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## Studies of male reproduction in captive African wild dogs (*Lycaon pictus*)

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### Abstract

Implementation of assisted breeding in the captive African wild dog is restricted by a current lack of knowledge on their reproductive physiology and the apparent difficulty of effectively manipulating the complex social dynamic of the pack in order to conduct reproductive procedures. In this study, we describe protocols for the safe and repeated capture and restraint of the African wild dog ( $n = 7$ ) as well as techniques for assessment of male reproductive function, semen collection and preservation. In a serendipitous finding, captive African wild dogs appeared to display significant seasonal change in male reproduction. Testicular volume and tone, spermatorrhea and the ability to collect semen by electroejaculation all increased significantly during late summer and then subsequently declined by early spring. While there were no detectable seasonal changes in testosterone concentration in the population as whole, the alpha-dominant male in both years of the study, had a highly elevated testosterone concentration compared to subordinate males. Semen collection by electroejaculation during the late summer was most effective in peri-pubertal males (15 months) when all seven electroejaculates were of adequate quality for cryopreservation. In the second breeding season (27 months), there were numerous changes in the pack hierarchy and electroejaculation was not as successful (3/7). The characteristics of electroejaculated semen collected in the breeding season are described for seven animals including the first descriptions and incidence of sperm abnormalities in the species. Semen ( $n = 7$ ) was frozen using a Tris–citrate fructose buffer and final egg yolk and glycerol concentration of 20% and 4%, respectively. Sperm were loaded into 0.25 mL straws, frozen in liquid nitrogen vapor and then thawed at 37 °C. Initial post-thaw survival of spermatozoa was encouraging (% motile:  $31.8 \pm 5.8\%$ ; rate:  $2.8 \pm 0.3$ ; % intact plasma membranes:  $33.4 \pm 5.3\%$  and the

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% of damaged acrosomes:  $4.4 \pm 1.5\%$ ) but following 2 h incubation at  $37^\circ\text{C}$ , post-thaw survival declined markedly.

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## 1. Introduction

The African wild dog or painted hunting dog is a “wolf-like” carnivore unrelated to the domestic dog. They represent a separate evolutionary line unique to Africa going back 15 million years. Painted dogs once occurred in large populations in ten of thousands across Africa but numbers in the wild have declined dramatically over the last thirty years due to habitat loss, predation, viral disease and persecution by man (Woodroffe and Ginsberg, 1997b). In 1997, only 3000–5000 dogs were estimated to be remaining in the wild and many of these were in small fragmented or genetically non-viable populations (Fanshawe et al., 1997). The African wild dog is currently considered by the world conservation union (IUCN) as one of most endangered canids in the world (McNutt et al., 2004).

Captive breeding of African wild dogs has been successfully established in a number of institutions both in and outside of Africa, resulting in what currently appear to be self-sustaining populations (Frantzen et al., 2001). There are approximately 300 wild dogs in captivity in 55 zoos, as listed on ISIS (International Species Information System) and as many as 200 animals occur in zoos or private collections, mostly in South Africa (McNutt et al., 2004). Nevertheless, these populations still need to be managed carefully to avoid inbreeding depression, genetic drift and stochastic events. As of December 2005, there were a total of 38 males and 37 female African wild dogs located across five Australasian zoos (Johnson et al., 2005), with the most abundant population based at Western Plains Zoo, Dubbo in central New South Wales. The effective genetic and reproductive management of this small population involves the regular movement of individual animals between institutions, a situation that is complicated in *Lycaon pictus* by its requirement for functioning in complex social groups.

Most African wild dog packs contain four to eight adults, a breeding pair with 2–6 yearlings and 5–11 pups (Woodroffe and Ginsberg, 1997a). The adaptive value to parents of incorporating post-pubertal young into the pack is that they can contribute to the parental care of their younger siblings (Courchamp and Macdonald, 2001). To overcome the managerial limitations of this complex social system, we propose the development of assisted breeding technologies such as artificial insemination, which would allow the movement of preserved sperm cells rather than live animals. This would result in improved animal welfare outcomes and reduce the cost and dangers of whole animal shipment and integration of an unrelated animal into an established pack. To date, there has been only one study that has examined assisted breeding technology in African wild dogs (Hermes et al., 2001) in which they developed protocols for the sonographical characterisation of the testicles and prostate and attempted to collect and cryopreserve semen from two males, one of which was subsequently found to have testicular pathology.

There are a number of limitations to the implementation of assisted breeding technology in African wild dog and these particularly concern the separation and re-introduction of individual males from the pack in order to conduct repeated anaesthesia and semen collection procedures. Animals that are separated from the pack should be drafted off and re-introduced to the pack with great care, in order to reduce subsequent aggressive encounters or rejection of pack members on

their re-introduction. In the current paper, we describe a strategy for overcoming this problem that may also be useful for canid species with similar social systems.

The normal technique for semen collection in domestic canids involves the use of digital manipulation of the penis in the presence of oestrous bitch and the manipulation of the ejaculate so that only the sperm rich fraction is collected (Hewitt et al., 2001). Development of such a technique in the African wild dog is highly unlikely, so that electroejaculation of the anaesthetised animal is the method of choice. While electroejaculation has been used to collect semen in a range of wild canids (Platz et al., 2001; Silva et al., 2004), the resulting ejaculate is often contaminated with urine (Hewitt et al., 2001) so that bladder catheterisation is recommended to improve ejaculate quality (Platz et al., 2001). Successful collection of semen in the African wild dog has been described by Hermes et al. (2001), but the study was based on the ejaculate of only one dog.

The development of assisted breeding technology in the African wild dog will ultimately involve the establishment of a genome resource bank or frozen repository of sperm and embryos that can be managed on a global basis for both captive and wild populations. Successful cryopreservation of African wild dog semen has been reported by Hermes et al. (2001) but for only one ejaculate divided into three fractions. Each fraction was frozen-thawed in a TEST buffer containing 7.5% (v/v) glycerol and 15% (v/v) egg yolk. The resulting post-thaw motility of fraction three, 10 min post-thaw was 40%, but this fell to 10%, 30 min after thawing.

The present study attempted to collect semen from seven African wild dogs in different seasons and under changing pack social dynamics. When semen was successfully obtained, the ejaculate was evaluated for seminal characteristics and cryopreserved using a standard canid freeze–thaw procedure (Gunn, 1988). During periods of semen collection, breeding soundness of all male dogs in the pack was systematically examined in terms of testicular size, testosterone secretion and semen quality. These observations were then interpreted in terms of seasonal breeding, peri-puberty and social dominance within the pack structure.

## 2. Materials and methods

### 2.1. Animals

At the commencement of this study in early Spring 2000, Western Plains Zoo (Dubbo, Australia) maintained a pack structure of seven males and one alpha female. The pack was a non-breeding group and the alpha female was receiving contraception [Delvosteron<sup>®</sup>, (Intervet, Australia) in May and October 2000, followed by a GnRH agonist (Deslorelin<sup>®</sup>, Peptech, Australia) in October 2001 and subsequently throughout the duration of the study]. Six of the seven male dogs were litter-mates born in 1999 and were approximately 15 months old at the start of the study; the remaining older male (D198) was approximately 27 months and was considered the dominant or alpha male. All males remained clinically healthy throughout the duration of the project. Table 1 documents the body weight (kg), body condition (0–5) and social status (top three ranked males) from early spring 2000 to early spring 2002. Body condition was based on a subjective visual assessment of fatness and the prominence of vertebrae, ribs, hips and pin bones (Gordon, 2005). Estimates of social hierarchy were based on independent unsolicited zookeeper records and observations. Reproductive assessment and semen collection procedures were conducted over four separate time periods; early spring 2000, late summer 2001, late summer 2002 and early spring 2002. These periods represented respective non-breeding and breeding seasons coincident with previous observations of African wild dog breeding at Western Plains Zoo. This research had animal ethics approval from the Zoological Parks Board of New South Wales.

Table 1

Changes in body weight (kg), body score (0–5) and social rank of captive African wild dogs housed at Western Plains Zoo, Dubbo from early spring 2000 to early spring 2002

Season	ID	Body weight	Body score	Social rank
Early spring 2000	D198	28	3	1
	D199	32	4	2
	D399	32	3	
	D499	32	4	
	D699	32	3	
	D799	31	3	
	D899	29	3	3
Late summer 2001	D198	32	3	3
	D199	35	4	2
	D399	34	4	
	D499	34	5	
	D699	34	4	
	D799	36	5	
	D899	30	3	1
Late summer 2002	D198	29.5	5	3
	D199	32	5	2
	D399	32	5	
	D499	32	5	
	D699	31.5	5	1
	D799	33	5	
	D899	30	5	
Early spring 2002	D198	28	4	3
	D199	31	3	2
	D399	31	3	
	D499	31	3	
	D699	30	3	1
	D799	32	3	
	D899	27	3	

## 2.2. African wild dog enclosure facilities at western plains zoo

The primary on-exhibit enclosure (Figs. 1 and 2) was a large grassed area of approximately 8300 m<sup>2</sup> comprised of a surrounding water moat (3 m wide and 2.5 m deep) and a rear perimeter fence constructed of chain-mesh measuring 3 m high. The exhibit contained a large shade tree, a sand pit and numerous smaller vegetation types along the moat edge. There was a central raceway from the rear of exhibit that led to a holding facility and breeding yards. Situated within the raceway was a purpose built restraint chute and “squeeze” cage that facilitated the drafting of individual animals, veterinary care and procedures (e.g. induction of anaesthesia and venipuncture; Fig. 1). The off-exhibit breeding yards consisted of two adjacent enclosures measuring approximately 100 m × 30 m containing two whelping dens. These dens were constructed from large stormwater culvert pipes elevated with rocks, covered in a mound of soil and protected by aluminium “carport” shelters (Fig. 1). The holding yards were joined by vertical slide gates that were used for re-introduction of new individuals to the pack, recovery from veterinary procedures and drafting. This enclosure design has proven extremely effective for the captive breeding of this species with over 60 puppies being born at the zoo since the start of the program in the early 1980s.



Fig. 1. African wild dog facility at Western Plains Zoo, Dubbo, New South Wales, Australia: (A) pack dynamics during a carcass feed; (B) breeding yards (white arrow shows whelping dens); (C) crush facility located in raceway used for drafting and induction of anaesthesia.

### 2.3. Capture and anaesthesia of African wild dogs

In preparation for semen collection, the entire pack was starved overnight and then enticed into the holding areas adjacent breeding enclosures by visual stimulation with food. These animals were then fed and housed in four separate holding pens overnight where they had visual contact with each other. On the morning of the first semen collection, one dog was drafted from the group into a passageway and placed into a squeeze crush prior to the induction of general

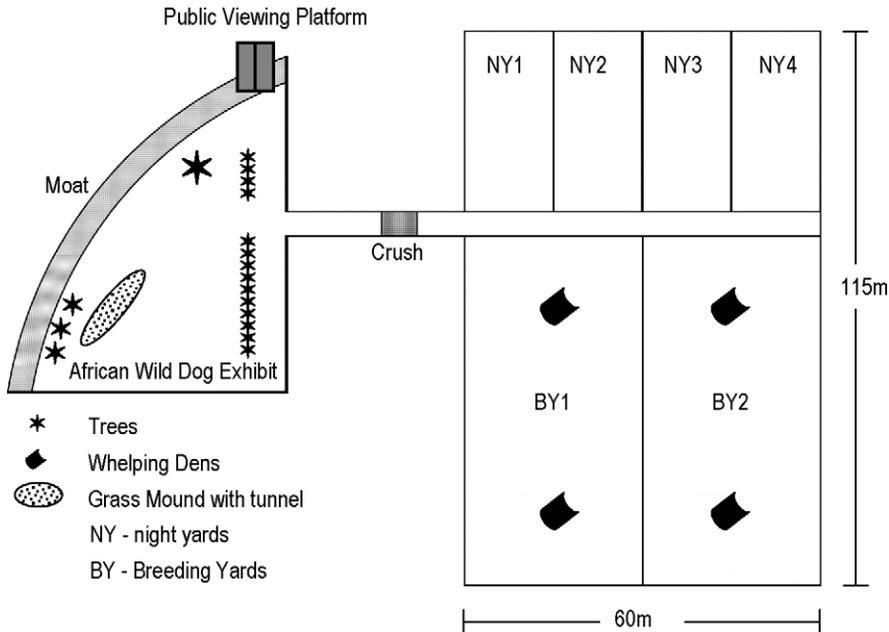


Fig. 2. Plan of African wild dog facility at Western Plains Zoo.

anaesthesia by the intramuscular injection of 35–50 mg (1.5 mg/kg i.m.) ketamine (Ketamine injection<sup>®</sup>, 100 mg/mL, Parnell Laboratories, Australia), 1.0–1.2 mg (0.04 mg/kg i.m.) medetomidine (Domitor<sup>®</sup>, 1 mg/mL, Novartis Animal Health, Australia) and 1.5 mg (0.05 mg/kg i.m.) atropine (Atropine injection<sup>®</sup>, Apex Laboratories, Australia). Time from injection to sternal recumbency and immobility was noted. Once the animal had attained a surgical plane of anaesthesia, it was transported to the veterinary surgery. A sterile ophthalmic ointment (Lacrilube, Allergan Pharmaceuticals, Johannesburg) was applied to the eyes of each dog prior to blind folding to prevent drying. Each dog was then intubated with a 10 mm or 12 mm endotracheal tube and anaesthesia maintained with 1.5% gaseous isoflurane (Abbott Pty Ltd, Australia) in oxygen (2L/min) administered by an isoflurotec vaporiser on a Bain-closed circuit system. Anaesthesia reversal was achieved by administration of 2.5–3 mg atipamezole (Antisedan<sup>®</sup>, 5 mg/mL, Novartis Animal Health, Australia; 0.1 mg/kg i.v. via right cephalic vein and 0.1 mg/kg i.m. via left cranial quadriceps muscle) and 2.5–3 mg atipamazole intramuscularly. Prior to reversal, 0.2 mg/kg meloxicam (Metacam<sup>®</sup>, Boehringer Ingelheim Animal Health, Australia) anti-inflammatory agent was given subcutaneously. Detailed monitoring of these anaesthetic procedures was the subject of a parallel study (Ward et al., 2006).

Upon completion of the first electroejaculation procedure, the dog was returned to the holding yard facility and placed into a separate holding yard from other members of the pack. A second and third dog, were subsequently separated under similar circumstances over the remainder of the first day of semen collection. The fourth yard held the remaining five (four males and one female) dogs of the pack. All semen collection procedures were completed by 2 p.m. and all dogs were standing within 45 min of the end of the procedure. At 5 p.m., the three dogs that had undergone procedures earlier in the day were reintroduced to one another but remained separated from the pack. On the second day of semen collection, the remaining four dogs were anaesthetised in a

similar manner. Shuffling of the dogs within the holding pens allowed all three animals to recover in individual pens while the fourth recovered in a purpose built crate adjacent the veterinary surgery. The three dogs that had been anaesthetised on day 1 were reintroduced to the alpha female following the separation of the final dog from which semen was collected on day 2. All four dogs that were anaesthetised on day 2 were fully standing within 45 min of the procedure. These animals were reintroduced to each other by 5 p.m. but remained separated from the rest of the pack. The following day all eight dogs were introduced to each other in the holding yards in the event that interaction problems could be appropriately managed. All gates between the yards were opened to allow dogs an escape refuge if required. Thirty minutes after introduction the pack was enticed into the primary exhibit with a carcass feed. The capture, restraint and re-introduction protocols described here were repeated another three times over the course of this study without incident.

#### 2.4. Testicular volume and tone

The African wild dog has a shallow non-pendulous scrotum so that measurement of scrotal circumference was not possible. Consequently, testicular volume was calculated using the following formula:  $V = 0.524 \times L \times W^2$ , where  $V$  = volume ( $\text{cm}^3$ ),  $L$  = length (cm) and  $W$  = width (cm) (Wildt, 1996). A subjective evaluation of testicular parenchymal tone was also determined using the following ranked scoring system: 1, very soft; 2, soft; 3, moderate; 4, firm and 5, very firm. All testicles were sonographed transcutaneously (Hermes et al., 2001) to check for parenchymal fibrosis, cysts or tumours but no abnormal pathology was noted.

#### 2.5. Blood collection and measurement of plasma testosterone

Three blood samples were recovered throughout the duration of each anaesthetic at approximately 15 min intervals to account for any variable testosterone secretion during the reproductive assessment. Blood (5 mL) was recovered using a 20-gauge intravenous indwelling catheter (Terumo, Surflo, Japan) placed into the right cephalic vein of each dog, prior to electroejaculation, during the semen collection procedure and post-electroejaculation. Blood was collected and centrifuged in heparinized tubes and the plasma stored at  $-20^\circ\text{C}$  until hormone analysis. Commercially available enzyme immunoassay (EIA) kits for testosterone (DSL-10-4000 ACTIVE™ TESTOSTERONE EIA; DSL Inc., Texas, USA) were validated for use in African wild dogs. Plasma samples were assayed for testosterone concentration according to the manufacturer's specifications. The EIA assay was validated for AWD plasma by demonstrating parallelism between dilutions of pooled serum and the standard curve (Fig. 3). The intra-assay and inter-assay coefficient of variation was 3.7% ( $n = 10$ ) and 4.4% ( $n = 10$ ), respectively. A recovery of 126% ( $n = 4$ ) was obtained when samples were spiked with a known amount of testosterone. The detection limit of the assay was 0.04 ng/mL. All samples were assayed in duplicate. The testosterone antibody used in this assay cross reacted with testosterone 100%,  $5\alpha$ -dihydrotestosterone 6.6%, 5-androstane- $3\alpha,17\beta$ -diol 2.2%, 11-oxotestosterone 1.8% and <1% with all other steroids tested (manufacturer's data).

#### 2.6. Semen collection

Upon obtaining a surgical plane of anaesthesia, a sterile lubricated (10F  $\times$  600 mm) urethral catheter (Sims Portex Ltd, UK) was inserted into the penis of the dog and passed into the blad-

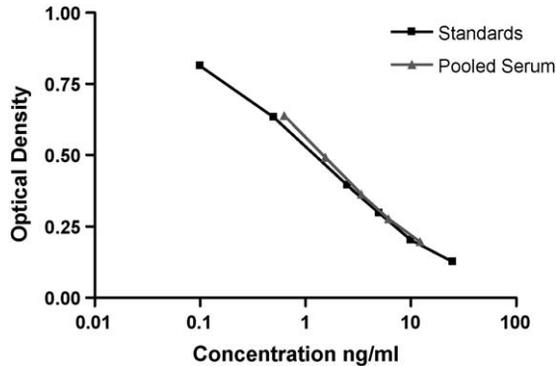


Fig. 3. The EIA testosterone assay was validated for African wild dog serum by demonstrating parallelism between dilutions of pooled serum and the standard curve.

der and urine decanted using a 50 mL syringe. Removal of urine from the bladder reduced the likelihood of urine contamination during electroejaculation (Platz et al., 2001). Urine was also recovered in a similar manner following electroejaculation in those species in which electroejaculation was not successful. Microscopic examination of urine for spermatozoa helped to confirm active spermatogenesis. Prior to electroejaculation the distal portion of the rectum was evacuated of faecal material and a 2.5 mL Microlax enema inserted (Pharmacia AB, Sweden). The electroejaculation probe used in this study was obtained from P-T electronics (Oregon; ik9sb@aol.com). It consisted of nylon core approximately 11 mm in diameter with three strip (30 mm) electrodes situated ventrally approximately 15 mm from the anterior tip of the probe. The electro-stimulation unit was a “Standard Precision Electronics” (Model—524; Colorado, USA); this machine produced a rounded square wave of approximately 22 Hz and had a maximum voltage output of 15 V. The stimulation protocol used was similar to that described by Platz et al. (2001). Briefly, the probe was lubricated with KY<sup>®</sup> Brand Jelly (Johnson and Johnson Inc., Australia) and inserted at progressive increments of 5 cm up to a maximum depth of 15 cm. At each depth approximately 15–20 sets of stimulations were administered. The stimulatory cycle was 3 s from zero to peak voltage (peak defined as when the hind legs were extended), 2 s at peak voltage, 1 s from peak to zero voltage and finally 2 s at zero voltage before the cycle began again. After electrostimulation at each depth within the rectum there was a rest period of approximately 5 min before the procedure was repeated. Semen was collected during electroejaculation into a pre-warmed (37 °C) collection glass and immediately assessed for the presence of spermatozoa. The procedure was terminated as soon as viable semen had been obtained or if semen had not been recovered after 20 min of stimulation. Once electroejaculation was complete, a second Microlax enema was administered and the penis lubricated with KY<sup>®</sup> Brand Jelly and placed back into its prepuce. All animals were observed for normal defaecation patterns for up to 48 h after the procedure.

### 2.7. Assessment of African wild dog seminal characteristics

African wild dog electroejaculates were assessed for seminal volume and pH, sperm concentration, % motile spermatozoa, the rate of sperm movement, % live spermatozoa and % normal sperm morphology. Ejaculate pH was estimated by placing semen on pH colour paper

strips in 0.3 pH divisions (Prolabo, France). Sperm concentration of the original semen sample was determined using a calibrated sperm chamber (Makler, Sefi-Medical Instruments, Israel). Motility of diluted spermatozoa was evaluated based on criteria developed for frozen-thawed bovine spermatozoa (Barth, 1995). A 10  $\mu$ L aliquot of semen was diluted 1:10 in phosphate buffered saline and placed onto a pre-warmed microscope slide (37 °C) with a cover-slip, and the motility evaluated using a phase-contrast microscope at a magnification of 400 $\times$  objective. Estimates of percentage forward motility and rate of sperm movement (0 = no sperm movement; 5 = very rapid sperm movement) were determined (Barth, 1995). The percentage of spermatozoa with intact plasma membranes was assessed using a dual fluorescent staining technique (sperm live/dead viability kit, Molecular Probes Inc., OR, USA) on an Olympus BX50 phase contrast/epifluorescent microscope fitted with a U-MWG filter cube with excitation wavelengths of between BP510-550 nm. This method used two vital nucleic acid stains, namely SYBR-14 (final concentration 100 nM), which permeates intact plasma membranes causing viable sperm to fluoresce green, and propidium iodide (PI; final concentration 12  $\mu$ M), which permeates membrane-damaged spermatozoa causing them to fluoresce red (Holt et al., 1999). Two hundred sperm were evaluated at 400 $\times$  magnification on a warm stage set at 35 °C. The percentage of morphologically normal African wild dog spermatozoa was evaluated after staining smears in eosin-nigrosin and determining the % of abnormal sperm morphotypes as defined by Barth and Oko (1989). Two hundred sperm cells were evaluated (1000 $\times$ ) using standard bright field microscopy.

## 2.8. Cryopreservation

In late summer 2001 ejaculate quality of the majority of dogs ( $n=7$ ) was sufficient to conduct a cryopreservation trial using a standard canine protocol (Gunn, 1988). Upon collection and immediate initial evaluation of semen (% motile, rate, % live and % intact acrosomes), the ejaculate was diluted 1:1 at 37 °C in Tris–citrate buffer (3.0 g Tris buffer, 1.7 g citric acid and 1.25 g fructose) containing 20% (v/v) egg yolk without glycerol and cooled slowly in a water bath to 5 °C for 2–3 h equilibration. Once the semen sample had equilibrated, it was further diluted 1:1 with Tris–citrate buffer containing 20% (v/v) egg yolk and 8% glycerol so that the final concentration of glycerol in the cryopreservation diluent was 4% (v/v). Freezing was conducted over liquid nitrogen vapor where the straws were layered on a metal rack. The liquid nitrogen was allowed to settle and stabilize in the freezing container before the procedure commenced. Initially the rack containing the layered semen straws was placed at 14 cm above 4 cm of liquid nitrogen for 25 min. The rack was then lowered to 4 cm above the liquid nitrogen for a further 5 min before the straws were plunged into the liquid nitrogen. This procedure was calculated to replicate a freezing rate of 5 to  $-120$  °C over 25 min and then  $-120$  to  $-196$  °C over the next 5 min. Once frozen, straws were stored overnight or for up to 1 month before thawing in a 37 °C water-bath for 30 s. Post-thaw motility, rate of sperm movement, % live spermatozoa and the % of intact acrosomes were all assessed on thawing (T0) and again after 2 h (T2) incubation at 37 °C post-thaw.

## 2.9. Statistical analysis

Log transformed [T] and TV data were analysed by a single factor repeated measures analysis of variance, with and ante-dependence error structure. Analysis was carried out using the MIXED procedure in SASR Version 8.2 (c).

### 3. Results

#### 3.1. Capture and anaesthesia of African wild dogs

Over the course of this study, seven male dogs were individually drafted, anaesthetised and subsequently reintroduced back into their packs on a total of 28 occasions. All procedures were uneventful and proceeded without harm or injury to animals or zoo staff. The mean ( $\pm$ S.E.M.) induction time to lateral recumbency using medetomidine/ketamine/atropin (only induction procedure reported) was  $7.0 \pm 0.3$  min. Animals were typically maintained on isoflurane for  $35.2 \pm 1.8$  min but the length of this anaesthetic was dependent on the success or otherwise of the electroejaculation procedure. African wild dogs recovered from the anaesthesia (capable of standing with only mild signs of ataxia) following administration with atipamizole (IV) and atipamizole (IM) (only recovery procedure reported) in  $14.9 \pm 1.1$  min. During anaesthesia all animals showed normal patterns of heart and respiratory rates and oxygen saturation was not compromised.

Approximately 3 h after the final procedure had been conducted, animals that had been processed that day, were re-introduced to each other within the holding pens. On introduction, all dogs greeted one another immediately with high-pitched vocalisation and sniffing behaviour. A subordinate male in one pack rolled over on to his back to display submissive behaviour to the alpha male in the first 10 min of introduction. Subsequent observations for approximately 40 min revealed that most dogs displayed territorial investigation and urinated frequently around the perimeters of the holding yards. When both day 1 and day 2 packs were re-introduced to each other on day 3, all males acknowledged the dominant alpha female with high-pitched calls and submissive sniffing and “pecking” of the alpha female’s muzzle which continued for approximately 10 min. Thirty minutes after re-establishment of the original pack, all dogs had settled to a normal level of interaction.

A carcass feed was introduced to entice the animals back into the primary exhibit from the holding yards and this proved successful. Carcass feeding appeared to facilitate the re-assessment of normal hierarchy and associated behaviours. The dominant alpha male and alpha female were first to feed; subsequently subordinate animals fed in order of dominance. These results suggest that temporary separation of captive African wild dogs from the pack to conduct reproductive assessment ( $n=4$ ) did not appear to have a disruptive effect on social hierarchy, nor promote social disharmony within the pack.

#### 3.2. Seasonal changes in the reproductive parameters of African wild dogs

Table 2 shows the seasonal change in testosterone secretion, average testicular volume and tone during the 3 years of this study. While there was no seasonal difference in the mean testosterone concentration, testicular volume was significantly greater during late summer compared to early spring. Table 2 indicates that the testes of dogs in summer can be up to four-fold larger than in early spring (Fig. 4). Particularly interesting, was the finding that testicular volume in early summer 2002 was lower ( $P<0.05$ ) compared to summer in 2001; it should be noted that the majority of dogs (199, 399, 499, 699, 799 and 899) were early pubescent (15 months) during summer 2001. Progressive measurements of testicular volume out of the breeding season in early spring 2000 and 2002 are likely to be indicative of normal testicular growth ( $P<0.05$ ). Testicular tone was highest during the summer months.

Table 2

Seasonal changes in African wild dog plasma testosterone concentration (ng/mL), testicular volume (cm<sup>3</sup>), testicular tone (0–5), frequency of electroejaculation success and spermatorrhea

	Early spring 2000	Late summer 2001	Late summer 2002	Early spring 2002
[Testosterone] (ng/mL) (mean ± S.E.M.)	3.2 ± 1.9a	5.6 ± 0.4a	8.9 ± 6.7a	1.6 ± 0.8a
Average testicular volume (cm <sup>3</sup> ) (mean ± S.E.M.)	4.3 ± 0.2a	17.4 ± 1.0b	12.8 ± 2.0c	6.8 ± 1.0d
Average testicular tone (0–5; mode)	3	4	5	3
Frequency of electroejaculation success	0/7	7/7	3/7	0/7
Frequency of spermatorrhea	1/7	6/7	6/7	0/7

Different letters within rows are significant ( $P < 0.05$ ).

Table 2 also reports the seasonal changes in the frequency of electroejaculation success and the occurrence of spermatorrhea. Clearly, semen collection in African wild dogs by means of electroejaculation is unlikely to be successful during early spring. Similar observations were noted for spermatorrhea suggesting that spermiogenesis is also seasonally dependent. In late summer 2001, it was possible to collect semen by electroejaculation from all seven animals, but in late summer 2002, semen was successfully recovered from only three dogs.

Table 3 shows the seminal characteristics of ejaculates collected in late summer 2001 and 2002. Unfortunately, given the limited ejaculates collected in 2002 ( $n = 3$ ) it was not possible to statistically compare this data to 2001 ( $n = 7$ ). Despite this limitation, it does appear that most

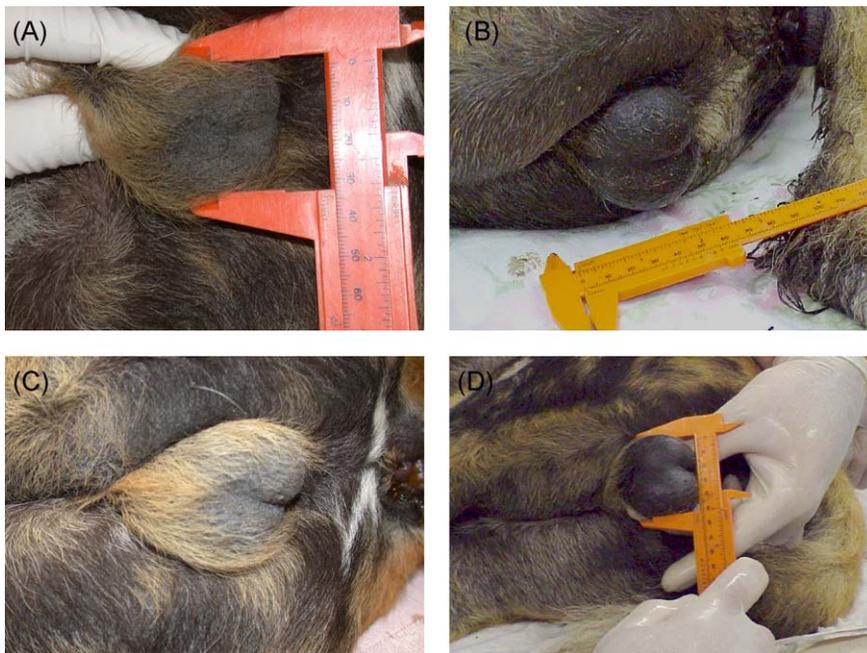


Fig. 4. Changes in African wild dog testicular volume during early spring (A and C) and late summer (B and D).

Table 3

Characteristics of African wild dog semen collected by electroejaculation in the breeding season (late summer)

	Late summer 2001 (mean $\pm$ S.E.M. (n))	Late summer 2002 (mean $\pm$ S.E.M. (n))	Total (mean $\pm$ S.E.M. (n))
Volume (mL)	0.7 $\pm$ 0.1 (7)	0.3 $\pm$ 0.1 (3)	0.6 $\pm$ 0.1 (10)
pH	7.3 $\pm$ 0.1 (7)	7.1 (2)	7.2 $\pm$ 0.1 (9)
Sperm concentration ( $\times 10^6$ mL <sup>-1</sup> )	268.0 $\pm$ 111.2 (7)	12.5 $\pm$ 6.3 (3)	212.3 $\pm$ 87.8 (10)
% motile	72.6 $\pm$ 3.1 (7)	62.3 $\pm$ 8.0 (3)	69.5 $\pm$ 3.3 (10)
Rate of sperm movement (0–5)	3.4 $\pm$ 0.2 (7)	3.2 $\pm$ 0.2 (3)	3.4 $\pm$ 0.3 (10)
% plasma membrane intact	81.4 $\pm$ 1.7 (7)	–	81.4 $\pm$ 1.7 (7)
% normal sperm morphology	78.9 $\pm$ 8.0 (7)	70.0 $\pm$ 10.8 (3)	76.2 $\pm$ 6.2 (10)

dogs were producing spermatozoa during the summer months but that ejaculate quality of some subordinate litter-mates may reduce after puberty.

Results thus far have been presented for the captive population as a whole and it may be more informative to analyse the salient parameters of male reproductive function individually and over time. Fig. 5 is a schematic diagram illustrating the changes in testicular volume, testosterone concentration, sperm production (spermatorrhea and EJ success) over the duration of the study; it also indicates the changes in alpha-male dominance.

During early spring in 2000 and 2002 testicular volume of all wild dogs, including the dominant males, was low and there was no clear relationship between social hierarchy and testosterone secretion. During the summer, testicular volume in all males increased dramatically but testosterone secretion in most animals remained low; interestingly, testosterone secretion of the alpha male was substantially increased during this period. In 2001, male 899 had a testosterone concentration that was 20 $\times$  higher than that of subordinate males, while in 2002 the testosterone concentration in male 699 was over 40 $\times$  higher than that of subordinate males. Testosterone secretion of the 2nd and 3rd most dominant males was not elevated but similar in concentration to other subordinate males. Clearly, the alpha male is capable of suppressing testosterone secretion in the subordinate males, but this does not appear to transpose to an equivalent ability to suppress testicular hyperplasia or sperm production. In fact, testicular volume of both dominant males in 2000 and 2002 was comparatively low with respect to the subordinate males.

Fig. 5 also shows that the social structure of the African wild dog pack at Western Plains Zoo was dynamic over the duration of the study indicating that dominance related behaviour might be mediated by testosterone secretion.

### 3.3. Electroejaculation and seminal characteristics of African wild dog semen

Electroejaculation was attempted on seven African wild dogs a total of 28 times, but resulted in semen samples adequate for analysis on only 10 occasions. The primary determinant of semen collection in this species, apart from urine contamination, appears to be breeding season but the suppressive effects of alpha-male dominance on sperm production cannot be ruled out. There were no adverse effects or injuries to dogs associated with electroejaculation and all animals were observed to behave and defaecate normally following the procedure. Seminal characteristics of the African wild dog electroejaculates are documented in Table 3. Table 3 only reports data from summer collections, highlighting ejaculate quality for each year (2001,  $n = 7$ ; 2002,  $n = 3$ ) and as a total ( $n = 10$ ). Ejaculates recovered in the 2001 summer were analysed further for incidence of abnormal spermatozoa (Table 4).

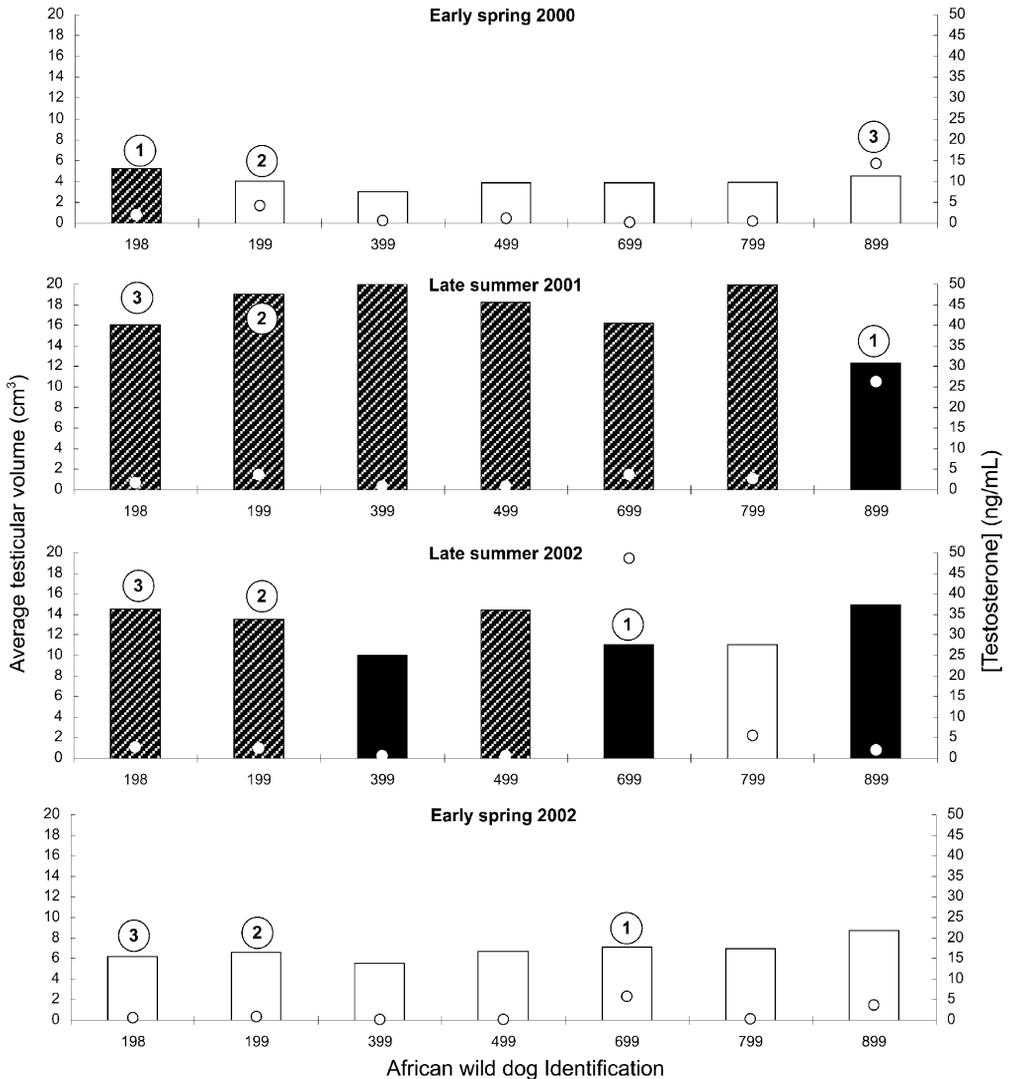


Fig. 5. Schematic diagram illustrating the changes in testicular volume, sperm production (spermatorrhea and electroejaculation success) and social status of individual animals over the duration of the study. Columns: testicular volume (cm<sup>3</sup>); shaded bars: animals that showed spermatorrhea; cross hatching: animals from which a electroejaculated semen sample was recovered; white dots: testosterone concentration (ng/mL); circle with numbers indicate alpha (1) and beta males (2 and 3) as determined by zoo-keeping staff.

### 3.4. Cryopreservation of African wild dog semen

Table 5 documents the pre and post-thaw survival of African wild dog semen following cryopreservation with a standard canine protocol using a final concentration of 20% egg yolk and 4% glycerol in a Tris–citrate fructose buffer (Gunn, 1988). Sperm evaluated immediately post-thaw showed an adequate tolerance to the cryopreservation procedure; however, when sperm were incubated for 2 h in a 37 °C incubator, all parameters of sperm survival declined markedly.

Table 4

Incidence of abnormal sperm morphotypes found in the electroejaculates ( $n=7$ ) of African wild dogs collected in late summer 2001

Abnormal spermatozoal morphotypes <sup>a</sup>	Mean $\pm$ S.E.M. (%)
Loose or damaged acrosome	1.7 $\pm$ 0.4
Detached sperm head	2.7 $\pm$ 1.0
Pyriform head defect	0.6 $\pm$ 0.4
Diadem defect	0.3 $\pm$ 0.3
Nuclear crater defect	0.1 $\pm$ 0.1
Microcephalic sperm	0.4 $\pm$ 0.3
Broken neck defect	0.7 $\pm$ 0.7
Distal midpiece reflex	5.3 $\pm$ 0.4
Thickened midpiece	0.4 $\pm$ 0.3
Segmental aplasia of the midpiece	0.1 $\pm$ 0.1
Proximal cytoplasmic droplets	7.9 $\pm$ 4.4
Distal cytoplasmic droplets	1.7 $\pm$ 0.8
Severely coiled principle-piece	1.4 $\pm$ 0.7
Double-tailed spermatozoa	0.1 $\pm$ 0.1
Teratoid spermatozoa	0.1 $\pm$ 0.1

<sup>a</sup> Sperm morphotypes as defined by Barth and Oko (1989).

Table 5

Mean ( $\pm$ S.E.M.) % motile, rate of motility, % live and % intact acrosomes of African wild dog semen pre-freeze, immediately post-thaw (T0) and 2 h post-thaw after incubation at 37 °C (T2)

Treatment	Pre-freeze	Post-thaw T0	Post-thaw T2
% motile	72.6 $\pm$ 3.1	31.8 $\pm$ 5.8	0.2 $\pm$ 0.1
Rate (0–5)	3.4 $\pm$ 0.2	2.8 $\pm$ 0.3	0.1 $\pm$ 0.1
% live	81.4 $\pm$ 1.7	33.4 $\pm$ 5.3	8.1 $\pm$ 2.2
% damaged acrosomes	1.5 $\pm$ 0.5	4.4 $\pm$ 1.5	7.9 $\pm$ 2.1

#### 4. Discussion

When this study was in the initial planning stages there was some concern expressed by zoo-keepers at Western Plains Zoo that separation of the dogs for individual reproductive procedures may present social disruption of the pack dynamics when the animals were reintroduced. Given the importance of pack dynamics to co-operative breeding in this species (Courchamp and Macdonald, 2001), these concerns were not unreasonable. Nevertheless, the repeated ( $n=4$ ) successful capture, restraint and re-introduction of African wild dogs in this study has provided a useful model for the future development and application of assisted breeding technology, reproductive management and routine veterinary examination.

Previous matings of African wild dogs at Western Plains Zoo (unpublished observations) have been observed between mid summer to early winter, a finding that was consistent with studies of captive and free-range animals in southern Africa (mid summer to late autumn; van Heerden and Kuhn, 1985) and as might be expected, 6 months out of phase with animals housed in captivity in North American Zoos (late summer to mid autumn; Monfort et al., 1997). The combined serum testosterone concentration of all seven males observed in the current study did not change significantly during observations of two breeding periods but there was a substantial increase in

testosterone secretion of individual alpha males during the breeding period. Testosterone secretion of all subordinate males remained low, both in and out of the breeding periods.

The episodic nature of plasma testosterone secretion in mammals requires that comparisons with studies using estimates of faecal testosterone should be made with caution. Nevertheless, while studying a free-range Tanzanian population, Creel et al. (1996) reported that the mean faecal testosterone concentration of males (irrespective of social rank) in breeding and non-breeding seasons was not significantly different. However, they did indicate that faecal testosterone concentration declined in both beta and subordinate males during the breeding season. Monfort et al. (1997) have reported a rise in faecal testosterone concentration during the breeding period of two captive animals in North America. Perhaps the isolation of males in small groups prevents any reproductive suppression induced by con-specifics and allows for a non-confounded expression of reproductive seasonality.

The most dramatic evidence of seasonal reproduction in the male African wild dogs housed at Western Plains Zoo were the changes associated with testicular volume and tone. Both alpha and subordinate males demonstrated up to a four-fold increase in testicular volume from mid spring to late summer. This increase in testicular volume and tone was also associated with an increase in sperm production as demonstrated by spermatorrhea and success of semen collection by electroejaculation. Interestingly, there appears to be a uncoupling of sperm production and testosterone secretion in the African wild dog testis; this phenomenon requires further investigation and confirmation as both testosterone and sperm production are typically controlled by the same hypothalamic support. This is the first description of a seasonal change in the testicular volume and sperm production in African wild dog, although similar changes have been reported in other wild canids living in temperate latitudes (Asa, 1999).

The results from the current study show that alpha males, as identified by zoo-keepers at Western Plains Zoo, had the highest levels of testosterone concentration both in and out of the breeding period. A change in the alpha-male status was clearly accompanied by an elevated testosterone secretion. While Creel et al. (1996) have demonstrated cooperative breeding and female reproductive suppression in free-range populations of African wild dogs, they have also reported on alpha male induced reproductive suppression of subordinates. For example, subordinate males showed a lower rate of mating, a lower testosterone concentration and less aggressive behaviour. In the current study, seasonal changes in testicular volume and sperm production were not under the same alpha-male suppressive control as that of testosterone secretion, as all males showed evidence of seasonal changes in testicular volume and sperm production. In fact, in late summer 2001, the testicular volume of the alpha male was the smallest of all dogs.

An interesting phenomenon reported in this study was the reduction in testicular volume observed during the breeding periods from summer 2001 to 2002. Leading into 2001, all littermates had just reached or were close to sexual maturity and showed a mean ( $\pm$ S.E.M.) testicular volume of  $17.4 \pm 1.0 \text{ cm}^3$ ; 12 months later in the next breeding period, testicular volume decreased significantly to  $12.8 \pm 2.0 \text{ cm}^3$ . Semen collection was also more successful in late summer 2001 than it was in 2002. Perhaps this is evidence that the suppressive effects of the alpha male do not become apparent on reproductive function, until the subordinate dogs reach sexually maturity.

The success of electroejaculation in the African wild dog was clearly dependent on the breeding period, urine contamination and possibly, also on the establishment of reproductive suppression by the alpha male. This study employed a technique to catheterise and empty the bladder of male dogs before electroejaculation was attempted as recommended by Platz et al. (2001). While urine contamination was not totally prevented during electroejaculation of the African wild dog, its

incidence was relatively low when compared to previous semen collection attempts in other wild Canidae (Platz et al., 2001).

The only previous description of the characteristics of African wild dog semen was that of Hermes et al. (2001) which was based on one ejaculate. Semen samples from the seven dogs in this study now provide the basis of a database for the seminal characteristics of this species. There are very few studies describing the electroejaculate characteristics of wild canid semen. The most informative of these from a comparative perspective was a study on semen collection and sperm cryopreservation from the red wolf (*Canis rufus*) (Goodrowe et al., 1998). While electroejaculate volume in the red wolf ( $4.7 \pm 0.7$  mL) was greater than that observed for the African wild dog ( $0.6 \pm 0.1$  mL), sperm concentration ( $146.5 \pm 25.7 \times 10^6$  mL<sup>-1</sup>) was less than that for the African wild dog ( $212.2 \pm 87.8 \times 10^6$  mL<sup>-1</sup>). The percentage of motile spermatozoa, viability (live) and % normal spermatozoa were similar for both species. The range of sperm abnormalities was also similar, although the terminologies used to describe African wild dog sperm abnormalities in the current study were based on the bovine spermatogram model (Barth and Oko, 1989), which has well-established criteria.

Hermes et al. (2001) have described an attempt to cryopreserve African wild dog spermatozoa which involved dilution 1:3 in TEST buffer with 7.5% glycerol (v/v) and 15% (v/v) hen egg yolk frozen in polypropylene biofreeze vials placed into a programmable freezer. This cryopreservation procedure was used to freeze two different fractions of the same ejaculate and resulted in an average post-thaw motility of approximately 35 and 8% when elevated at 10 and 30 min post-thaw, respectively. Results from the current study using seven ejaculates were similar in terms of the initial post-thaw motility ( $31.8 \pm 5.8\%$ ); however, incubation of African wild dog semen for 2 h at 37 °C following cryopreservation resulted in essentially no motility and indicates that other cryopreservation protocols should be investigated.

Cryopreservation of electroejaculated red wolf spermatozoa using a commercial egg yolk-based glycerolated extender (International Canine Semen Bank, ICSB; Sandy, OR, USA) and a pelleting protocol resulted in a post-thaw motility of approximately 30% and a statistically significant decrease in the percentage of intact acrosomes (Goodrowe et al., 1998, 2001). Interestingly, the cryopreservation protocol employed in the current study resulted in only a minor increase in the percentage of damaged acrosomes. While cryopreservation of wild canid spermatozoa is likely to require a species-specific approach, successful AI programs in domestic dogs and the commercial fox industry (Farstad, 1996) should provide enough encouragement to pursue the application of this technology in species such as the African wild dog.

## 5. Conclusion

This study was conducted to explore the possibility of assisted breeding technology in the African wild dog, particularly the development of semen collection and sperm cryopreservation protocols for use in AI. We have demonstrated that it is possible to safely and reliably capture and restrain African wild dogs for the purposes of reproductive evaluation and semen collection. We have also determined that under the husbandry conditions currently employed at Western Plains Zoo, male reproduction in the African wild dog population appears to demonstrate seasonality and an apparent degree of reproductive suppression from the dominant male. Both these phenomena will need to be considered when attempting to collect semen by electroejaculation. Electroejaculation of African wild dogs within the breeding season, resulted in the production of spermic ejaculates, the majority of which could be used for cryopreservation. While cryopreservation in 0.25 mL straws using a Tris–egg yolk-based extender and final concentration of 4% glycerol

resulted in a post-thaw motility of approximately 30%, further studies are required to improve the longevity of African wild dog semen post-thaw before AI of frozen-thawed semen is attempted.

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