Social rank does not affect sperm quality in male African wild dogs (Lycaon pictus)

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Abridged title: Social rank and African wild dog sperm quality

Abstract

Sperm banking and artificial insemination could benefit endangered African wild dog conservation. However, it is unclear whether their dominance hierarchy causes a decrease in reproductive and sperm quality parameters in subordinate males that typically do not breed. We investigated the effect of social rank on male reproductive parameters including faecal androgen and glucocorticoid metabolite concentrations, prostate and testes volume, preputial gland size, semen collection success, and sperm quality. Samples were obtained from captive males (pre-breeding season: n=12 from 4 packs; breeding season: n=24 from 7 packs) who were classified as alpha (dominant), beta, or gamma (subordinates) based on the frequency of dominant *vs.* submissive behaviours. In the pre-breeding season, semen was successfully collected from all alpha but only half the subordinate males; with urine contamination (associated with lower rank) significantly reducing total and progressive motility, sperm motility index, normal sperm morphology and the integrity of the acrosome. The breeding season was associated with a significant increase in faecal androgens, prostate and testis volume, as well as progressive motility and total number of ejaculated sperm. However, with the exception of prostate volume (alpha: 12.5 ± 4.5 *vs.* beta: 7.1 ± 1.0 *vs.* gamma: 7.3 ± 1.0, $P = 0.035$), all other reproductive and sperm quality parameters did not differ between males of each social rank. In conclusion, reproductive suppression of subordinate males appears to be behaviourally mediated, as males of all social rank produce semen of similar quality; making them suitable candidates for sperm banking, particularly during the breeding season when sperm quality improves.

1. Introduction

The African wild dog (AWD; *Lycaon pictus*) has a complex social structure which includes a cooperative breeding system where subordinate animals usually do not breed but help in pup rearing (Creel and Creel 2002). This species, classified as endangered by the IUCN (Woodroffe and Sillero-Zubiri 2012), requires effective management of the captive and free-living population (Frantzen *et al.* 2001). Development of artificial insemination and sperm banking are key elements for the overall conservation management of this species (Van den Berghe *et al.* 2012). Subordinate males are generally directly related to the dominant or alpha male (brothers or juvenile offspring) but share only 50% of their alleles at best; making the preservation of their genetic diversity equally important for sperm banking initiatives.

However, given that the alpha pair reproduces, it is not clear whether subordinate males are reproductively suppressed by behavioural or physiological mechanisms as seen in other species (Creel 2005; Young *et al.* 2006; Barja *et al.* 2008; Van den Berghe *et al.* 2012). Behavioural suppression of subordinate reproduction through mate guarding of the alpha female by the alpha male is one of the mechanisms seen in AWDs (Van Heerden and Kuhn 1985). Moreover, it is known that most subordinate AWD females ovulate, as shown by non-invasive faecal hormone monitoring (Van der Weyde *et al.* 2015), the high degree of glandulo-cystic endometrial hyperplasia and pyometra evident in captive individuals (Asa *et al.* 2014), as well as the occasional litter (Spiering *et al.* 2009); suggesting that reproductive suppression in females is behaviourally controlled (i.e. they are fertile but prevented from mating). Similarly, both dominant and subordinate males within a pack show an increase in testis size and sperm production during the breeding season (Johnston *et al.* 2007; Newell-Fugate *et al.* 2012). In fact, subordinate paternity has been widely documented in wild packs (Girman *et al.* 1997; Mouiex 2006; Spiering *et al.* 2009).

However, physiological suppression of reproduction via the hypothalamic-pituitary-gonadal axis through hormonal or pheromonal signals cannot completely be excluded. Higher testosterone levels in the dominant male during the breeding season (Creel *et al.* 1997; Monfort *et al.* 1997; Johnston *et al.* 2007; Newell-Fugate *et al.* 2012) and an overall decrease in sperm quality and quantity after the establishment of a hierarchy (Johnston *et al.* 2007), suggest that subordinate males may exhibit some form of physiological subfertility. At this point however, the exact extent of subordinate male subfertility is not clear. Stress, either chronic or acute, can decrease semen quality, including increased sperm DNA damage due to a rise in cortisol levels (Sasagawa *et al.* 2001; Ren *et al.* 2010). Sperm DNA damage can reduce fertilization success and impair embryo development (Seli *et al.* 2004; Lewis and Aitken 2005). However, 'physiological castration' through higher stress levels in subordinate animals may be unlikely in AWDs as it is usually the dominant male and female that show higher glucocorticoid levels without exerting any obvious negative effects on their fertility (Creel *et al.* 1997; Van den Berghe *et al.* 2012).

Thus, differences in reproductive parameters between dominant and subordinate AWD males warrants further investigation to determine whether that may help target sperm banking initiatives for the species; which can significantly assist their conservation (Van den Berghe *et al.* 2012). Our study, therefore, aimed to evaluate the effect of social rank on reproductive and sperm quality parameters during the pre-breeding and breeding season in male AWDs.

2. Materials and methods

2.1. Animals and husbandry

This study was approved by James Cook University Animal Ethics Committee and by the Institutional Animal Care and Use Committees (IACUC) of the participating institutions. A total

of n=28 AWD males were used, n=15 of which were housed in 5 different zoological institutions in the US (ABQ, Albuquerque BioPark, Albuquerque, NM; TOP, Topeka Zoo, Topeka, KS; BRK, Brookfield Zoo, Chicago, IL; BIN, Binder Park Zoo, Battle Creek, MI; and OKC, Oklahoma City Zoo, Oklahoma City, OK), while the remaining n=13 were males housed in 3 different packs at Harnas Wildlife Foundation, Gobabis, Namibia (BRU, Brutus pack; PLA, Platform pack; SAN, San pack; Table 1).

As described in Van den Berghe *et al.* (2018a), US packs consisted of 3 males with 1 female (BRK pre-breeding season, OKC) or 3 males alone (BRK breeding season, ABQ, TOP, BIN). All males were reproductively mature (range: 2.8 - 7.8 years; Table 1); with 2 from the BRK pack (ID 2413 and 2499) siring litters in previous years, and all 3 from the OKC pack observed mating with the female 3 weeks prior to sample collection (26th Aug - 8th Sep 2014), with puppies born 7th Nov 2014 (exact paternity unknown). Males were immobilised for health assessment and sample collection during the 2014 pre-breeding season (ABQ, BRK, BIN, TOP; May – early Jul 2014) and breeding season (ABQ, BRK, BIN, OKC; Aug - Sep 2014; Table 1). All animals had access to water *ad libitum* and were individually fed with ground horsemeat (Central Nebraska Packing Inc., NE, USA), occasionally replaced by bones, whole pig or goat carcass. All AWDs were housed on outside public display (range 634 - 1226 m^2) during the day with no access to off-exhibit holding areas. These holding areas were open to animals late afternoon, permitting free access to both areas overnight, except for the BIN pack that was confined to their holding area (consisting of 4 separate huts each with a small outside area, connected to each other). Natural daylight was available to all AWDs in the project at all times.

Namibian packs were of mixed-sex and held in large enclosures of natural habitat consisting of dense trees, scrub and an artificial waterhole. Animals were group-fed with donkey and

Table 1. *Pack composition, social rank, observation and sampling times of African wild dogs included in this study.*

Pack	ID - Name	Sex	Social rank*	Age (y)	Relationship with conspecifics	Total observation time (h)		Immobilisation date	
						Pre-breeding	Breeding season	Pre-breeding	Breeding season
ABQ	2393 - Mooseface	♂	α	7.8	*siblings*	7.83	9.33	15 May 2014	8 Aug 2014
	2394 - Digger	♂	β	7.8	*siblings*			15 May 2014	7 Aug 2014
	2395 - Growlly	♂	γ	7.8	*siblings*			15 May 2014	7 Aug 2014
TOP	2500 - Kipaku	♂	α	3.8	*siblings*	6.66		28 May 2014	-
	2492 - Minzi	♂	β	3.8	*siblings*			28 May 2014	-
	2496 - Hunter	♂	γ	3.8	*siblings*			28 May 2014	-
BRK	2413 - Digger	♂	α	7.6	*sire*	9.36	10.44	27 Jun 2014	-
	2494 - Nar	♂	β	3.6	*offspring*			27 Jun 2014	21 Aug 2014
	2499 - Jack	♂	γ	3.6	*offspring*			27 Jun 2014	21 Aug 2014
	2278 - Kim-ly	♀	α	9.6	*dam*				†
BIN	2428 - Blacktail	♂	α	6.8	*siblings*	10.47	9.45	08 Jul 2014	17 Sep 2014
	2383 - Victor	♂	β	7.8	*siblings*			08 Jul 2014	18 Sep 2014
	2427 - Verizon	♂	γ	6.8	*siblings*			08 Jul 2014	17 Sep 2014
OKC	T1 - Dojo	♂	α	2.8	*siblings*		14.13	-	30 Sep 2014
	T3 - Chipata	♂	β	2.8	*siblings*			-	30 Sep 2014
	T2 - Juma	♂	γ	2.8	*siblings*			-	30 Sep 2014
	2516 - Xena	♀	α	2.8	*dam*				
BRU	M1 - Brutus	♂	α	7-9#	*sire*		15.50	-	17 Nov 2015
	M2 - Apollo	♂	β	1.8	*offspring*			-	-
	M3 - Ares	♂	β	1.8	*offspring*			-	-
	M4 - Heracles	♂	β	1.8	*offspring*			-	-
	F1 - Saddleback	♀	α	7-9#	*dam*				
	F2 - Gaia	♀		1.8	*offspring*				
	F3 - Artemis	♀		1.8	*offspring*				
PLA	M1 - Zevon	♂	α	3-5#			12.34	-	14 Jan 2016
	M6 - Styx	♂	α	3-5#				-	16 Jan 2016
	M8 - Harrison	♂	α	3-5#				-	16 Jan 2016
	M12 - Simon	♂	α	3-5#				-	-
	M7 - Hendrix	♂	β	3-5#				-	-
	M9 - Cohen	♂	β	3-5#				-	-
	M10 - Garfunkel	♂	β	3-5#				-	15 Jan 2016
	M11 - Lennon	♂	β	3-5#				-	15 Jan 2016
	M2 - Marley	♂	γ	3-5#	*siblings from several litters*			-	14 Jan 2016
	M3 - Zeppelin	♂	γ	3-5#				-	14 Jan 2016
	M4 - Dylan	♂	γ	3-5#				-	14 Jan 2016
	M5 - Ozzy	♂	γ	3-5#				-	15 Jan 2016
	M13 - Wilson	♂	γ	3-5#				-	16 Jan 2016
	F1 - Yoko Ono	♀	α	3-5#					
	F2 - Neko	♀		3-5#					
	F3 - Susie Q	♀		3-5#					
	F4 - Joni	♀		3-5#					
SAN	M1	♂	α	5.0	*sire*		7.77	-	20 Mar 2016
	M2	♂	γ	5.0	*brother α* ♂ and ♀			-	19 Mar 2016
	M3	♂	β	1.7	*offspring*			-	-
	M4	♂	γ	1.7	*offspring*			-	-
	F1	♀	α	5.0	*dam*				
	F2	♀		5.0	*sister α* ♂ and ♀				
	F3	♀		5.0	*sister α* ♂ and ♀				
	F4	♀		1.7	*offspring*				
	F5	♀		1.7	*offspring*				

USA: ABQ, Albuquerque BioPark Zoo; TOP, Topeka Zoo; BRK, Brookfield Zoo; BIN, Binder Park Zoo; OKC, Oklahoma City Zoo; Harnas Wildlife Foundation, Namibia: BRU, Brutus pack; PLA, Platform pack; SAN, San pack.

*Social rank based on behavioural observations; †Dog euthanized between pre-breeding and breeding season evaluations; # Estimated age.

horsemeat on the bone or intestines, occasionally replaced by dog pellets (Hill's Pet Nutrition, Kansas, United States), or goat, sheep or wild game meat. The BRU pack was held in a 0.7 ha enclosure and consisted of an alpha male and female of unknown age and their offspring (2 females and 3 males; Table 1). The PLA pack consisted of siblings from different litters, in total 13 males and 4 females of unknown age (Table 1). These AWDs were held in a 14.4 ha enclosure but were moved into 4 smaller adjacent enclosures (each 0.1 ha) for the period of study to facilitate observations, faecal sample collection, and capture (Fig. 1). To habituate the AWDs to these pens, access was granted from 5 days prior to the start of sample collection. During this time, AWDs were seen continuously in these smaller pens, and the door to their original enclosure was locked the day before behavioural observations began. The 4 enclosures could be isolated from each other using doors in the centre (Fig. 1) but remained open during the days of observations. The SAN pack was held in a 3.6 ha enclosure and consisted of 10 AWDs (Table 1). The oldest 5 AWDs (2 males and 3 females) were siblings brought to Harnas as puppies in 2011. The alpha couple of this pack had a litter at Harnas in July 2014 containing 2 males and 3 females.

2.2. Classification of social hierarchy

Behaviour was analysed by filming interactions within each pack for a total of 10.3 ± 0.8 h over 3 days prior to the first immobilisation (Table 1). In the US, animals were filmed from outside the enclosures, either from the public viewing area or from the zookeeper section. In Namibia, the SAN and BRU packs were filmed from within their original enclosures from the top of a car, which was moved when necessary, while the PLA pack was translocated to 4 smaller pens adjacent to their enclosure (Fig. 1) and filmed from a car parked in pen 1. Behaviour was recorded at times AWDs were most active in the individual settings; generally,

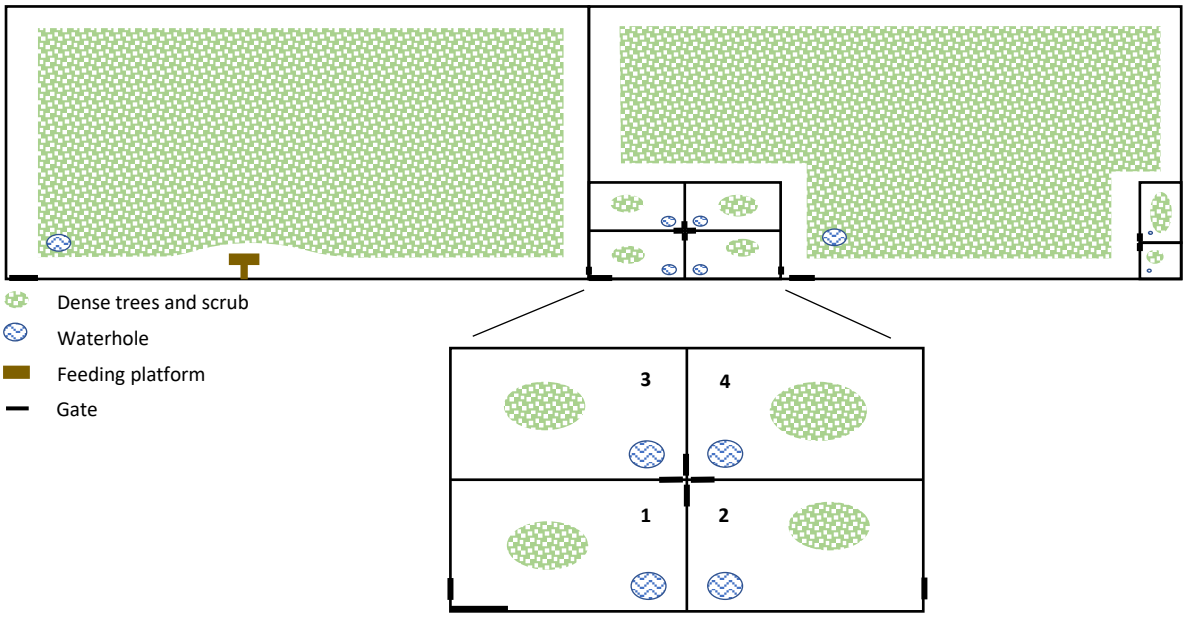


Figure 1. Layout of the 14.4 ha Platform pack enclosure and 4 adjacent 0.1 ha pens used for behavioural observations, faecal sample collection and capture.

around feeding time, in the early morning or late afternoon. To determine hierarchy, videos were analysed by noting the actor and recipient of all dominant and submissive behaviours and interactions between males (Van den Berghe *et al.* 2018b; Table 2). Males were then classified as alpha, beta, or gamma male within the pack based on the frequency of dominant *vs.* submissive behaviours respectively (Fig. 3 and Table 3).

2.3. Faecal sample collection and steroid hormone analysis

In the US, individually marked (by coloured plastic beads in feed) faecal samples were collected daily in the morning from all animals during enclosure cleaning, from 3 days before the immobilisation of males as described previously (Van den Berghe *et al.* 2018b). Samples were oven-dried and transported to the University of Pretoria (South Africa) for steroid analysis (Van den Berghe *et al.* 2018b). In Namibia, individual marking of faeces from group-housed individuals was not possible due to large enclosures and the method of group feeding. Faecal samples were collected opportunistically during fixed observation periods within a few minutes after AWDs were seen defecating, sealed in plastic bags and kept in a cooler box on ice until the end of observation, then frozen at -20ºC. Samples were then transported to the University of Namibia (Windhoek, Namibia) on dry ice, where they were oven-dried (Scientific Engineering, Stormill, South Africa). In both US and Namibian animals, a faecal sample was also collected during immobilisation directly from the rectum.

Dried samples were pulverised, and ethanol extracted as described by Van den Berghe *et al.* (2018b). For Namibian samples, 1 ml of faecal extract was oven dried at 45°C for transport to the University of Pretoria (Pretoria, South Africa). The dried extracts were later reconstituted by adding 1 ml of 80% ethanol, vortexing together with glass beads at high speed for 15 seconds, followed by 30 min sonication. The reconstituted extracts were stored at -20°C until

Table 2. *Dominant and submissive social interactions used for behavioural analysis. Modified from Vlamings (2011).*

Behaviour	***Description***
Dominant behaviour	
Aggressive vocalisation	Growling.
Scruff orientated approach	The actor approaches the scruff of the recipient without biting.
Stalk approach	The actor slowly approaches the recipient with a prowling posture; that is with the head and neck in a straight line below the shoulder, the ears folded back, the tail relaxed or in a straight horizontal line and without losing eye-contact with the recipient.
Food approach	The actor approaches the recipient while looking at him in the context of food acquisition.
Intervention by approach, stand or threat	The actor stops an interaction between two interactants by approach, stand in between or threat towards one of the recipients respectively.
Fixating	The actor looks straight at the recipient from a distance, motionless, in a high posture and with the ears forward.
Mark over urine or food	The actor secretes, with one (or both) feet lifted from the ground, a small amount of urine over a previous urine mark or food item on the ground.
Freezing	The actor stands stiff with the head straight to the ground and the eyes fixated, either on the ground or on the recipient; the behaviour is shown mostly as a reaction to 'food approach'.
Inguino-genital inspection	The actor initiates an inguinal contact and investigates the genitals of the recipient while the latter remains passive.
Point	The actor directs, with an abrupt movement of his head or a short jump, towards the recipient.
Mount	The actor places both its forepaws on the back of the recipient. It may do so from behind or from the side.
Stand over position	The actor stands across a lying recipient.
Approach in high posture	Moving towards the recipient in a high posture, while looking at him.
High posture snout	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a high posture.
High posture face lick	The actor licks the nose, lips and mandibular region of the recipient while being in a high posture.
Submissive behaviour	
Escape/flight	The actor runs away from the recipient, often seen during conflicts.
Retreat	The actor moves away from the recipient in a low position after having been approached by him. This also includes a retreat in the context of food acquisition.
Shrink back	The actor jumps back from the recipient, after being approached by him.
Avoid	The actor stands aside for the recipient, after being approached by him.
Active submission	A behavioural complex in which the actor actively seeks contact with a recipient by approaching him in a crouched manner with curved back and bent legs, while the tale is curled down, often wagging, and while the ears are folded back. From this position, the actor tries to contact the recipient by licking its nose.
Passive submission	The actor pushes himself down in front of the recipient.
Head turning	The actor turns his head and avoid eye contact with the recipient, exposing the neck region towards the recipient.
Low posture standing	Stand in a low position, with the ears pulled back.
Approach in low posture	The actor moves towards the recipient in a low posture while looking at him.
Low posture snout contact	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a low posture.
Submissive vocalisation	Twittering, whimpering, yelping, whining vocalisations.
Present body	The actor rolls on his side in front of the recipient or rolls towards him, awaiting his inspection.
Food solicit	The actor approaches or walks in parallel with the recipient while begging for food and trying to reach for his mouth corners. There is some resemblance with 'greeting', which is an affiliative behaviour, but the context is different and the behaviour is not likely to be reciprocated.
Hoo call	Indicative for distress.
Low posture face lick	The actor licks the nose, lips and mandibular region of the recipient while being in a low posture.

Table 3. *Classification of social hierarchy based on the frequency of dominant and submissive behaviour in the PLA pack of Namibia during the breeding season.*

Social rank	***ID - Name***	***Dominant behaviour***		***Submissive behaviour***	
		Given	***Received***	***Given***	***Received***
α	M1 - Zevon	13 (5)	6 (4)	6 (4)	12 (5)
	M6 - Styx	15 (8)	11 (5)	4 (3)	13 (8)
	M8 - Harrison	8 (5) + 3[a]	8 (4)	3 (2)	12(5)
	M12 - Simon	9 (5)	13 (4)	8 (3)	6 (4)
β	M7 - Hendrix	3 (3)	2 (2)	5 (3)	2 (2)
	M9 - Cohen	8 (3)	4 (3)	2 (2)	7 (3)
	M10 - Garfunkel	4 (3)	3 (3)	1 (1)	2 (2)
	M11 - Lennon	3 (3)	5 (3)	5 (3) + 1[b]	2 (1)
γ	M2 - Marley	2 (2)	8 (3)	10 (4)	3 (2)
	M3 - Zeppelin	1 (1)	3 (3)	3 (3)	1 (1)
	M4 - Dylan	1 (1)	6 (5)	6 (4)	1 (1)
	M5 - Ozzy	0 (0)	0 (0)	1 (1)	0 (0)
	M13 - Wilson	0 (0)	4 (2)	3 (1)	1 (1)

α = dominant to ≥ 5 dogs; β = dominant to 3-4 dogs; γ = dominant to ≤2 dogs.

[a]Marking behaviour. [b]Hoo calling. Numbers in parenthesis indicate number of dogs to/from which behaviours were given or received respectively.

analysis. All steroid extracts were measured for immunoreactive faecal glucocorticoid metabolite (fGCMs) and faecal androgen metabolite (fAMs) concentrations (Van den Berghe *et al.* 2018b) using established enzyme-immunoassays (EIAs; Palme and Mostl 1994; Palme and Mostl 1997). Sensitivities (90% binding) of the assays were 1.2 ng/g dry faeces (DW) for fGCM and 4.8 ng/g DW for the fAM EIA, respectively. Intra-assay coefficients of variation (CV), determined by repeated measurements of high and low value quality controls ranged between 4.8% and 5.6% for fGCM, and 5.0% and 5.1% for fAM measurements. Inter-assay CV ranged between 12.2% and 13.8% for fGCM and 8.9% and 10.9% for the fAM EIA.

2.4. Immobilisation

In the US, all male AWDs in each pack were starved for at least 12h then immobilised over a 1- or 2-day period to minimise potential aggression during reintroduction. AWDs were separated into individual holding pens and either darted or hand injected in a crush cage (Van den Berghe *et al.* 2018b). Anaesthetic protocols were as described in Van den Berghe *et al.* (2018a). In Namibia, AWD males were either darted by CO_2 dart gun (Daninject No. 2587 MOD JM, Dorkop, Denmark) in their enclosure and transported to the veterinary clinic for sample collection, or trapped in a cage and hand injected through the cage after transport to the clinic. Due to the size of the pack, it was not possible to complete all immobilisations within a 2-day interval for the PLA pack. To facilitate capture, the 4 smaller adjacent pens were used to separate animals from each other (Fig. 1). On Day 1, M2, M3 and M4 were isolated in pen 1 and darted, while M1 was cage-trapped in pen 3 and hand injected. The doors of the pens were reopened in the evening during feeding to enable remaining AWDs access to each other. On Day 2, M5 was isolated in pen 4 and darted, while M7 and M9 were trapped in pen 2 and hand injected. On the third and last day of immobilisations, M10 got darted and M6 and M8

were trapped & hand injected. The three remaining subordinate males from this pack were not evaluated. To minimize the risk of pack disruption due to the intervention/reintroduction, AWDs sedated on Day 1 were held overnight in cages before release into pen 3 the following day (separate from the remaining animals), AWDs sedated on Day 2 were released into pen 2 the same day and doors were opened, and AWDs sedated on Day 3 were released into pen 2 the following day then given access to other pack members after a habituation period of approximately 1.5 hours. SAN pack males were darted from within their original enclosure. One subordinate male (M2) was darted on Day 1 and the alpha male (M1) was darted the day after. The remaining subordinate males (M3 and M4) were not evaluated as they were still pre-pubertal (Table 1). After sample collection, the immobilised males were kept in cages next to the veterinary clinic with visual and olfactory contact between each other until simultaneous release back to their pack the next day.

2.5. Physical and reproductive examination

All AWDs were weighed and subjected to a full physical and reproductive examination. Testes, prepuce and penis were visually inspected, palpated and any abnormalities noted. Androgen-dependant preputial gland swelling was ranked using a score from 0 (no swelling) to 3 (large swelling; Fig. 2a-d). Testis tone was characterised as either flaccid, normal or hard. The prostate was palpated by digital rectal examination to check the position, size, consistency and symmetry. Thereafter, prostate and testes were visualised using an Ibex Portable ultrasound (EI Medical Imaging, Loveland, CO, USA) with an 6MHz transducer, except for the PLA pack where a CTS-900V V1.39 ultrasound (SIUI, Guandong, China) with a 5MHz transducer was used. Prostate length (L) and height (H) where measured in a longitudinal, and width (W) in a transverse plane. Prostate volume was calculated as L x W x H x 0.523 (Ruel *et al.* 1998).

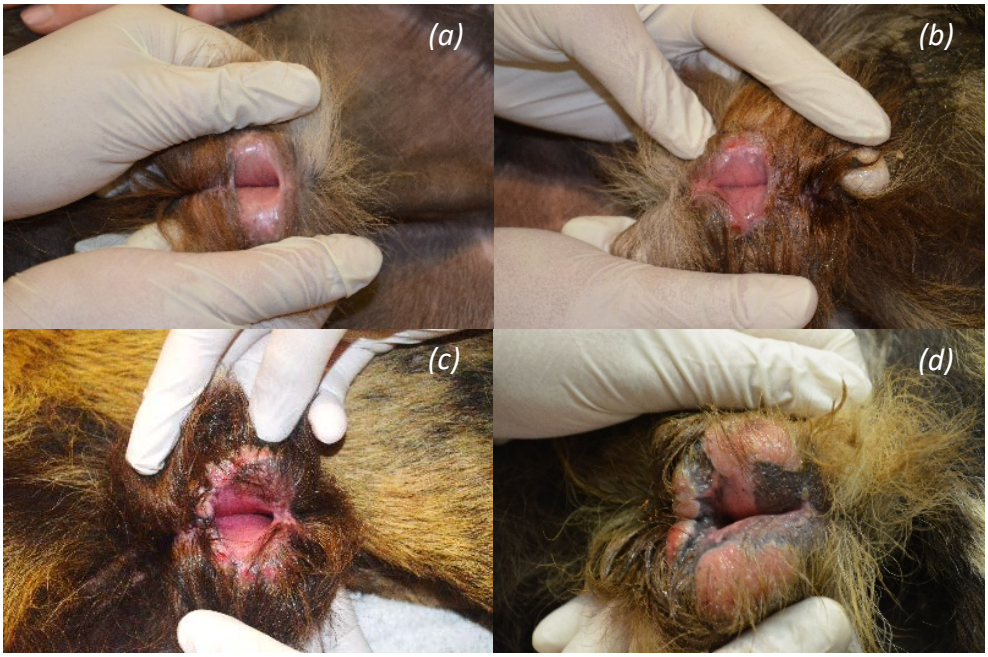


Figure 2. Classification of African wild dog preputial gland development:
(a) no swelling (score 0);
(b) mild swelling (score 1);
(c) medium swelling (score 2); and
(d) large swelling (score 3).

Testis length (L) and height (H) where measured in a longitudinal, and width (W) and height (H) in a transverse plane, and mean value taken for height (Newell-Fugate *et al.* 2012). Testis volume was calculated as L x W x H x 0.523 (Newell-Fugate *et al.* 2012) and the mean value of right and left testis of each dog was used for analysis.

2.6. Semen collection and evaluation

Semen was collected using a custom-built 20 Hz sine wave electro ejaculator (CGS Products Pty. Ltd., Trafalgar, Victoria, Australia) with a 20 or 25 mm diameter probe as described in Van den Berghe *et al.* (2018a). After each of 3 stimulation series, the semen collection tube was kept at 37°C until analysis, which started within 5 min after the end of the last series. Each fraction was evaluated for volume, colour, presence of motile spermatozoa and pH, after which all fractions were combined. Detailed sperm analysis (motility, viability, morphology, sperm number, acrosome status and DNA integrity) was performed as described in detail in Van den Berghe *et al.* (2018a). In short, motility and sperm motility index were calculated by classifying at least 100 spermatozoa at 400 X magnification as grade 0 (non-motile sperm), 1 (motile non-forward progression), 2 (poor forward progression), 3 (moderate straight-line forward progression) or 4 (fast straight-line forward progression) by placing 10 µl of the sample on a pre-warmed glass slide with cover-slip. The percentage of viable and morphological normal spermatozoa *vs.* those with primary or secondary defects were evaluated by eosin-nigrosin smear (≥ 100 cells, 1000 X magnification; Johnston *et al.* 2001). Sperm concentration and total number of sperm ejaculated were calculated using a haemocytometer (BLAUBRAND® Neubauer improved bright-line, Brand GMBH, Wertheim, Germany). Acrosome integrity was evaluated using a fluorescent Pisum Sativum Agglutinin (PSA) conjugated with FITC (Sigma-Aldrich, St-Louis, MO, USA) and DNA integrity was

evaluated with the *In-Situ* Cell Death Detection Kit, Fluorescein™ (Roche Diagnostics, Basel, Switzerland).

2.7. Statistical analysis

Data were analysed using a linear mixed effect analysis using R statistical software (V3.5.1, R Foundation for Statistical Computing, Vienna, Austria) with pack and AWD ID as random effects. 'Social rank' and 'season' were considered as fixed effects for all evaluated parameters. In addition, 'age' was considered as a fixed effect for analysis of fAM and fGCM concentrations, and for prostate and testis volume. 'Country' (US *vs.* Namibia) was considered as a fixed effect for analysis of fAM and fGCM concentrations, and 'body weight' and 'fAM concentrations' were considered as fixed effects for analysis of prostate and testes volume. Statistical analysis was not possible for sperm collection success and urine contamination rate, due to limited data. Prostate volume was compared between animals with and without urine contamination of the semen sample during the pre-breeding season using an independent sample t-test. To enable statistical analysis of preputial gland size, scores were subdivided into 2 groups ('scores 0-1' and '2-3'). Due to limited data, beta and gamma males were combined into one subordinate group for analysis of sperm quality parameters and compared to the dominant males. With the exception of motility rating and total number of ejaculated sperm, sperm quality parameters were also analysed using 'urine contamination' as a fixed effect. The combined effect of 'social rank' and 'season' was evaluated for body weight, preputial gland size and sperm quality parameters. $P \leq 0.05$ was considered significant and all data are presented as mean ± SEM unless otherwise noted.

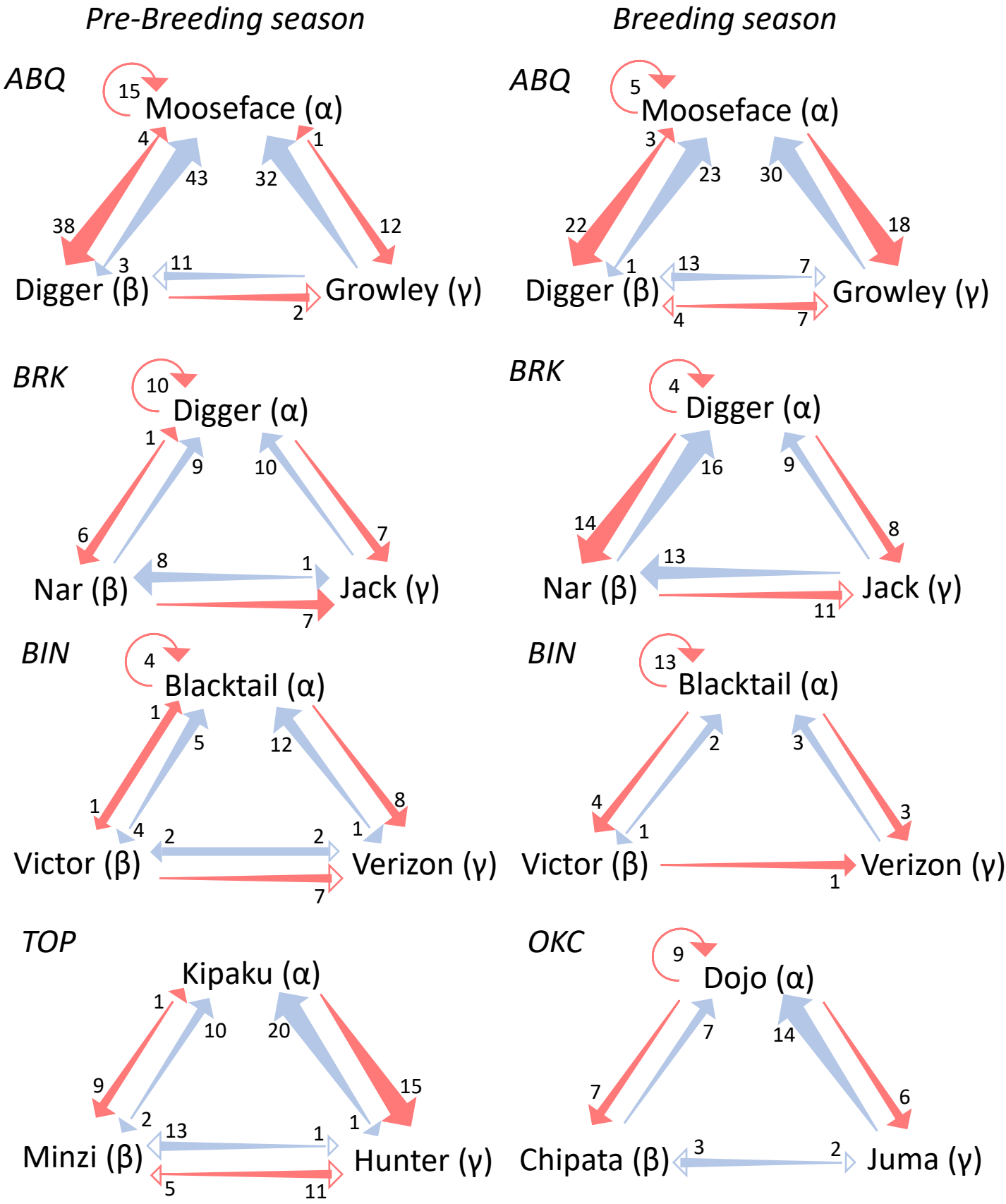
3. Results

3.1. Classification of hierarchy

In the US, ABQ, BRK and TOP packs showed a clear hierarchy with the alpha male exhibiting marking behaviour and/or frequent dominant behaviour toward subordinate males, as well as receiving clear submissive behaviour from both subordinates (Fig. 3a). There was also a clear hierarchy between beta and gamma males in these three packs, with frequent dominant behaviour from the beta toward the gamma male and conversely, frequent submissive behaviour from the gamma toward the beta male. The OKC alpha male also showed marking and clear dominant behaviour towards, and received submissive behaviour from the 2 subordinate males (Fig. 3a). However, the relationship between the 2 subordinate males could only be determined based on their frequency of submissive behaviour toward the dominant male since there were no dominant interactions and similar amounts of submissive behaviour between them (Fig. 3a). The relationship between the alpha and beta male in the BIN pack during the pre-breeding season was not obvious as they showed similar amounts of dominant and submissive behaviour towards each other. However, one was classified as the dominant alpha male based on frequent marking behaviour, which was subsequently confirmed by more a pronounced set of behaviours in the breeding season (Fig. 3a). The gamma male received dominant behaviour from both the alpha and beta male and exerted a high level of submissive behaviour towards the alpha male. Social rank did not change from the pre-breeding to the breeding season in any pack.

In Namibia, the BRU alpha male showed frequent marking and dominant behaviour towards all subordinate males (his pre-pubertal offspring) and received submissive behaviour from them. The relationship between subordinate males was unclear and all were classified as beta males because they exhibited similar amounts of dominant and submissive behaviour toward

a.


Pre-Breeding season
Breeding season
ABQ
Mooseface (α)
Digger (β)
Growley (γ)
BRK
Digger (α)
Nar (β)
Jack (γ)
BIN
Blacktail (α)
Victor (β)
Verizon (γ)
TOP
Kipaku (α)
Minzi (β)
Hunter (γ)
OKC
Dojo (α)
Chipata (β)
Juma (γ)

b.

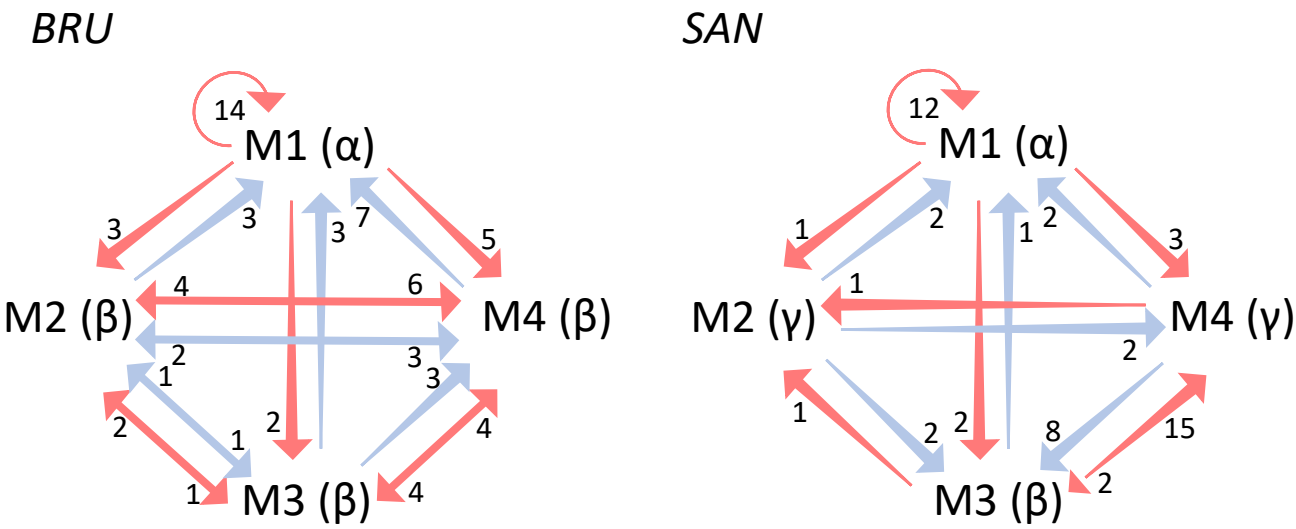


Figure 3. Classification of social hierarchy based on the frequency of dominant (red arrows), submissive (blue arrows) and scent marking (round red arrows) behaviour in the
(a) US packs during the pre-breeding and breeding season, and
(b) BRU and SAN packs of Namibia during the breeding season. Arrow heads indicate direction of behaviour from actor to recipient. Numbers next to arrows indicate the frequency of a behaviour within the total observation time.

each other (Fig. 3b). SAN pack males showed fewer dominant-submissive interactions, but one of two older males (M1) was classified as alpha male based on marking behaviour, mate-guarding of the dominant female, and no submissive behaviour toward any other males (Fig. 3b). The other older male (M2 and brother of M1), was never seen with the pack (frequently chased away by the group) and was considered an outcast together with two older females (sisters of M1, M2 and the alpha female). M2 never showed dominant behaviour toward other males and was classified as gamma male. Among the two younger males, M3 was clearly dominant over M4 and they were classified beta and gamma male respectively (Fig. 3b). No clear hierarchy was present in the PLA pack, with most males both giving and receiving dominant and submissive behaviours (Table 3). However, some males showed more dominant and received more submissive behaviour than others. As such, each male was grouped as either alpha, beta or gamma based on the number of other males toward which they showed dominant behaviour (Table 3).

3.2. Effects on male reproductive and sperm quality parameters

Mean body weight of all collected dogs significantly decreased from the pre-breeding to breeding season (Table 4; χ^2 = 4.038, *df* = 1, *P* = 0.044). However, social rank had no significant effect on body weight (Table 5; χ^2 = 0.218, *df* = 2, *P* = 0.897), irrespective of season (Table 6; χ^2 = 0.475, *df* = 2, *P* = 0.816). Although AWDs in the US appeared heavier than those in Namibia, this effect was not significant (χ^2 = 0.408, *df* = 1, *P* = 0.523).

fAM concentrations were significantly higher in the breeding season (Table 4; χ^2 = 3.865, *df* = 1, *P* = 0.050), but were not affected by social rank (Table 5; χ^2 = 0.654, *df* = 2, *P* = 0.721), country (χ^2 = 0.019, *df* = 1, *P* = 0.891) or age (χ^2 = 0.315, *df* = 1, *P* = 0.575). fGCM concentrations were not affected by season (Table 4; χ^2 = 1.708, *df* = 1, *P* = 0.191), social rank (Table 5; χ^2 =

Table 4. *Mean (± SEM) reproductive parameters grouped by season*

Parameter	***Pre-breeding (n)***	***Breeding (n)***	***P-value***
Weight (kg)	30.9 ± 0.5 (12)	28.6 ± 0.9 (24)	0.044
fAM (µg/g)	0.46 ± 0.06 (12)	0.47 ± 0.06 (25)	0.050
fGCM (ng/g)	38.1 ± 2.8 (12)	31.0 ± 2.7 (25)	0.191
Prostate volume (cm^3)	4.6 ± 0.6 (12)	11.2 ± 2.2 (24)	<0.001
Testes volume (cm^3)	10.2 ± 1.3 (12)	13.4 ± 0.8 (24)	<0.001
Preputial gland size (score 0-3)	1.5 ± 0.4 (12)	1.9 ± 0.2 (24)	0.494
Total motility (%)	17.3 ± 10.2 (8)	47.4 ± 6.7 (20)	0.315
Progressive motility (%)	12.8 ± 8.5 (8)	30.5 ± 5.8 (20)	<0.001
Sperm motility index (0-400)	45.7 ± 29.2 (8)	112.7 ± 20.0 (20)	0.486
Motility rating (1-4)	1.2 ± 0.6 (8)	2.0 ± 0.4 (20)	0.398
Total no. ejaculated sperm (x 10^6)	27.4 ± 11.5 (8)	32.3 ± 9.2 (20)	<0.001
Normal sperm morphology (%)	40.6 ± 9.8 (8)	50.9 ± 5.2 (20)	0.381
Sperm viability (% alive)	63.1 ± 5.1 (8)	74.4 ± 4.2 (20)	0.575
Sperm acrosome (% intact)	72.6 ± 5.2 (8)	85.6 ± 3.0 (19)	0.271
DNA integrity (%)	-	99.7 ± 0.1 (16)	-

Table 5. *Mean (± SEM) reproductive parameters grouped by social rank*

Parameter	***Alpha (n)***	***Beta (n)***	***Gamma (n)***	***P-value***
Weight (kg)	29.6 ± 1.03 (12)	30.1 ± 1.2 (10)	28.7 ± 1.1 (14)	0.897
fAM (µg/g)	0.45 ± 0.08 (14)	0.40 ± 0.10 (11)	0.53 ± 0.07 (12)	0.721
fGCM (ng/g)	30.8 ± 2.9 (14)	34.3 ± 5.1 (11)	35.6 ± 2.9 (12)	0.739
Prostate volume (cm^3)	12.5 ± 4.5 (12)	7.1 ± 1.0 (10)	7.3 ± 1.0 (14)	0.035
Testes volume (cm^3)	12.7 ± 11.5 (12)	12.2 ± 1.7 (10)	12.0 ± 1.1 (14)	0.477
Preputial gland size (score 0-3)	2.4 ± 0.2 (12)	1.5 ± 0.3 (10)	1.4 ± 0.3 (4)	0.674
	Alpha (n)	***Subordinate***		
Total motility (%)	40.9 ± 10.0 (10)	37.6 ± 7.9 (18)		0.832
Progressive motility (%)	27.9 ± 8.2 (10)	24.0 ± 6.4 (18)		0.857
Sperm motility index (0-400)	100.3 ± 28.6 (10)	89.8 ± 22.2 (18)		0.827
Motility rating (1-4)	1.9 ± 0.5 (10)	1.7 ± 0.4 (18)		0.611
Total no. ejaculated sperm (x 10^6)	27.6 ± 11.2 (10)	32.7 ± 9.6 (18)		0.890
Normal sperm morphology (%)	53.4 ± 8.5 (10)	45.0 ± 5.5 (18)		0.465
Sperm viability (% alive)	72.0 ± 4.2 (10)	70.7 ± 4.8 (18)		0.914
Sperm acrosome (% intact)	83.2 ± 3.8 (9)	81.0 ± 3.8 (18)		0.753
DNA integrity (%)	99.8 ± 0.13 (3)	99.7 ± 0.1 (13)		0.768

0.606, *df* = 2, *P* = 0.739), or country (χ^2 = 1.250, *df* = 1, *P* = 0.263), but were significantly affected by age with higher concentrations seen in younger animals (χ^2 = 5.837, *df* = 1, *P* = 0.016).

Mean prostate and testes volume both significantly increased from pre-breeding towards breeding season (Table 4; χ^2 = 16.516, *df* = 1, *P* < 0.001 and χ^2 = 68.494, *df* = 1, *P* < 0.001 respectively). Social rank had a significant effect on prostate volume (Table 5; χ^2 = 4.424, *df* = 1, *P* = 0.035) but not on testes volume (Table 5; χ^2 = 0.507, *df* = 1, *P* = 0.477) with larger prostates in dominant animals. Body weight or fAM concentrations did not significantly affect prostate (χ^2 = 3.060, *df* = 1, *P* = 0.080 and χ^2 = 0.150, *df* = 1, *P* = 0.699 respectively) or testes volume (χ^2 = 0.211, *df* = 1, *P* = 0.646 and χ^2 = 0.263, *df* = 1, *P* = 0.608 respectively), however a significant effect of age was seen with older males having larger prostates and testes (χ^2 = 5.072, *df* = 1, *P* = 0.024 and χ^2 = 57.274, *df* = 1, *P* < 0.001 respectively). Preputial gland size was not significantly affected by season (Table 4; χ^2 = 0.468, *df* = 1, *P* = 0.494) or social rank (Table 5; χ^2 = 0.177, *df* = 1, *P* = 0.674), however a strong significant effect between dominance and season was observed (Table 6; χ^2 = 73.462, *df* = 1, χ^2 = 1.250, *df* = 1, *P* < 0.001), as preputial gland size predominantly increased in subdominant animals from pre-breeding to breeding season.

Spermatozoa could be collected from 8 out of 12 dogs (67%) in the pre-breeding season, including all dominant males but only half of the beta and gamma males (Fig. 4a). Urine contamination of the sperm sample during electro ejaculation was a major issue in the pre-breeding season (8 out of 12 dogs, 67%) and tended to be higher in subordinate males (Fig. 4a). Although prostate volume tended to be smaller in males with urine contamination, this difference was not significant (3.9 ± 0.5 *vs.* 6.1 ± 1.3 cm^3; *P* = 0.085). In the breeding season,

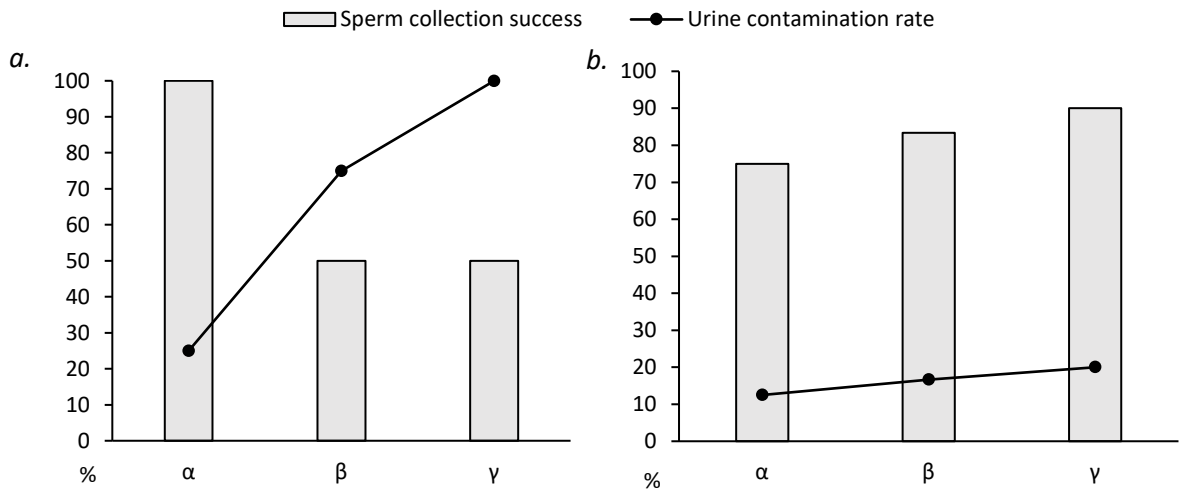


Figure 4. Collection success and rate of urine contamination in sperm samples from African wild dogs grouped by social rank during the (a) pre-breeding and (b) breeding season.

Table 6. *Mean (± SEM) reproductive parameters grouped by social rank and season*

	Pre-breeding season			***Breeding season***			
Parameter	***Alpha (n)***	***Beta (n)***	***Gamma (n)***	***Alpha (n)***	***Beta (n)***	***Gamma (n)***	***P-value***
Weight (kg)	30.1 ± 1.2 (4)	31.2 ± 0.9 (4)	31.2 ± 0.7 (4)	29.3 ± 1.5 (8)	29.3 ± 1.9 (6)	27.7 ± 1.5 (9)	0.815
fAM (µg/g)	0.45 ± 0.12 (4)	0.46 ± 0.16 (4)	0.46 ± 0.05 (4)	0.45 ± 0.10 (10)	0.36 ± 0.13 (7)	0.57 ± 0.11 (8)	-
fGCM (ng/g)	37.3 ± 4.0 (4)	41.5 ± 6.9 (4)	35.6 ± 4.1 (4)	27.8 ± 3.5 (10)	30.2 ± 6.8 (7)	35.6 ± 4.1 (8)	-
Prostate volume (cm^3)	5.6 ± 1.5 (4)	4.6 ± 0.7 (4)	3.7 ± 0.5 (4)	16.0 ± 6.5 (8)	8.9 ± 1.1 (6)	8.7 ± 1.0 (10)	-
Testes volume (cm^3)	12.3 ± 2.3 (4)	8.7 ± 2.1 (4)	9.8 ± 2.4 (4)	13.0 ± 1.4 (8)	14.6 ± 1.9 (6)	12.9 ± 1.1 (10)	-
Preputial gland size (score 0-3)	2.5 ± 0.5 (4)	1.25 ± 0.5 (4)	0.8 ± 0.8 (4)	2.4 ± 0.2 (8)	1.7 ± 0.3 (6)	1.6 ± 0.3 (10)	<0.001
	Alpha (n)	***Subordinate (n)***		***Alpha (n)***	***Subordinate (n)***		
Total motility (%)	32.1 ± 18.4 (4)	2.5 ± 2.5 (4)		46.7 ± 12.3 (6)	47.7 ± 8.4 (14)		0.927
Progressive motility (%)	27.7 ± 16.8 (4)	0.0 ± 0.0 (4)		29.4 ± 10.4 (6)	30.9 ± 7.3 (14)		<0.001
Sperm motility index (0-400)	88.9 ± 52.3 (4)	2.5 ± 2.5 (4)		107.9 ± 36.4 (6)	114.7 ± 24.8 (14)		0.849
Motility rating (1-4)	2.3 ± 1.0 (4)	0.3 ± 0.3 (4)		1.6 ± 0.6 (6)	2.1 ± 0.5 (14)		0.067
Total no. ejaculated sperm (x 10^6)	26.1 ± 20.6 (4)	28.8 ± 13.8 (4)		28.6 ± 14.4 (6)	33.8 ± 12.0 (14)		0.062
Normal sperm morphology (%)	59.8 ± 13.0 (4)	21.4 ± 5.7 (4)		49.1 ± 11.9 (6)	51.7 ± 5.7 (14)		0.123
Sperm viability (% alive)	70.3 ± 4.4 (4)	55.9 ± 8.1 (4)		73.1 ± 6.7 (6)	74.9 ± 5.4 (14)		0.694
Sperm acrosome (% intact)	77.9 ± 7.4 (4)	67.3 ± 7.2 (4)		87.4 ± 3.0 (5)	85.0 ± 4.0 (14)		0.902
DNA integrity (%)	-	-		99.8 ± 0.1 (3)	99.7 ± 0.1 (14)		-

spermatozoa could be collected from 20 out of 24 dogs (83.3%), with more than 75% success from males of all social ranks (Fig. 4b). In addition, urine contamination of the sperm sample only occurred in 4 out of 24 males (17%) at similar levels across the different social ranks.

Overall sperm quality was low in the pre-breeding season and improved in the breeding season, with a significant increase in progressive motility and total number of ejaculated spermatozoa (Table 4; χ^2 = 25.735, *df* = 1, *P* < 0.001 and χ^2 = 53.979, *df* = 1, *P* < 0.001 respectively). Social rank did not directly affect any sperm quality parameters (Table 5). However, urine contamination had a significant effect on several sperm quality parameters; which was most prevalent in subordinate animals during the pre-breeding season. Total (8.1 ± 5.5% *vs.* 53.3 ± 6.33%; χ^2 = 9.822, *df* = 1, *P* = 0.002) and progressive motility (3.6 ± 3.3% *vs.* 35.8 ± 5.82%; χ^2 = 31.359, *df* = 1, *P* < 0.001), sperm motility index (3.6 ± 3.3% *vs.* 130.9 ± 19.7%; χ^2 = 8.130, *df* = 1, *P* = 0.004), normal morphology (25.6 ± 4.15 *vs.* 58.5 ± 4.93; χ^2 = 8.560, *df* = 1, *P* = 0.003) and acrosome integrity (69.9 ± 5.84 *vs.* 86.7 ± 2.43; χ^2 = 4.144, *df* = 1, *P* = 0.042) were lower in all urine contaminated (n = 9) vs. non-contaminated samples (n = 19).

4. Discussion

Our study is the first to investigate the effect of social rank on sperm quality and several other male reproductive parameters in the context of seasonal changes in AWDs. Although dominant animals had larger prostates, no effect of social rank on sperm quality was observed. However, urine contamination tended to be higher in subordinate animals during the pre-breeding season, which in turn negatively affected total and progressive motility, sperm motility index, normal sperm morphology and acrosome integrity. These results imply that subordinate males are not physiologically subfertile in response to social mechanisms of reproductive suppression in this species. Moreover, it suggests that all adult AWD males,

whether dominant or subordinate, are suitable candidates for sperm banking programs when collected during the breeding season.

In some social species such as meerkats, elevated cortisol in subordinate animals is a common mechanism for reproductive suppression of the hypothalamic-pituitary-gonadal axis (Creel 2001; Van den Berghe *et al.* 2012). We did not see differences in fGCM concentrations between dominant and subordinate AWDs, which is consistent with previous studies conducted in semi-natural captivity (de Villiers *et al.* 1997), and the wild (Van der Weyde 2013). Moreover, another study showed free-living dominant AWDs actually have higher stress levels than subordinate animals (Creel *et al.* 1997). This discrepancy could possibly be explained by differences in pack structure, behaviour, or threats faced in the wild (Van der Weyde 2013). Thus, using fGCMs and sperm quality data, our results coupled with previous studies, dismiss the role of the stress hormone cortisol as a putative mechanism for reproductive suppression in AWDs.

Androgen levels, testis size and sperm production are usually positively correlated in mammals (Preston *et al.* 2001; Gomendio *et al.* 2007). Previous research performed in AWDs showed that alpha males have higher androgen concentrations compared to subordinate males in the breeding season (Monfort *et al.* 1997; Johnston *et al.* 2007; Newell-Fugate *et al.* 2012), which could positively influence spermatogenesis and the size and secretory activity of accessory sex glands (Paris *et al.* 2005; Gomendio *et al.* 2007). In addition, another study showed a reduction in overall semen quality within the pack after establishment of a social hierarchy, suggesting dominance could induce subfertility in subordinate AWD males (Johnston *et al.* 2007). Our study however, did not show any differences in fAM concentrations between males of different social rank, which is in agreement with Van der

Weyde (2013). Moreover, this corresponded to testis size and sperm quality that did not differ between social ranks, indicating that the hypothalamic-pituitary-gonadal axis is functioning normally in all pack males. In addition, with the exception of prostate volume, there was no significant difference in any other reproductive or semen quality parameter between males of different social rank. Therefore, we conclude that reproductive suppression must be behaviourally mediated in male AWDs; established by mate guarding of the female. Such behavioural mechanisms of reproductive suppression appear to be limited in their effectiveness since multiple paternity in AWD litters is quite common in the wild, with as little as 50% of pups sired by the alpha male (Spiering *et al.* 2009).

During the pre-breeding season however, we found subordinate males tended to show very high rates of urine contamination of the sperm sample (7 out of 8 males). Urine contamination negatively affected total and progressive motility, sperm motility index, normal sperm morphology and the integrity of the acrosome, similar to that observed in other species (Chen *et al.* 1995; Kim and Kim 1998; Blanco *et al.* 2002; Santos *et al.* 2011; O'Brien *et al.* 2013). This is likely to be related to prostate size, which was affected by social rank and season. When prostate size is smaller, there is a higher risk of bladder stimulation, contraction and urine release during the electroejaculation procedure, since the rectal probe (with 3-4 cm long electrodes) is normally positioned at the level of the prostate proximal to the bladder (Van den Berghe *et al.* 2018a).

Seasonality has been investigated previously in male AWDs, on a quarterly (Newell-Fugate *et al.* 2012) and 6-monthly (Johnston *et al.* 2007) basis. We examined changes in reproductive parameters from 2 months prior to the breeding season, as we also wanted to investigate the effect of social hierarchy from the early stages of reproductive activation through to full

breeding condition. Concentrations of fAM marginally increased during this transition. Some studies showed highest fAM values in December (Southern hemisphere) or from July to September (Northern hemisphere), followed by a gradual decrease towards the non-breeding season (Monfort *et al.* 1997; Newell-Fugate *et al.* 2012), while in others, androgen concentrations did not increase towards the breeding season (Creel *et al.* 1997; Johnston *et al.* 2007). We did not see changes in fGCM concentrations from the pre-breeding to breeding season, similar to Van der Weyde (2013), in which fGCMs were higher around the denning, but not the mating period. This confirms that the period of peak-breeding (mating) itself is not perceived as more stressful than the pre-breeding season. Newell-Fugate *et al.* (2012) reported an increase in prostate and testis volume in the breeding (January to April) compared to non-breeding (August), but not pre-breeding season (November). An increase in prostate and testis size occurred in our study from the pre-breeding to breeding season. This was accompanied by an increase in sperm motility and number, similar to that found by other researchers (Johnston *et al.* 2007; Newell-Fugate *et al.* 2012).

In the breeding season, overall sperm quality was low. Mean progressive motility was just above 30%, with only 32 x 10^6 ejaculated spermatozoa and 51% normal morphology, which is much lower than the 85% progressive motility, 150 x 10^6 ejaculated spermatozoa and 72% normal morphology reported in n=4 AWD males collected at the beginning of the South African breeding season (January; Newell-Fugate *et al.* 2012). However, sperm quality in this study dropped considerably over the breeding season with only 44% progressive motility and 31.4% normal morphology left by the end of the breeding season (April), while the number of sperm ejaculated increased to 301 x 10^6 spermatozoa. Our sperm motility and normal morphology results were also lower than previously reported by Johnston *et al.* (2007), in

which high sperm quality was observed during the first breeding season, but this declined considerably during the second breeding season, coupled with a decline is testis volume and semen collection success. It was argued that since the hierarchy of this pack was established between the first and second breeding season, the decline in sperm quality was related to physiological suppression of subordinate reproduction. Our study however found no evidence of physiological suppression of reproduction in subordinates during the breeding season. Thus, it is unclear what caused the differences in sperm quality between the different studies. The electroejaculation protocol differed between each study, which has been shown in humans to result in semen of lower quality than found in natural ejaculates (Restelli *et al.* 2009). In domestic dogs however, apart from lower sperm count, no differences in semen quality could be seen after collection using digital manipulation or electroejaculation (Ohl *et al.* 1994; Christensen *et al.* 2011).

Concentrations of fGCM were higher in younger compared to older adult males. Sub-adults (to approximately 2 years) have been reported to have higher circulating glucocorticoid concentrations compared to adults, presumably caused by a more active dominance style exerted by younger animals compared to more passive dominance in older males with more experience (de Villiers *et al.* 1997). However, in our study, 3 out of 5 younger males in the US did not exert any dominant behaviour and none were alpha males, so it is unlikely that active dominance in these males caused their higher stress levels. Alternatively, it's possible that their higher fGCM levels were induced by a combination of hierarchy establishing behaviours, including receiving dominance and exerting submissive behaviours, particularly in captivity.

In our study, the breeding season was defined as the time mating occurs. In captive AWDs in the northern hemisphere, this is around August/September (late summer), with most births

occurring in November (Van den Berghe *et al.* 2012). Therefore, AWDs in the US were collected in August and September for the breeding season evaluation. In the Southern hemisphere, the major breeding season is 6 months earlier (February/March), with the majority of successful matings seen around February. Newell-Fugate *et al.* (2012) reported the best male reproductive and sperm quality parameters in South Africa from January to April; 3-5 months after the initial testosterone rise. Our PLA and SAN pack males from Namibia were observed to mate in January and March respectively when we collected samples. By contrast, the BRU pack male was observed to mate the alpha female yearly around October-November; confirmed by the birth of pups in January 2014. It is unclear why this pack showed a consistently altered breeding season, but consequently, we performed the breeding season evaluation of males in this pack during mid-November.

In conclusion, subordinate AWD males do not appear to be subfertile due to physiological suppression, since their sperm is of similar quality to dominant males in the pack. Thus, during the breeding season when sperm quality is best, males of all social ranks can be considered suitable candidates for sperm banking programs. However, additional research is needed to improve the reliability and quality of sperm collection (e.g. chemical ejaculation protocols) and other possible factors affecting sperm quality (e.g. timing, nutrition, presence of females, latitude).

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Conflict of interest

The authors declare no conflict of interest

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