F382
F429
F431
F433
F434
M184
M185
M288
M383
M384
M428
M430
M432

48
M186
M369

COMPOUND
F322 ; M333
M357 ; M421
M422 ; F423
F424

TOILET CAMPS
A
B
F363
M380

DATE: 18/08/2005
= poo collection (n=11)
= behavioral study (n=8)
= other dogs (n=8)

APPENDIX B: Data sheet used to capture measurements during immobilizations

WILD DOG CONTRACEPTION STUDY DATA SHEET

General information:

Date:_______________________

Enclosure name: _______________________________________

Dog id number (and color scheme id): ___________________________

Weight: ____________________________

Physical exam: ____

Group (poo, behavior or other): ______________________________

Blood parameters (mls drawn and any other relevant info):

CBC: _____________ Serum testosterone: _______________

Chem: ____________

Anatomical measurements:

Prostate measurements (ultrasonic):

L W H

Transverse____________________________________

Longitudinal___________________________________

Testicular measurements (calipers): RIGHT (l x w) ____________________________

LEFT (l x w)______________________________

Preputial gland measurements (calipers): RIGHT________________________________

LEFT_________________________________

Distance from abdomen_____________________

Orifice_________________________

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APPENDIX C: Semen evaluation—data capture sheet

Semen evaluation					
Method of collection:	EE	**Ejaculated**	Into sheath	From protruded penis	
Interval since previous ejaculate:		Fraction of ejaculate collected:	< ½	> ½	all
Macroscopic evaluation	**Volume:** ml	**Colour:**	**Consistency:**		
	Marbling:	Distinct	Weak	Absent	
	Is mucus or pus or debris present?				
	pH:	**Odour:**			
Motility	**% Progressive:**	**% Aberrant:**	**% Immotile:**		

Place identification sticker here

Sperm morphology (eosin nigrosin)

Identification of smear		
Percentage sperm with one or more nuclear (head) defects (defects 1-12)		
1	Teratoid	
2	Double	
3	Macrocephalic	
4	Microcephalic	
5	Rolled/crested head	
6	Pyriform	
7	Tapered (narrow) head	
8	Diadem (nuclear crater)	
9	Narrow base	
10	Abnormal base	
11	Other abnormal head shapes	
12	Abnormal loose heads	
Sperm with nuclear defects (Types 1-12) as well as acrosomal or tail defects (Types 13-28)		
13	Knobbed acrosome	
14	Stump tail	
15	Pseudodroplet	
16	Segmental aplasia of mitochondrial helix or malpositioned mitochondria	
17	Cork screw	
18	Dag	
19	Other midpiece defects	
20	Coiled principle piece	
21	Proximal droplet	
22	Midpiece reflex	
23	Normally shaped loose heads	
24	Fractured flagellum	
25	Distal droplet	
26	Damaged / degenerate acrosome	
27	Bent midpiece	
28	Bent principle piece	
Percentage sperm with one or more acrosome or tail defects (no's		
Percentage morphologically normal sperm		

Number of nuclear defects (Types 1-12) in excess of the number of sperm affected by them

Number of acrosomal or tail defects (Types 13-28) in excess of the number of sperm affected by them

The shaded spaces need not be used, but using them will save a lot of time, because the percentages of sperm with nuclear defects, acrosomal or tail defects, or normal sperm can then easily be calculated

Final conclusion	