A Conservation Management Toolkit: Developing Assisted Breeding and Behavioural Management Tools for the African Wild Dog (Lycaon pictus)

Thesis submitted by

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DECLARATIONS

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Femke Van den Berghe

03 March 2018

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ABSTRACT

The African wild dog (AWD; *Lycaon pictus*) is endangered with the current population estimated at 6,600 animals, scattered over several subpopulations in Southern and Eastern Africa. They show a complex social structure including a separate male and female hierarchy and a cooperative breeding system where subdominants usually do not breed but help in raising the pups. To maintain a viable captive population and genetic diversity, animals are often translocated between institutions to form a new breeding pack. Similarly, a metapopulation management plan has been introduced in South Africa, involving the reintroduction of AWDs in small protected areas and regular translocations of individuals between subpopulations. However, due to their complex social structure, new pack formations can often lead to aggression between animals resulting in injury or even mortality.

Sperm freezing, and development of artificial insemination (AI) techniques, can aid species management and conservation of the AWD. The use of semen cryopreservation and AI could overcome problems of intra-pack aggression associated with new pack formations by supplementing genetic diversity without disrupting existing pack structure; and thereby facilitating captive breeding and metapopulation management. In addition, transporting spermatozoa instead of live animals reduces the risk of disease transmission and has ecological and economic benefits. Sperm from free-roaming males could be used to increase genetic diversity in captivity, avoiding the removal of animals from the wild. Lastly, establishing a sperm bank of genetically valuable animals will provide a genetic back-up of the remaining population, providing a buffer against possible threats. Therefore, the aim of this thesis was to develop assisted breeding and behavioural management techniques to enable the application of AI in this species, through the following objectives*: (i) determine the effect of social rank on subordinate male fertility (Chapter 2); (ii) develop a sperm freezing protocol (Chapter 3); (iii) determine if Dog Appeasing Pheromone (DAP) can reduce stress and aggression associated with temporary pack separation (Chapter 4); and (iv) validate the use of behaviour and faecal steroid hormone profiles as a non-invasive way to time the fertile period in AWD females for timed AI (Chapter 5).*

The study included n=15 males from 5 packs housed in 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) and n=13 males and n=3 females from 3 packs housed at Harnas Wildlife Foundation, Gobabis, Namibia (BRU, Brutus pack; PLA, Platform pack; SAN, San pack). Males were immobilised during the pre-breeding season (n=12; ABQ, BRK, BIN, TOP) and breeding season (n=24; ABQ, BRK, BIN, OKC, BRU, PLA, SAN) and male fertility parameters including hormones (faecal androgen - fAM and glucocorticoid metabolite - fGCM concentrations), prostate and testes volume, preputial gland size, semen collection success, and multiple measures of sperm quality were recorded *(objective 1)*. Sperm samples of sufficient quality collected in the breeding season were split and frozen using 2 canine freezing protocols: Protocol 1: a one-step dilution in TRIS-20% egg yolk containing 8% glycerol; and Protocol 2: a two-step dilution in TRIS-20% egg yolk containing a final extender concentration of 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution *(objective 2)*. In addition, males from US packs were treated topically either with DAP (n=11; 4 packs) or placebo solution (n=12, 4 packs), applied at the end of anaesthesia prior to reintroduction into the pack *(objective 3)*. Behavioural interactions as well as fGCM and fAM were examined from 3 days before *(objective 1)* until 4-6 days after immobilisation *(objective 3)*. Moreover, behavioural interactions, faecal progesterone (fPM) and estrogen (fEM) metabolite concentrations were examined for n=3 alpha females during their periovulatory period. Furthermore, each female was immobilised on 2 or 3 occasions at this time to evaluate vulvar size, and serum progesterone and oestrogen levels as well as perform vaginal cytology, vaginoscopy, and ovarian ultrasound *(objective 4)*.

Semen could be collected successfully from all alpha males but from only half the subordinate males in the pre-breeding season, with higher urine contamination in lower ranking animals. Fertility parameters did not differ between social ranks, except for a lower sperm progressive motility and normal morphology in subordinates. In the breeding season, preputial gland size increased with social rank, but no difference between ranks was observed in any other parameter, including sperm quality *(objective 1)*. Eight ejaculates of sufficient quality were frozen in the breeding season. Sperm motility persisted for up to 8h after thawing for Protocol 2, while it dropped to nearly 0% after 2h incubation for Protocol 1. In addition, viability and acrosome integrity of spermatozoa were higher for Protocol 2 throughout post-thaw incubation *(objective 2)*. The application of DAP to males at the end of anaesthesia and prior

to reintroduction, did not alter the rise in fGCM levels after intervention. However, fAM increased in placebo but was prevented in DAP treated animals. On the day of reintroduction, DAP treated packs tended to show lower rates of contact-dominance and active-submission behaviour, but higher rates of non-contact dominance behaviour *(objective 3)*. In females, late oestrus (fertile period) could be clearly distinguished from early oestrus by an increase in frequency of certain types of behavioural interactions between the alpha male and female (affiliative behaviour, sexual follow, male initiating behaviour, ride-up, and copulation). One female exhibited an anovulatory cycle while the remaining 2 showed a significant 2.5- to 3-fold increase in fPM levels and similar declining fEM levels (after a rise in pro-oestrus) compared to baseline. However, vaginal cytology and vaginoscopy results deviated from patterns seen in domestic dogs, and blood progesterone showed lower levels. Moreover, ovarian structures were difficult to visualise by ultrasound. As such, without frequent sampling, these invasive diagnostic techniques are unsuitable to determine the fertile phase in wild canids such as the African wild dog *(objective 4)*.

In conclusion, reproductive suppression of subordinate males appears to be behaviourally mediated, given that AWD males of all social ranks produce semen of similar quality during the breeding season, making them suitable candidates for sperm banking *(objective 1)*. In addition, a two-step dilution in TRIS-egg yolk-glycerol extender containing Equex STM yields significantly improved post-thaw quality and longevity of AWD spermatozoa, making it appropriate for sperm banking and artificial insemination initiatives *(objective 2)*. The observed effects of DAP on AWD hormones and behaviour could decrease the risk of agonistic interactions, making it a useful tool to help manage temporary pack separation, needed when performing semen freezing and AI *(objective 3)*. Lastly, distinctive behaviours during late oestrus, together with an increase in faecal progesterone and decline in faecal oestrogen in AWD females, could potentially be used to determine the timing of the fertile period *(objective 4)*. These results combined facilitate sperm banking and the application of AI in the African wild dog, thereby assisting management and conservation of the species.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii
STATEMENT OF CONTRIBUTIONS OF OTHERS vii
ABSTRACT ix
TABLE OF CONTENTS xiii
LIST OF FIGURES xvi
LIST OF TABLES xviii
LIST OF ABBREVIATIONS xix
PREFACE xxi
PUBLICATIONS ARISING FROM THIS THESIS xxii
Chapter 1: General introduction 1
Abstract 3
1. The African wild dog – General information 3
1.1. Species description, distribution and status 3
1.2. Social structure 4
1.3. Threats 5
1.4. Conservation efforts 6
2. The role of Assisted Reproductive Technology (ART) in wildlife conservation 8
2.1. AI in wild carnivores 9
2.2. AI in wild canids 10
2.3. AI in the AWD 11
3. Question 1: Reproductive suppression in AWD males 13
4. Question 2: Sperm Cryopreservation: Towards the development of an AWD sperm bank 15
4.1. Semen collection methods 15
4.2. Semen evaluation 16
4.3. Semen freezing 18
4.3.1. Cold shock and sperm membrane lipids 18
4.3.2. Cryoinjury 19
4.3.3. Canine semen freezing 20
5. Question 3: Dog appeasing pheromone: A tool for behavioural management? 26
6. Question 4: Timing of artificial insemination in the AWD. 28
6.1. Reproductive cycle of the domestic dog 28

6.2. Current knowledge in female AWD reproduction 30
6.3. Timing of AI in domestic dogs 31
6.3.1. Analysis of blood hormones 31
6.3.2. Vaginal cytology 32
6.3.3. Vaginal endoscopy 34
6.3.4. Transabdominal ovarian ultrasound 34
6.3.5. Alternative approaches 35
7. Aims of the thesis 37
Chapter 2: The effect of social rank on male fertility 39
Abstract 41
1. Introduction 42
2. Materials and methods 43
2.1. Animals and husbandry 43
2.2. Classification of social hierarchy 45
2.3. Faecal sample collection and steroid hormone analysis 47
2.4. Immobilisation 47
2.5. Physical and reproductive examination 48
2.6. Semen collection and evaluation 49
2.7. Statistical analysis 50
3. Results 52
3.1. Classification of hierarchy 52
3.2. Effect of social rank on male fertility in the pre-breeding season 54
3.3. Effect of season on male fertility 57
3.4. Effect of social rank on male fertility in the breeding season 58
4. Discussion 60
Chapter 3: Improved sperm freezing in the African wild dog 65
Abstract 67
1. Introduction 68
2. Materials and methods 71
2.1. Animals 71
2.2. Anaesthesia 71
2.3. Semen collection 73
2.4. Semen evaluation 73
2.5. Sperm freezing 75

2.6. Statistical analysis 76
3. Results 77
4. Discussion 82
Chapter 4: Dog appeasing pheromones to manage temporary pack separation 87
Abstract 89
1. Introduction 90
2. Materials and methods 93
2.1. Experimental design, animal capture and release 93
2.2. Faecal sample collection 95
2.3. Faecal steroid extraction and analysis 96
2.4. Effect of time and temperature on steroid metabolite concentration post-defecation 97
2.5. Behavioural observations 97
2.6. Statistical analysis 98
3. Results 103
3.1. EIA validation and steroid degradation experiment 103
3.2. fGCM and fAM hormone concentrations 105
3.3. Behaviour 107
4. Discussion 112
Chapter 5: Timing of artificial insemination 117
Abstract 119
1. Introduction 121
2. Materials and methods 124
2.1. Animals, husbandry and behavioural observations 124
2.2. Faecal sample collection and steroid hormone analysis 126
2.3. Immobilisation and sample collection 127
2.4. Statistical analysis 130
3. Results 131
3.1. Behaviour 131
3.2. Faecal steroid hormone metabolites 136
3.3. Other reproductive parameters 139
4. Discussion 145
Chapter 6: General discussion 151
REFERENCES 159

LIST OF FIGURES

Figure 1.1. African wild dog (*Lycaon pictus*). 3

Figure 1.2. Schematic overview of questions to be answered to enable the application of sperm banking and AI in the African wild dog 12

Figure 1.3. Phases and corresponding hormone profiles during the oestrus cycle of the domestic dog. E2, oestrogen; LH, luteinising hormone; P4, progesterone. 29

Figure 1.4. Vaginal cytology of a domestic dog. 33

Figure 1.5. Vaginal endoscopy of a domestic dog during early pro-oestrus, pro-oestrus, oestrus (angulation) and early dioestrus (metoestrus). 34

Figure 2.1. 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. 45

Figure 2.2. Classification of African wild dog preputial gland development. 49

Figure 2.3. Classification of social hierarchy based on the frequency of dominant (red arrows), submissive and scent marking 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. 53

Figure 2.4. Prostate and testes volume, and fAM and fGCM concentrations in African wild dogs grouped by social rank during the (a) pre-breeding and (b) breeding season.. 56

Figure 2.5. 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. 56

Figure 2.6. Seasonal changes in prostate and testis volume in African wild dogs combined and grouped by social rank (paired data). 58

Figure 3.1. (a) Acrosome intact and incomplete African wild dog sperm showing evidence of damaged membranes stained with PSA-FITC; (b) DNA fragmented and intact AWD sperm evaluated by TUNEL assay. 75

Figure 3.2. Post-thaw (a) motility, (b) sperm motility index, (c) viability, (d) acrosome integrity and (e) morphology of African wild dog spermatozoa over a period of 6-8h after cryopreservation using Protocols 1 and 2. 81

Figure 4.1. Example of faecal sample and behavioural data collection from each African wild dog pack. 96

Figure 4.2. Social behaviours of captive African wild dogs. 100

Figure 4.3. (a) Relative fGCM and (b) fAM profiles from n=2 African wild dog females injected with ACTH; (c) Mean (± SEM) relative hormone profiles of fGCM and (d) fAM concentrations measured over 72h after storage of fecal samples at refrigerated or ambient temperatures. 104

Figure 4.4. Mean (± SEM) relative hormone profiles before (pre-SIR) and after (SIR, SIR+1, SIR≥2) separation, immobilisation and reintroduction for (a) fGCM and fAM in non-immobilised animals; (b) fGCM and (c) fAM in n=11 DAP or n=12 placebo treated males. 106

Figure 4.5. Mean (± SEM) relative hourly rates of (a) total, (b) contact and (c) non-contact dominance behaviour, and (d) marking in DAP and placebo treated packs throughout the observation period. 109

Figure 4.6. Mean (± SEM) relative hourly rates of (a) total, (b) passive and (c) active submissive behaviour in DAP and placebo treated packs throughout the observation period 110

Figure 4.7. Mean (± SEM) relative hourly rates of (a) affiliative and (b) aggressive behaviour in DAP and placebo treated packs throughout the observation period. 111

Figure 5.1. Measurement of vulva thickness (a) and height (b) using callipers in female African wild dogs. 128

Figure 5.2. Classification of African wild dog vaginal epithelial cells stained with Diff-quick or Harris-Shorr. 129

Figure 5.3. (a, b) Hourly rates of sexual behaviour and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of oestrus for the alpha African wild dog female BRU F1 ♀. 133

Figure 5.4. (a, b) Hourly rates of sexual behaviour and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of oestrus for the alpha African wild dog female PLA F1 ♀. 134

Figure 5.5. (a, b) Hourly rates of sexual behaviour and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of oestrus for the alpha African wild dog female SAN F1 ♀. 135

Figure 5.6. Faecal progesterone, oestrogen and glucocorticoid metabolite concentrations relative to the onset of oestrus for the alpha African wild dog female (a) BRU F1 ♀, (b) PLA F1 ♀, and (c) SAN F1 ♀. 138

Figure 5.7. Swollen vulva and vestibule of PLA F1 ♀. 140

Figure 5.8. Vaginal cytology in African wild dog females during (a,b) anoestrus (Pla F2 ♀); (c, d) late pro-oestrus (Bru F1 ♀ day -1); (e,f) early oestrus (Bru F1 ♀ day +6); (g,h) late oestrus (Bru F1 ♀ day +9); (i,j) late oestrus (last day oestrus, San F1 ♀ day +4); (k,l) early dioestrus (San F1 ♀ day +8)..... 144

Figure 5.9. Ultrasound image of the right ovary of the African wild dog alpha female SAN F1 ♀ during late oestrus, showing an anechogenic structure 5 mm in diameter. 144

LIST OF TABLES

Table 1.1. Classification of the different Families within the Order Carnivora, the total number of species within the Family, and their classification according to the IUCN Red List of Threatened Species. 8

Table 1.2. Important progesterone concentrations during the peri-ovulatory period in the domestic dog bitch. 32

Table 2.1. Pack composition, social rank, observation and sampling times of African wild dogs included in this study. 46

Table 2.2. Classification of social hierarchy based on the frequency of dominant and submissive behaviour in the PLA pack of Namibia during the breeding season. 54

Table 2.3. Mean (± SEM) sperm quality between dominant and subordinate African wild dogs during the pre-breeding and breeding season 55

Table 2.4. Seasonal changes in mean (± SEM) reproductive parameters and sperm quality in African wild dogs (paired data). 57

Table 3.1. Animal details and semen quality of African wild dogs included in this study. 72

Table 3.2. Sperm quality (mean ± SEM) in fresh and thawed African wild dog semen frozen using Protocol 1 and 2 for good quality samples and poor quality samples (characterized by a rapid decrease in motility just prior to freezing). 78

Table 4.1. Pack composition, treatment schedule and mean daily observation times (h) per pack in the pre-breeding and breeding season 93

Table 4.2. African wild dog ethogram showing social interactions used for behavioural analysis. 101

Table 5.1. Pack composition, age, observation and analysis time, and reproductive history of the n=3 alpha females sampled in this study. 124

Table 5.2. African wild dog ethogram characterising sexual behaviour used in behavioural analysis. 126

Table 5.3. Mean (± SEM) hourly rates of sexual and non-sexual behaviour and fractional resting patterns in n=3 African wild dog alpha females during pro-oestrus, early oestrus, late oestrus and dioestrus. 136

Table 5.4. Mean ± SEM faecal progesterone, oestrogen and glucocorticoid metabolite concentrations in n=3 African wild dog alpha females during pro-oestrus, early oestrus, late oestrus and early dioestrus. 139

Table 5.5. Other reproductive parameters in female African wild dogs during different phases of the reproductive cycle 142

LIST OF ABBREVIATIONS

ABQ	Albuquerque BioPark
AI	Artificial insemination
ANOVA	Analysis of variance
ART	Assisted reproductive technologies
AWD	African wild dog
BIN	Binder Park zoo
BRK	Brookfield zoo
BRU	Brutus (pack)
BSA	Bovine serum albumin
CASA	Computer-assisted sperm analysis
CDV	Canine distemper virus
CL	Corpus luteum
CV	Coefficients of variation
DAP	Dog appeasing pheromone
DHA	Docosahexaeonic acid
DPA	Docosapentaenoic acid
DW	Dry faecal weight
EEJ	Electroejaculation
EIA	Enzyme-immunoassay
ELISA	Enzyme-linked immunosorbent assay
ET	Embryo transfer
EY	Egg yolk
fAM	Faecal androgen metabolite
fEM	Faecal oestrogen metabolite
fGCM	Faecal glucocorticoid metabolite
FITC	Fluorescein isothiocyanate
fPM	Faecal progesterone metabolite
H-P-A	Hypothalamic-pituitary-adrenal
H-P-G	Hypothalamic-pituitary-gonadal
IACUC	Institutional animal care and use committee
ICSI	Intra-cytoplasmic sperm injection
IUCN	International Union for Conservation of Nature
IVF	*In vitro* fertilization
LDL	Low-density lipoprotein

LH	Luteinising hormone
LSD	Least significant difference
OKC	Oklahoma City zoo
PLA	Platform (pack)
PNA	Peanut agglutinin
PSA	Pisum sativum agglutinin
PUFA	Polyunsaturated fatty acid
RIA	Radioimmunoassay
ROS	Reactive oxygen species
SAN	San (pack)
SCSA	Sperm chromatin structure assay
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SIR	Separation, immobilisation and reintroduction
TOP	Topeka zoo
TUNEL	Terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling

PREFACE

This thesis consists of six chapters, four of which (Chapter 2, 3, 4 and 5) have been written as stand-alone publications prior to incorporation in the thesis. As such, there might be some overlap in this manuscript. These chapters are currently published, submitted or in preparation for publication in peer-reviewed journals and have been presented at international scientific conferences (see publications arising from this thesis).

PUBLICATIONS ARISING FROM THIS THESIS

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Chapter 1

General introduction



Abstract

The African wild dog (AWD; *Lycaon pictus*) is an endangered canid with, as of 2012, less than 6,600 animals remaining in the wild. Despite different strategies undertaken to preserve the species, numbers of free-living animals are still in decline. Artificial insemination (AI) and semen banking techniques could not only overcome some of the current problems encountered in captive breeding programs but also help sustain the genetic diversity of the current free-living population. This introductory chapter provides a general background to the species and discusses the benefits of AI in wildlife conservation, with emphasis on the AWD. Four important questions are proposed that need to be answered before sperm banking and AI techniques can be applied; leading to the objectives of this thesis.

1. The African wild dog – General information

1.1. Species description, distribution and status

The African wild dog (AWD; *Lycaon pictus*), also known as the painted hunting dog or Cape hunting dog, is an African carnivore belonging to the Canidae. It has an unusual appearance, characterised by large ears, long legs, and a distinctive black, brown and white coat pattern that is unique to each individual (Fig. 1.1.). Height and weight are comparable with that of a medium-sized domestic dog (75-110 cm and 18-36 kg respectively).



Figure 1.1. *African wild dog (Lycaon pictus).*

The Canidae consists of about 36 different species including wolf-like, jackal-like and fox-like carnivores (Wayne *et al.* 1997). The AWD is quite taxonomically distinct (the only species in its genus *Lycaon*) and relatively distantly related to the other canids. Its position within this

Family is not precisely known. Depending on the criteria used for classification, the AWD is either most closely related to two South American canids: the bush dog (*Speothos venaticus*) and maned wolf (*Chrysocyon brachyuris*); or to the group of wolf-like carnivores: including wolves, coyotes, jackals (*Canis spp.*) and the Dhole (*Cuon alpinos*; Wayne *et al.* 1997).

The AWD used to be distributed over a wide range in Africa, covering most regions south of the Sahara (Woodroffe & Sillero-Zubiri 2012). The current population is greatly reduced with the largest populations remaining in Southern Africa and the southern regions of East Africa (Woodroffe & Sillero-Zubiri 2012). In the wild, numbers are estimated at 6,600 individuals of which only 1,400 are mature (Woodroffe & Sillero-Zubiri 2012). AWDs have very large home ranges of up to 2,500 km^2 (Woodroffe & Ginsberg 1997) and low population densities (Creel & Creel 1998), making them difficult to observe in the wild. Current population numbers continue to decline making the AWD endangered on the IUCN red list of threatened species (Woodroffe & Sillero-Zubiri 2012).

1.2. Social structure

As a highly social species, AWDs live in permanent packs of most commonly 5-15 adults and yearlings (Creel & Creel 2002). They show a complex social structure consisting of a separate male and female hierarchy and a cooperative breeding system (Creel & Creel 2002). Typically, only the dominant male and female reproduce, and subdominant animals are reproductively suppressed but help to rear the pups. Due to this complex social system, there is a positive relationship between pack size and successful breeding, hunting and survival (Courchamp & Macdonald 2001, Buettner *et al.* 2006), with a critical pack-size of at least 5 adult animals being necessary for survival of the pack, thereby enabling pup-guarding while still having sufficient numbers to hunt efficiently (Courchamp *et al.* 2002). Young adult animals disperse usually within a single-sex group at the age of 28.1 months in males and 21.8 months in females (McNutt 1996). A new pack is formed when two such opposite-sex groups join to develop a new stable reproductive unit. The success of these newly-formed groups depends on different factors such as group size, mate choice and competition (McCreery & Robbins 2001).

In captivity, this complex social system introduces considerable challenges to effective breeding management. To maximise genetic diversity, juvenile or adult animals often need to be translocated between zoological institutions to form a new breeding pair or pack. These translocations and introductions are very stressful to the animals and very challenging for the people involved. Introductions often induce aggression between animals that can result in serious injury or mortality (Scheepers & Venzke 1995, Foster 2014, Quick 2014). Thus, new strategies to manage behaviour, stress and aggression are urgently needed in this species in order to facilitate their captive management.

1.3. Threats

Free-living AWDs face numerous challenges. The most significant threats are habitat fragmentation and loss, anthropogenic mortality (e.g. persecution and road accidents), infectious diseases, and intra- and interspecies competition (mostly with lions, *Panthera leo*, and hyenas, *Crocuta crocuta*; Creel & Creel 1998, Vucetich & Creel 1999, Woodroffe *et al.* 2007, Groom *et al.* 2017).

Habitat fragmentation greatly affects the viability of AWD populations, particularly as they have large home ranges and low population densities (Fanshawe *et al.* 1991). Firstly, the chances for successful dispersal decrease, leading to population declines, subdivision and genetic isolation (Marsden *et al.* 2012). Secondly, there is a higher probability of encountering humans, thereby increasing the risk of persecution, road accidents and accidental snaring. AWDs have been persecuted on a large scale in the past, even within national parks until the nineteen seventies, as they had an image of being 'vermin' and 'cruel murderers' (Fanshawe *et al.* 1991, Woodroffe & Ginsberg 1997). Thankfully such targeted persecution is declining but anthropogenic mortality is still considerable, especially outside protected areas (Woodroffe *et al.* 2007) where they are viewed as a pest by farmers and regularly persecuted for predating livestock (Woodroffe & Ginsberg 1997, Fraser-Celin *et al.* 2017). Although livestock losses to carnivores may be important in some areas (Holmern *et al.* 2007, Gusset *et al.* 2009), AWDs generally prefer wild prey (Woodroffe *et al.* 2005). The percentage of predation reports caused by AWDs is low and not always evidence-based, making the actual economic loss caused by this species minimal in most areas (Rasmussen 1999, Gusset *et al.* 2009).

AWDs are susceptible to disease, and epizootic outbreaks have led to drastic population declines in the past (Woodroffe & Ginsberg 1997). The expanding human population and habitat fragmentation also implies an increased contact with domestic dogs and their diseases (Fanshawe *et al.* 1991, Flacke *et al.* 2013). During the late eighties in the Masai Mara and Serengeti National Parks bordering Kenya and Tanzania, a rabies outbreak resulted in the disappearance of 8 entire AWD study packs (Kat *et al.* 1996, Woodroffe & Ginsberg 1997, Cleaveland *et al.* 2007). Recent genetic profiling suggests however that some AWDs from the original population have managed to survive (Marsden *et al.* 2012). Rabies outbreaks resulting in the disappearance of AWD packs have also been reported in Namibia and South Africa (Scheepers & Venzke 1995, Hofmeyr *et al.* 2004). Moreover, this species is also susceptible to Canine Distemper Virus (CDV; Flacke *et al.* 2013), as demonstrated by an outbreak of CDV in captivity in 2000, which resulted in the death of 49 out of 52 animals within two months (van de Bildt *et al.* 2002). Furthermore, in the early nineties, CDV caused the disappearance of a free-living AWD pack in Botswana and potentially several packs in Kenya (Alexander & Appel 1994, Alexander *et al.* 1996).

1.4. Conservation efforts

Different programs are currently in place to aid the conservation of the AWD. The IUCN Species Survival Commission has established different regional conservation strategies (IUCN/SSC 2007a, 2007b). These strategic plans have been developed through the cooperation of representatives from the concerned countries and outline specific objectives and clear targets to meet these objectives. Aims include (i) the promotion of the coexistence of AWDs with people and domestic animals (e.g. through awareness programs, reducing the impact on livestock, and canid disease management); (ii) developing capacities for conservation (e.g. through the development of law enforcement); (iii) improving knowledge in the conservation biology of the species; and (iv) minimizing the negative effects of land development by encouraging land-use planning. From these regional plans, different countries have developed, or are currently developing, specific national action plans (e.g. South Africa; Lindsey & Davies-Mostert 2009).

Two very interesting and important conservation strategies currently in practice in South Africa include reintroduction of captive bred animals and metapopulation management (Davies-Mostert *et al.* 2009). In reality, the only area large enough to maintain a viable population of AWDs in South Africa is Kruger National Park. Thus, the idea of reintroduction and metapopulation management is to release animals in smaller protected areas and manage them through regular translocations. These translocations are designed to mimic the natural dispersal pattern of the species and sustain a single population composed of different isolated subpopulations (Davies-Mostert *et al.* 2009, Davies-Mostert *et al.* 2015). Initial reintroduction attempts date from 1975, but these early trials were unsuccessful for many years primarily because captive bred dogs didn't have the necessary skills to survive in the wild (Scheepers & Venzke 1995, Woodroffe & Ginsberg 1999). Subsequent reintroductions had greater success when wild-caught AWDs were released together with the captive bred animals (Woodroffe & Ginsberg 1999). Ultimately, the management of this metapopulation has proven successful, although labour intensive (Davies-Mostert *et al.* 2009, Davies-Mostert *et al.* 2015). In 2009, the metapopulation in South Africa comprised of 14 packs with more than one hundred individuals across different reserves; which is about 30% of the South African AWD population (Lindsey & Davies-Mostert 2009). Unfortunately, despite such conservation efforts, numbers of free-living animals are still declining. Additional conservation strategies are urgently needed to help sustain and increase current population numbers and genetic diversity.

2. The role of Assisted Reproductive Technology (ART) in wildlife conservation

The decrease of wildlife populations is a global problem. According to the IUCN Red List of threatened species, only 175 of the 295 carnivore species are listed as 'least concern', 6 species are already extinct, and 113 are classified between 'near threatened' to 'critically endangered' (Table 1.1). Within this grouping, the AWD specifically is classified as endangered.

Table 1.1. *Classification of the different Families within the Order Carnivora, the total number of species within the Family (Total), and their classification according to the IUCN Red List of Threatened Species (IUCN 2017). LC, Least Concern; NT, Near Threatened; VU, Vulnerable; EN, Endangered; CR, Critically Endangered; EW, Extinct in the Wild; E, Extinct; DD, Data Deficient.*

FAMILY	***Total***	***LC***	***NT***	***VU***	***EN***	***CR***	***EW***	***E***	***DD***
Ailuridae	1				1				
Canidae	37	25	5		4	1		2	
Eupleridae	10	1		5	3			1	
Felidae	38	13	7	13	5				
Herpestidae	35	29	3	2					1
Hyaenidae	4	2	2						
Mephitidae	12	10		2					
Mustelidae	64	39	7	6	7	1		1	3
Nandiniidae	1	1							
Odobenidae	1			1					
Otariidae	16	9	1	1	4			1	
Phocidae	19	14		1	3			1	
Prionodontidae	2	2							
Procyonidae	14	10	2		1	1			
Ursidae	8	2		6					
Viverridae	33	18	3	6	3	1			2
Totals	**295**	175	30	43	30	4		6	6

The primary cause for this decline of biodiversity and ultimately species extinction is the loss of habitat (Gonzalez-Suarez & Revilla 2014, Martinez-Ramos *et al.* 2016, McCormick & Allan 2017). To ensure the survival of many species and to maintain current levels of genetic diversity, captive breeding has become extremely important. However, traditional captive breeding programs are often limited by for example inappropriate animal behaviour in captivity and space limitation (Lasley *et al.* 1994). The development and use of Assisted Reproductive Technologies (ART) can be an important aid to overcome some of the limitations inherent in the captive breeding of non-domestic animals (Lasley *et al.* 1994). ART

includes a series of different tools such as gamete (sperm and oocyte) freezing, artificial insemination (AI), *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI), embryo transfer (ET), and ovarian tissue grafting. AI and semen freezing are considered the most rudimentary and least invasive techniques and are already well-established tools in the breeding management of domestic animals (Durrant 2009, Mastromonaco *et al.* 2011). Gradually, these techniques have been applied into the captive breeding programs of a wide range of wildlife species (Paris & Mastromonaco 2009).

2.1. AI in wild carnivores

Currently there are only an estimated 2,060 giant pandas (*Ailuropoda melanoleuca)* left in the wild (Swaisgood *et al.* 2016). In this species, natural breeding alone cannot support a self-sustaining captive population due to poor fecundity, predominantly caused by male aggression and motivational deficits (Swaisgood *et al.* 2000, Olson *et al.* 2003, Zhang *et al.* 2004). AI using fresh and frozen semen has substantially improved captive breeding success (Olson *et al.* 2003, Zhang *et al.* 2004).

Another species with a poor record of captive breeding is the African cheetah (*Acinonyx jubatus*). Only 25% of captive females in North America ever reproduce, likely to be related to a suboptimal environment (Comizzoli *et al.* 2009, Howard & Wildt 2009). Non-domestic species generally require anaesthesia to undertake semen collection, cryopreservation and AI. The cheetah however, is less aggressive than most wild species and can in some circumstances be trained for semen collection (Durrant 2009). Consequently, it is one of the most thoroughly studied wild felids in reproduction (Comizzoli *et al.* 2009). Like many other wild felids examined, teratospermia (<60% morphologically normal spermatozoa) is common in the cheetah (Goodrowe *et al.* 2000, Howard & Wildt 2009). Evidence suggests that this problem is directly related to low genetic diversity and inbreeding (Goodrowe *et al.* 2000, Howard & Wildt 2009). As a partial solution to this problem, a cheetah genome resource bank has already been established, also containing semen from free-roaming males. Coupled with targeted AI, this can offer a strategy to increase the genetic heterozygosity of captive cheetah populations without the need to remove males from their natural habitat (Comizzoli *et al.* 2009). In fact, AI has already been conducted successfully in North-American captive cheetahs using frozen-thawed semen of wild males (Howard & Wildt 2009).

Black-footed ferrets are probably the best example in which AI has made an invaluable contribution to the preservation of a species (Howard and Wildt, 2009). In the mid-eighties, only 18 animals remained in the wild. These individuals were captured, and a massive captive breeding and recovery program was established. By 2005, over 139 kits where born with the help of AI, while semen collected from males that died in the 1990s is still being used to maximise genetic diversity (Comizzoli *et al.* 2009, Howard & Wildt 2009). Following the re-introduction of captive bred black-footed ferrets, there are currently approximately 1000 animals surviving in the wild (Howard & Wildt 2009).

2.2. AI in wild canids

Apart from the domestic dog and some species of foxes, AI in canids is still poorly developed or largely missing for most species. In foxes, particularly the red and blue fox, AI with both fresh and frozen semen is a commonly used technique in the fur industry in Northern Europe, where thousands of vixens are artificially inseminated each year (Thomassen & Farstad 2009). In terms of wild canids, several wolf species are threatened by inbreeding and human interference (Thomassen & Farstad 2009). AI can permit the dispersal of genetics without breaking up their complex monogamous social structure (Asa & Valdespino 1998, Zindl *et al.* 2006). Despite the major advantages AI could have on breeding management of captive wolves, published case reports or research publications in this area are limited. Two publications describe the birth of live puppies after AI in generic grey wolves, one using frozen semen (Seager *et al.* 1975) and one using fresh semen after oestrus induction of the female (Asa *et al.* 2006). In addition, 3 hybrid pregnancies could be achieved after AI in western coyotes (*C. latrans*) using sperm from grey wolves (Mech *et al.* 2014). In Mexican grey wolves (*C. lupus baileyi*), semen has been cryopreserved (Zindl *et al.* 2006) but until now, there are only reports of AI in 3 females using fresh semen (Thomassen & Farstad 2009). The red wolf (*C. rufus*) was once considered extinct in the wild and captive breeding must be carefully managed to sustain current levels of genetic diversity (Goodrowe *et al.* 1998). Several publications describe semen freezing in this species (Goodrowe *et al.* 1998, Lockyear *et al.* 2009), but AI with both fresh and frozen semen has been unsuccessful (Walker *et al.* 2002). Lastly, semen freezing has been reported in coyotes (Minter & DeLiberto 2005), but again AI has not been attempted.

2.3. AI in the AWD

The benefits of AI in AWD conservation and management have been described in detail in Van den Berghe *et al.* (2012). Although AI can have tremendous potential for the conservation of AWDs, this technique remains undeveloped. Despite existing conservation efforts, numbers of free-living wild animals are still decreasing. However, this is not caused by a decline in fertility or reproductive success (Comizzoli *et al.* 2009). AWDs usually breed well in the wild (Courchamp & Macdonald 2001), and mostly also in captivity, with the production of sufficient pups to maintain an effective population size. However, the long-term captive propagation of this species requires careful regulation of breeding partners to maintain a population size that will efficiently maximize and preserve genetic diversity (Frantzen *et al.* 2001, Van den Berghe *et al.* 2012). To achieve this and to avoid inbreeding depression, current strategies involve the regular translocation of live animals between groups. In captivity, new packs are formed by introducing male and female single-sex groups (males from one pack and females from another pack) to each other, in an attempt to mimic natural dispersal patterns and avoid inbreeding (Van den Berghe *et al.* 2012). However, the complex social pack structure seen in AWDs together with an unnatural space-limited environment often present in zoos make such introductions difficult, often leading to aggression, injuries or even mortality (Van den Berghe *et al.* 2012).

The use of semen cryopreservation and AI could overcome problems of intra-pack aggression associated with new pack formations by infusing genetic diversity (foreign male sperm) while maintaining existing pack hierarchies, and thereby facilitate captive breeding and metapopulation management (Van den Berghe *et al.* 2012). Transporting spermatozoa instead of live animals will not only improve animal welfare by decreasing the risk of aggression, but also reduces the risk of disease transmission and has important ecological and economic benefits (Van den Berghe *et al.* 2012). Sperm from free-roaming males could be used to increase genetic diversity in captivity, avoiding the removal of animals from the wild. In addition, establishing a sperm bank using genetically valuable animals will provide a genetic back-up of the remaining population, providing a buffer against possible threats such as fires or disease outbreaks in captivity and in the wild (Pukazhenthi *et al.* 2007, Van den Berghe *et al.* 2012). Therefore, it is crucial to develop these techniques now, while the remaining AWD population is still viable. To do so, four important questions need to be answered, as indicated

in Fig. 1.2. The remainder of this introductory chapter will elaborate further on these questions, as to why they are important, and will provide current background knowledge in AWDs and domestic dogs.

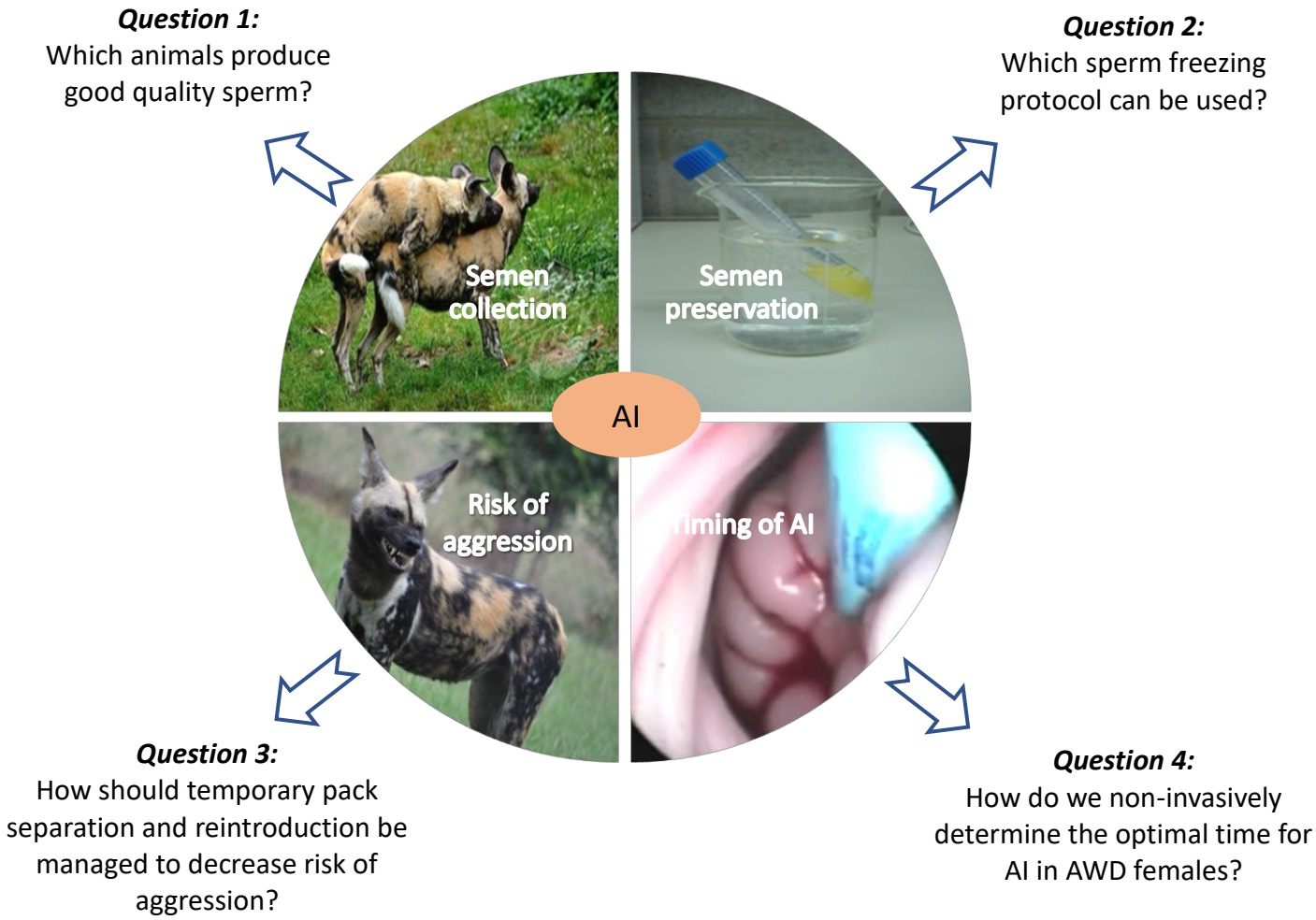


Figure 1.2. *Schematic overview of questions to be answered to enable the application of sperm banking and AI in the AWD*

3. Question 1: Reproductive suppression in AWD males

Understanding the effect of dominance on male fertility is crucial to establish a robust semen bank containing high quality sperm collected from a diversity of valuable individuals during their peak fertility. As mentioned previously, it is usually the dominant pair that reproduces within an AWD pack and the subdominant animals help in raising the pups. This raises questions about the fertility of subdominant AWD males. Possible mechanisms of reproductive suppression in AWD males have been discussed in detail in Van den Berghe *et al.* (2012). In short, reproductive suppression might be behaviourally mediated through mate guarding and aggression as seen for example in grey wolves (*Canis lupus*). Alternatively, hormonal or pheromonal signals might be involved in partial suppression of the hypothalamic-pituitary-gonadal axis of AWD subdominant males.

There are several indications in the literature that reproductive suppression of subdominant males is mostly behaviourally controlled. Firstly, subdominant males also copulate, but at a lower rate than the dominant male (Creel *et al.* 1997). Multiple paternity seems to be quite common and is seen in at least 10% of litters in a variety of free-roaming packs, with in some litters only half of the pups being sired by the dominant male (Girman *et al.* 1997, Mouiex 2006, Spiering *et al.* 2009, Van den Berghe *et al.* 2012). Secondly, in the breeding season, testicular size also increases in subdominant males, indicating spermatogenesis is not likely to be arrested, and most males within a captive pack produced spermatozoa (Johnston *et al.* 2007, Van den Berghe *et al.* 2012). Lastly, most subdominant females ovulate (Van der Weyde *et al.* 2015), indicating reproductive suppression in females is behaviourally mediated.

However, there are also some indications that suppression of subdominant male reproduction could be physiologically mediated at least to some degree. Alpha males have higher levels of testosterone during the breeding season compared to subdominant males (Creel *et al.* 1997, Monfort *et al.* 1997, Johnston *et al.* 2007, Newell-Fugate *et al.* 2012). This could have a positive impact on spermatogenesis and prostate volume and secretory activity (Paris *et al.* 2005, Gomendio *et al.* 2007), making dominant males possibly more fertile. In addition, in one study, semen collections were performed on four occasions in a captive wild dog pack consisting of 7 males. Results showed that mean sperm quality decreased after a stable hierarchy was established (Johnston *et al.* 2007). This suggests that dominance may

affect subordinate male fertility, but more research is needed to investigate this in further detail.

In other animals with a complex social structure such as rats and primates, it has been shown that subdominant animals can experience 'social stress', leading to a chronic increase in glucocorticoid secretion which is thought to induce a 'physiological castration' (Creel 2001, Van den Berghe *et al.* 2012). In rats, chronic or acute stress 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 & Aitken 2005). In free-living AWDs however, the dominant male and female show higher glucocorticoid levels without exerting any obvious negative effects on their fertility (Creel *et al.* 1997). Also in a captive semi-natural environment, it has been shown that cortisol levels were higher in young dominant animals than in older subdominants (de Villiers *et al.* 1997). It has recently been shown that basal faecal cortisol levels are higher in captive than in free-living female AWDs (Van der Weyde 2013) but this information is missing for captive males. Moreover, the mechanisms responsible for causing the relative difference in testosterone in dominant (high) versus subdominant (low) animals, and the effect this has on semen quality (including sperm DNA integrity) in both captive and wild individuals need to be further investigated.

4. Question 2: Sperm Cryopreservation: Towards the development of an AWD sperm bank

The preservation of spermatozoa is an important strategy that permits the uncoupling of semen collection from the male with timing of AI in the female, which offer considerable advantages. In domestic dogs for example, the use of AI with refrigerated semen (4°C) enables easy, short-distance transport of genetic material, bringing considerable economic benefits to breeders (Rijsselaere *et al.* 2011). Furthermore, frozen semen (liquid nitrogen, -196°C) can be transported worldwide and prolongs the reproductive life of an important stud dog, as litters can still be obtained after the dogs' fertility has decreased in their old age or even after their death (Rijsselaere *et al.* 2011). The current status of semen collection, evaluation and prolonged storage is discussed for canids with an emphasis on current developments in the domestic dog and their application to AWDs.

4.1. Semen collection methods

In the domestic dog, semen can be voluntarily collected by digital manipulation; massaging the penis at the level of the bulbus glandis, retracting the prepuce caudally to the bulbis once partially erection is established, and thereafter holding a constant pressure at this level (Kutzler 2005). This technique is ideally performed in the presence of a teaser bitch in oestrus (Kutzler 2005). Unfortunately, this simple semen collection method cannot routinely be used in wild canids, as they are often intractable, making such procedures impossible to perform. However, in some cases of wild canids the possibility exists to train captive animals to submit semen samples (grey wolf, Seager *et al.* 1975; maned wolf, Teodoro *et al.* 2012).

Alternatively, semen can be collected in most wild mammals by means of electroejaculation (EEJ) under anaesthesia, a technique currently used in a variety of wild canids such as the red wolf (Lockyear *et al.* 2009), generic and Mexican grey wolves (Zindl *et al.* 2006), the coyote (Minter & DeLiberto 2008) and the AWD (Hermes *et al.* 2001, Johnston *et al.* 2007, Newell-Fugate *et al.* 2012). In the domestic dog, this technique is mainly used in cases of insufficient libido in important stud dogs but, due to the risks associated with anaesthesia, is not performed routinely (Kutzler 2005). During EEJ, a probe is placed rectally at the level of the prostate. Electric stimulations of the pelvic, hypogastric and pudendal nerves mimic the natural processes of erection, spermatozoa and seminal emission, and ejaculation (Kamischke & Nieschlag 1999). As discomfort may arise during the procedure, general anaesthesia is

necessary. Catheterization and drainage of the bladder should be performed before the procedure as urine contamination of semen samples (Pineda & Dooley 1984) or retrograde ejaculation (Dooley *et al.* 1986) is sometimes encountered. In humans, EEJ is used to obtain semen samples from men with anejaculatory infertility (e.g. after spinal cord injury) and may result in semen of lower quality than found in natural ejaculates (Restelli *et al.* 2009). This could be explained by either the neurological injury and alterations of seminal gland excretions (Hirsch *et al.* 1991, Brackett *et al.* 1996), a suboptimal testicular environment (Restelli *et al.* 2009), or a direct electrical effect of the EEJ procedure in these patients (Linsenmeyer *et al.* 1989). In domestic dogs however, no significant differences in the quality of routine semen parameters could be seen immediately after collection between semen obtained by digital manipulation or EEJ (Ohl *et al.* 1994, Christensen *et al.* 2011). However, semen collected by EEJ had lower motility after 4h incubation at room temperature without extender (Christensen *et al.* 2011). According to the authors, the higher concentration of prostatic fluid in EEJ collected samples might explain this difference.

Another possible but less common technique is the collection of epididymal spermatozoa. This technique can be crucial to salvage genetic material after castration or sudden death of a genetically valuable animal. Semen can be recovered by flushing or mincing the cauda epididymis and vas deferens (Sirivaidyapong 2002). Successful pregnancies have been obtained after AI with fresh (Klinc *et al.* 2005) and even frozen-thawed (Hori *et al.* 2004, Hori *et al.* 2005) epididymal spermatozoa.

4.2. Semen evaluation

The ejaculate of the dog consists of three parts, evident when semen is collected by digital manipulation (Linde-Forsberg 1991, Kutzler 2005, Rijsselaere *et al.* 2011). The first fraction consists of a small amount of prostatic fluid, and its physiological importance is to “clean” the urethral tube at the start of the ejaculation. The second fraction is sperm-rich and is the most important for semen freezing and/or AI. The third fraction, also consisting of prostatic fluid, represents in volume the most important part of the dogs’ ejaculate and can be used for evaluating the presence of prostatic diseases (Van den Berghe 2012). When semen is collected using EEJ, this separation of the ejaculate in three fractions is absent, and a mixture of the sperm-rich fraction and prostatic fluid occurs.

A series of relatively simple techniques can be performed to evaluate the quality of collected semen. In domestic dogs, the quality of semen is usually evaluated prior to performing AI, semen refrigeration/freezing, or as a diagnostic tool (e.g. stud dogs with a history of poor fecundity; Payan-Carreira *et al.* 2011). Conventional semen evaluation consists of macroscopic and light microscopic assessment. Macroscopically, the second fraction has a cloudy white to opalescent colour and is variable in volume (typically from 1.0 to 4.0 ml) depending on breed, age and sexual activity of the dog (Johnston *et al.* 2001). By comparison, semen volume in AWDs, has been reported to be 0.6 ± 0.1 ml in Australia (n=10; Johnston *et al.* 2007) and 3 ml in Europe (n=1; Hermes *et al.* 2001). Since urine contamination is common in ejaculates collected by EEJ, semen volume should be evaluated with care, and macroscopic evaluation (e.g. colour and odour) will help determine the presence of urine. Light microscopy is typically used to evaluate the motility, concentration, viability and morphology of spermatozoa (Johnston *et al.* 2001). In domestic dogs, progressive motility is evaluated by placing a drop of semen on a pre-warmed glass slide and is normally ≥70% (Freshman 2002). During the breeding season, AWDs seem to have a similar level of progressive motility, but have lower motility when semen is collected at other times of the year (Johnston *et al.* 2007, Newell-Fugate *et al.* 2012). Sperm concentration is normally determined using a haemocytometer (WHO 2010) and varies between 4-400 x 10^6 sperm/ml in domestic dogs, depending on dilution with prostatic fluid. Sperm concentration is then used to calculate the total number of sperm cells in an ejaculate which usually is between 300 and 2000 x 10^6 sperm in the dog (Johnston *et al.* 2001). Sperm output during or at the end of the breeding season is much lower in AWDs ranging from 12.5 ± 6.3 x 10^6 (n=3; Johnston *et al.* 2007) to 301.4 ± 39.1 x 10^6 sperm (n= 5; Newell-Fugate *et al.* 2012). Viability and morphology of spermatozoa can be assessed using an eosin-nigrosin stain. Viability is usually higher than 80% in domestic dogs (Johnston *et al.* 2001), similar to that reported for AWDs during the breeding season (Johnston *et al.* 2007, Newell-Fugate *et al.* 2012). These techniques for semen evaluation can provide a relatively accurate measure of semen quality in the field, zoo veterinary clinic or general veterinary practice. However, as the measurement of motility in particular can be somewhat subjective, more objective techniques should be considered wherever feasible.

One such technique, computer-assisted sperm analysis (CASA), provides a rapid and standardized analysis of a large series of motility parameters (Ellington *et al.* 1993, Rijsselaere

et al. 2004). In addition, fluorescent stains that provide specific information on sperm integrity are available and have been validated for use in the dog. Large numbers of fluorescently stained spermatozoa can thus be rapidly analysed by flow cytometry (Martínez-Pastor *et al.* 2010). For example, membrane integrity can be evaluated using a combination of different stains such as propidium iodide and SYBR-14 (Rijsselaere *et al.* 2002). Acrosome status can be assessed by staining with lectins such as Peanut Agglutinin (PNA) or Pisum Sativum Agglutinin (PSA) conjugated with fluorescein isothiocyanate (Kawakami *et al.* 1993, Sirivaidyapong *et al.* 2000, Kawakami *et al.* 2002). Sperm DNA integrity can be evaluated by Terminal deoxynucleotidyl transferase biotin-dUTP Nick End Labelling (TUNEL; Lange-Consiglio *et al.* 2010) or Sperm Chromatin Structure Assay (SCSA; Koderle *et al.* 2009). Other assays such as the hypo-osmotic swelling test, which allows evaluation of sperm membrane integrity (Kumi-Diaka 1993), and the zona pellucida binding assay, which can assess the fertilizing potential of the sperm cells (Peña *et al.* 2004), are also available.

4.3. Semen freezing

The first domestic dog litter born after AI with frozen semen was reported by Seager in 1969 (cited by Seager *et al.* 1975). Since then, different semen freezing protocols have been described for use in the dog. The semen freezing process consists of several steps that include semen dilution with an appropriate extender, cooling and equilibration, freezing in liquid nitrogen and ultimately a thawing step that results in viable spermatozoa (Eilts 2005). All of these steps can have detrimental effects on post-thaw semen quality due to cellular damage and loss of function. The exact mechanisms resulting in cell death or loss of function of the spermatozoa seen after cryopreservation are not completely understood but can be either morphological, biochemical or physical.

4.3.1. Cold shock and sperm membrane lipids

Sperm membranes including the plasma membrane, the outer acrosomal membrane and the mitochondrial membranes are relatively sensitive to the cryopreservation process (Watson 1995). Compared to somatic cell plasma membranes, sperm plasma membranes contain a unique combination of lipids, phospholipids and cholesterol (Watson 1995), with the phospholipids making up 70% of the total lipids (Holt 2000).

At physiological temperatures, membrane lipids are organized in different domains and can show lateral rearrangements within the phospholipid bilayer (fluid state; Holt 2000). Cold shock or damage due to cooling above 0°C, can be present in different mammals, reflected by a loss of metabolic function and damage to cellular membranes (Watson 1995). Decreasing temperature causes a transition in the membrane phase from a fluid to a gel-like state, due to aggregations of phospholipid fatty acid chains. This can not only lead to physical damage of the sperm membranes, but can also increase membrane permeability thereby causing excessive passage of water and ions (Watson 1995, Holt 2000).

One factor influencing sperm cryogenic success is species, breed and even individual differences in the resistance of the sperm cell to cold shock and the freeze-thaw process. This ability can be correlated with the lipid composition of the sperm plasma membrane. Initially it was thought that resistance to cold shock was related to a low ratio of unsaturated/saturated membrane fatty acids and to high levels of membrane sterols (White 1993). However, it seems that polyunsaturated fatty acids (PUFAs), especially docosahexaeonic acid (DHA; 22:6, n-3), are actually important elements of sperm plasma membranes that determine both the quality of fresh semen and cryogenic success. This is not surprising as they influence sperm membrane fluidity (Wathes *et al.* 2007).

4.3.2. Cryoinjury

Further temperature decrease below the freezing point initiates the formation of extracellular ice (Watson 2000). This increases the concentration of extracellular salts, thereby drawing water from the cell by osmosis causing gradual dehydration and cell shrinking. Damage can be caused at this stage by using a suboptimal freezing curve. If the semen sample is frozen too slowly, excessive dehydration and cell shrinking occurs. Freezing too quickly causes the formation of intracellular ice crystals and direct cell damage (Watson 1995, Holt 2000). Increased water permeability of the cell induced during cold shock also increases the risk of intracellular ice formation during freezing (Farstad 2009). During thawing, water influx in the cell can disrupt cell membranes (Holt 2000). Moreover, if a suboptimal thawing rate is used, intracellular ice crystals may form from microcrystals originating during the freezing process (Holt 2000).

Refrigeration and freezing can also induce capacitation-like changes to sperm cells and promote pre-mature capacitation (Rota *et al.* 1999). Capacitation is a prerequisite for the acrosome reaction, which is necessary for recognition and penetration of the oocyte. The normal process involves physiological changes to sperm membranes and an increased influx of ions (Toshimori 2011). During freeze-thawing, spermatozoa are subjected to similar processes that induce premature capacitation-like changes which decrease the longevity of the cell (Watson 1995).

Another potential source of damage during cryopreservation is the presence of excessive levels of reactive oxygen species (ROS) that increase during the cryopreservation process (Chatterjee & Gagnon 2001). The two major sources of ROS in semen are leucocytes and the spermatozoa themselves (Tremellen 2008) In humans, excessive ROS in semen causes infertility due to both DNA damage (Kodama *et al.* 1997, Aitken *et al.* 1998) and damage to the sperm plasma membrane. This damage causes a decrease in motility by peroxidation of the sperm plasma membrane (Kao *et al.* 2008) and by direct damage to the mitochondria (de Lamirande & Gagnon 1992). Moreover, it also causes peroxidation of the sperm acrosomal membrane and decreases fusion with the oocyte (Zalata *et al.* 2004, Jedrzejczak *et al.* 2005).

4.3.3. Canine semen freezing

To minimize damage caused by cold shock and cryoinjury, a species-specific cryopreservation protocol should be utilised that incorporates an optimised semen extender. This is particularly important in canids, with their relatively limited sperm-rich fraction and low number of total spermatozoa. Moreover, a relatively large number of functional spermatozoa are needed for successful AI in dogs, making the need for a semen freezing protocol that yields high post-thaw semen quality extremely important. Several canine semen freezing extenders and protocols have been empirically established over the years that result in good post-thaw semen quality (Farstad 1996, Peña & Linde-Forsberg 2000, Thomassen *et al.* 2006, Bencharif *et al.* 2010).

- *Semen preparation*

When semen is collected by digital manipulation, only the second fraction is used for semen freezing. After evaluation, semen is directly diluted with the semen extender. Since contamination with prostatic fluid can be detrimental to semen quality after thawing

(Sirivaidyapong *et al.* 2001), the semen extender ensures prostatic fluid is sufficiently diluted (Farstad 1996). However, in certain cases, the spermatozoa in the second fraction are centrifuged before adding the semen extender to the sperm pellet to eliminate traces of prostatic fluid (Rijsselaere *et al.* 2002, Kutzler 2005, Schafer-Somi *et al.* 2006). As mentioned above, separation into 3 ejaculate fractions does not occur when semen is collected by electroejaculation, thus centrifugation should be considered in cases of low sperm concentration, excess prostate fluid or urine contamination.

- *Semen extenders*

The goal of the semen freezing extender is to protect the spermatozoa during the cryopreservation process. A good extender should (i) provide energy to the spermatozoa; (ii) protect them against pH changes, cold shock and cryoinjury; (iii) inhibit bacterial growth; and (iv) have an optimal osmolarity (Farstad 1996).

A series of adaptations to bovine semen freezing extenders have been trialled on dog sperm (Farstad 1996), but after decades of canine semen freezing, the most commonly used laboratory-prepared extenders are still the TRIS-citrate-fructose or -glucose based extenders (England & Ponzio 1996, Rota *et al.* 1999, Peña & Linde-Forsberg 2000, Thomassen *et al.* 2006, Varesi *et al.* 2014). These types of extenders are also now commercially available (e.g. CaniPlus Freeze®, Minitüb, Tiefenbach, Germany).

Egg yolk (EY) is added to the semen extenders of most species, usually at a concentration of 20% for domestic dogs (Rota *et al.* 1997, Silva *et al.* 2002, Martins-Bessa *et al.* 2006). EY is a complex biological compound containing proteins, phospholipids, vitamins, glucose, antibacterial compounds and antioxidants (Farstad 2009). The exact mechanism(s) responsible for its beneficial effects during semen refrigeration and freezing are not completely understood, but its active component is a low-density lipoprotein (LDL; Watson 1976). It is suggested that EY protects the sperm plasma membrane against cold shock by interaction with the bilayer thereby preventing or restoring the loss of phospholipids (Watson 1995, Farstad 2009). As EY is a biological substance, it may be a potential source of disease transmission during international transit. Therefore, other sources of lipids have been tested as alternatives to EY. The substitution of whole EY with 6% LDL together with 20 mmol glutamine, yielded an improved post-thaw semen quality (Bencharif *et al.* 2010), resulting in

its marketing as a commercial extender, CANIFreeze® (IMV Technologies, L'Aigle, France). In addition, the use of soybean lecithin gave immediate post-thaw results similar to TRIS-EY extender (Beccaglia *et al.* 2009), but viability in the long term was not investigated. Canine semen freezing has also been trialled with a skim milk-glucose-based extender. Post-thaw semen quality was similar to the results obtained using an EY-based extender, and AI of two bitches resulted in the birth of 6 puppies (Abe *et al.* 2008). Unfortunately, milk is also a biological substance, so the risks of disease transmission are not eliminated. Further research that closely examines pregnancy rates after AI using these type of extenders is required.

The type of cryoprotectant added to the semen extender may vary for different species but for most, glycerol yields the best freezing outcomes (Holt 2000). One of the most important roles of glycerol is probably its intracellular mechanism of action, in which it replaces water during dehydration thereby inhibiting excessive cell shrinkage or intracellular ice formation (Holt 2000). At an extracellular level, glycerol decreases the concentration of electrolytes in the unfrozen fraction thereby reducing its freezing point. Glycerol also integrates in the sperm plasma membrane and might in this way change the lipid packing structure and thus the stability and permeability of the membrane (Hammerstedt & Graham 1992, Holt 2000, Leahy & Gadella 2011). In high concentrations, glycerol can exert toxic effects on spermatozoa (Curry 2000, Holt 2000). The amount of glycerol added to the extender, and the contact time with the spermatozoa should be high enough to enable its cryoprotective effect but low enough to limit its potential toxicity. In domestic dogs, optimal post-thaw semen quality has been achieved using a glycerol concentration of between 2-8% (Olar *et al.* 1989, Peña *et al.* 1998, Rota *et al.* 1998).

The dilution of semen with the extender can be done using a one- or two-step process. When using a one-step dilution protocol, semen is diluted immediately after collection (37°C), and frozen after cooling and equilibration. Two-step dilution protocols consist of the first dilution of semen immediately after collection (37°C), followed by the second dilution after the sample is first cooled and equilibrated (4°C). This two-step procedure has been proposed to enable cooling and equilibration with lower glycerol concentrations, minimizing its toxic effect (Peña & Linde-Forsberg 2000). However, several reports in domestic dogs have shown no beneficial effects on post-thaw semen quality when glycerol was added at a later stage during or after the cooling and equilibration period, indicating that glycerol is not toxic at the

concentrations used for dog semen freezing (Peña *et al.* 1998, Peña & Linde-Forsberg 2000, Silva *et al.* 2003).

The addition of sodium dodecyl sulphate (SDS), an anionic detergent, to EY-based extenders can be beneficial to post-thaw semen quality and longevity (Rota *et al.* 1997, Peña *et al.* 1998, Peña & Linde-Forsberg 2000, Tsutsui *et al.* 2000, Nizanski *et al.* 2001). The exact mechanism by which SDS exerts this effect is unknown, but it has been suggested that it solubilises active molecules in the egg yolk, improving their contact with the sperm membranes (Rota *et al.* 1997). SDS has been added to extenders in its pure form (Schwarz/Mann Biotech, Division of ICN Biomedicals. Inc., Cleveland, OH, USA; Peña *et al.* 1998), or as a component of Equex STM paste (Nova Chemical Sales, Scituate, Inc., MA, USA; Rota *et al.* 1997), Equex paste (Minitüb, Tiefenbach, Germany; Peña *et al.* 2003), or Orvus ES paste (Nova Chemical Sales, Inc., MA, U.S.A; Tsutsui *et al.* 2000). However, the beneficial effect of SDS is influenced by additional factors in these additives since Equex STM paste yields better freezing outcomes than Equex paste when using the same dosage in the domestic dog (Peña *et al.* 2003), or generic grey and Mexican grey wolves (Zindl *et al.* 2006). This beneficial effect of Equex STM paste was greatest when added at the end of the equilibration period (Peña & Linde-Forsberg 2000), making a two-step dilution important when using this additive.

- *Cooling, equilibration and semen packaging*

To prevent cold shock and prepare semen for the cryopreservation process, it must be slowly cooled to 4°C, followed by a short-term storage at this temperature (equilibration). The cooling and equilibration period in domestic dogs usually takes about 1 to 3 hours, but research investigating the optimal equilibration time is limited. Olar *et al*. (1989) found no differences in post-thaw motility when semen was cooled for 1, 2 or 3 hours prior to freezing. Another study examined cooling and equilibration times using a two-step dilution protocol with only the second extender containing glycerol (Okano *et al.* 2004). The best post-thaw semen quality was obtained with 2 to 3 hours of cooling, with no improvement when glycerol-containing extender was added for up to 4 hours of additional equilibration (Okano *et al.* 2004).

After cooling and equilibration, semen can either be packed in 0.25 or 0.5 ml semen straws and frozen in liquid nitrogen vapour or frozen as pellets on dry ice. Research comparing post-

thaw semen quality in dogs using pellets or straws is limited but results seem to be most influenced by the type of semen extender used (Thomas *et al.* 1993). However, two studies show better post-thaw semen quality when using 0.5 ml instead of 0.25 ml straws (Thomas *et al.* 1993, Nöthling & Shuttleworth 2005).

- *Semen freezing and thawing*

Programmed semen freezing systems have been described for use in the dog. However automatic freezers are expensive and therefore not available to everyone. Thus, the 'classic method' for freezing is still often used, that involves suspending semen straws in supercooled vapour in a styrofoam box above liquid nitrogen (Peña & Linde-Forsberg 2000). Several studies have attempted to determine an optimal freezing rate for dog sperm, but results have been inconsistent (Olar *et al.* 1989, Peña & Linde-Forsberg 2000, Rota *et al.* 2005, Hori *et al.* 2006). A simple and commonly used freezing protocol consists of suspending the straws 4 cm above liquid nitrogen vapour for 10 min (rate of -30°C/min to -50°C), before plunging them into the liquid nitrogen (Peña & Linde-Forsberg 2000). Thawing of straws at 70°C for 8 sec yields better results than at 37°C for 15 or 30 sec (Olar *et al.* 1989, Peña & Linde-Forsberg 2000), but is practically more challenging so is less widely used.

- *Semen freezing in the AWD*

Sperm cryopreservation has been attempted in AWDs (Hermes *et al.* 2001, Johnston *et al.* 2007), but these methods will need to be greatly improved to establish a protocol that will allow the creation of a robust and reliable sperm bank. Hermes et al. (2001) collected and froze 3 ejaculates of one AWD male by diluting 1:3 in TEST buffer containing 7.5% glycerol and 15% EY, and using a freezing rate designed for freezing cat and fox sperm. This protocol initially gave promising results 10 min after thawing (sperm motility of 0, 30 and 40% respectively), but motility rapid decreased after 30 min (0, 5 and 10% respectively). Johnston *et al.* (2007) collected and froze semen from 7 AWDs using a canine two-step dilution protocol in which semen was first diluted 1:1 in Tris-citrate buffer containing 20% EY without glycerol, cooled to 5°C for 2-3 h, then finally diluted a further 1:1 with the same buffer now containing 8% glycerol. Semen was loaded into straws and frozen over liquid nitrogen vapour (14 cm above liquid for 25 min followed by 4 cm for 5 min), before being plunged into liquid nitrogen. Again, initial results immediately after thawing were encouraging (31.8 ± 5.8% sperm motility)

but dramatically declined after 2 h incubation (0.2 ± 0.1% motility). Thus, more research is urgently needed to improve the freezing of AWD sperm to develop a robust sperm bank for the species.

5. Question 3: Dog appeasing pheromone: A tool for behavioural management?

Dog appeasing pheromone (DAP) might be a valuable alternative method to reduce aggression during new pack formations or reintroduction of individuals to existing packs after veterinary interventions. Currently, drugs such as fluoxetine hydrochloride (a selective serotonin uptake inhibitor) are sometimes used to minimize these risks in captivity but the results of such drugs are not always ideal, with possible undesirable side effects (Vlamings 2011). In domestic dogs, lethargy during fluoxetine treatment can be as high as 39% (Irimajiri *et al.* 2009). Lethargy is detrimental during AWD (re)introductions as decreased responsiveness to the environment might increase the risk of aggression by untreated conspecifics during or after treatment. Appeasing pheromones could be a valuable alternative to reduce stress and aggression in captivity.

Pheromones are a mixture of volatile components secreted as a way of chemical communication between animals of the same species. In carnivores, pheromones are secreted by a series of glands in the facial area, on the feet, in the perianal, genital and mammary region, or secreted in the urine or faeces (Pageat & Gaultier 2003). Pheromones are perceived by conspecifics through the inspiration of molecules into the vomeronasal organ which then induce either a physiological or behavioural response in the receiver (Pageat & Gaultier 2003).

In several mammals, including dogs and cats, the structure and function of some pheromones are known, and synthetic forms are already being used to modify unpleasant behaviour. In dogs, the mammary appeasing pheromone is secreted by sebaceous glands in the intermammary sulcus of lactating bitches from 3-4 days post-partum until 2-5 days post-weaning (Pageat & Gaultier 2003). Synthetic analogues of this pheromone, known as DAP (Adaptil^{TM}, CEVA), can reduce stress and unwanted behaviour in fear-inducing situations such as fireworks (Sheppard & Mills 2003), car travel (Gandia Estelles & Mills 2006), and surgery (Siracusa *et al.* 2010) in adults, as well as social isolation (Gaultier *et al.* 2008), unfamiliar people and surroundings (Gaultier *et al.* 2009), and training (Denenberg & Landsberg 2008) in puppies. DAP has also been shown to be as effective as clomipramine in the treatment of separation anxiety (Gaultier *et al.* 2005). One of the major advantages of the use of

pheromones in behavioural treatment is the absence of toxicity or side effects, as there is no penetration of the substance into the organism (Pageat & Gaultier 2003).

A preliminary study indicates that AWDs might be capable of perceiving DAP (Vlamings 2011). In several trials, DAP infused collars reduced baseline stress levels of females. Moreover, the use of DAP collars and sprays before new pack formation resulted in low levels of aggression (Vlamings 2011). However, due to the small sample size, more research is needed into the effectiveness of DAP for the behavioural management of captive AWDs. If proven effective, DAP could be used during interventions associated with the development of assisted reproductive techniques that necessitate short-term isolation and anaesthesia of a pack-member (e.g. for semen collection or AI), as well as assist in translocations and new pack formations.

6. Question 4: Timing of artificial insemination in the AWD.

Although the domestic dog is distantly related to the AWD, it can be used as an important model for the development of AI technology in this and other wild canids. Basic reproductive physiology is well known in dogs and considerable research has been undertaken to improve semen freezing and AI success. The demand for AI in domestic dogs has increased significantly over the last decades, predominantly using fresh semen to prevent (i) disease transmission or physical injury to valuable animals during natural mating, (ii) mating incompatibility due to differences in height between the male and female or the presence of anatomical abnormalities, or (iii) insufficient libido in the male or female dog (Payan-Carreira *et al.* 2011).

While establishing a semen bank for an endangered species, it is necessary to also develop AI to re-infuse valuable genetics back into the population. To do so, it is crucial to have extensive knowledge of the reproductive anatomy and physiology of the female. This will permit an appropriate insemination technique to be developed, and the appropriate timing relative to ovulation to be determined for spermatozoa to be deposited in the female reproductive tract. In most wild species, detailed information on reproductive physiology is absent making the development of such techniques challenging. Basic reproductive physiology can vary extensively between species and thus, information cannot always be easily extrapolated from one species to another (Paris *et al.* 2007). That said, the domestic dog can be an important model for developing AI in the AWD since most canids studied to date have similar reproductive cycles (Asa & Valdespino 1998). This includes a mono-oestrous cycle with a long pro-oestrus and oestrus, a pregnant or non-pregnant (pseudopregnant) period of dioestrus, and an obligatory period of anoestrus (Asa & Valdespino 1998, Concannon 2009, Van den Berghe *et al.* 2012). In the following section, the reproductive cycle and methods for timing of AI are discussed for the domestic dog and supplemented with currently available information on African wild dog reproduction.

6.1. Reproductive cycle of the domestic dog

Depending on breed size, puberty typically occurs at 6-14 months in the domestic dog and adult females exhibit a monoestrus cycle which usually takes about 5-12 months (Concannon 2011). The reproductive cycle consists of 4 distinct phases: a long pro-oestrus and oestrus,

together described as “heat”, a pregnant or non-pregnant (pseudopregnant) period of dioestrus, and an obligatory period of anoestrus (Fig. 1.3; Concannon 2009).

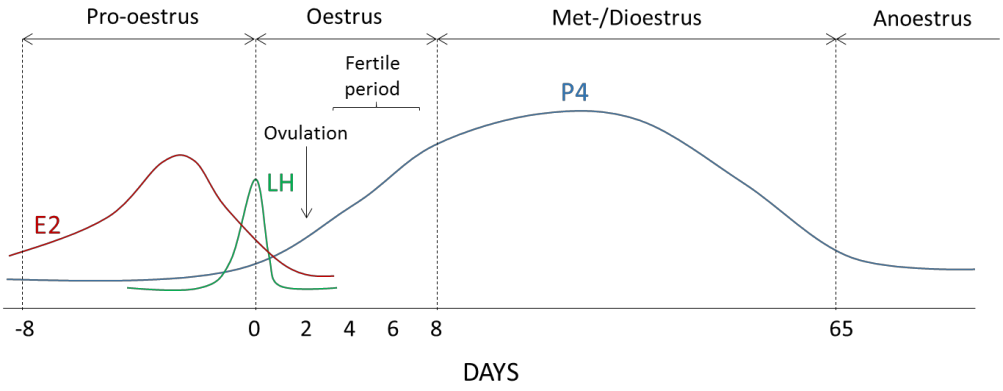


Figure 1.3. *Phases and corresponding hormone profiles during the oestrus cycle of the domestic dog. E2, oestrogen; LH, luteinising hormone; P4, progesterone.*

Pro-oestrus is the first stage of heat characterized by follicular growth and rising oestrogens, responsible for a visible swelling of the vulva and haemorrhagic discharge, originating from the uterus (Concannon 2011). Due to the vaginal secretion of pheromones (methyl p-hydroxybenzoate; Goodwin *et al.* 1979), the female exhibits proceptivity to male dogs but is not yet receptive to mating. This period takes about 8 days but can vary significantly (3 days to 3 weeks) between females and between cycles. The final stage of heat (oestrus) starts with the LH (luteinising hormone) surge. Unusually, in dogs most oocytes are ovulated in metaphase I 48-60h later, but need to mature to metaphase II for a further 48-60h in the distal oviduct before fertilization can occur (England 2010). Oestrus extends for a variable period of typically 8 days during which time the female is generally receptive to mating. Vulvar swelling and discharge diminish from the onset of oestrus due to decreasing levels of oestrogen (Concannon 2011). The subsequent period of dioestrus takes around 57 days in pregnant females (pregnancy takes 65 ± 1 day when counted from LH surge; Concannon *et al.* 1983) during which time the source of progesterone comes exclusively from the corpus luteum. In non-pregnant females, dioestrus is of similar duration but can vary from 50 to 80 days (Concannon 2011). The first few days of dioestrus are sometimes called metoestrus, based on changes visible in the cytology of the vagina (Fig. 1.4). Anoestrus, the period of

reproductive dormancy, occurs for about 4 months, but is shorter (2 months) in breeds such as the German shepherd and Rottweiler, and longer (10 months) in more primitive breeds such as the basenji (Jochle & Andersen 1977).

6.2. Current knowledge in female AWD reproduction

AWDs are seasonal breeders with the majority of successful matings seen around February (McNutt 1996, Buettner *et al.* 2006), which shifts by up to 6 months for animals living in the northern hemisphere (August/September; Verberkmoes 2008, Van den Berghe *et al.* 2012). Most details about the timing and profiles of reproductive hormones around the peri-ovulatory period are still largely missing, but the reproductive cycle appears very similar to that observed in domestic dogs. Puberty is reached near the age of 2 years (Van Heerden & Kuhn 1985, Van der Weyde 2013), which is relatively late compared to the domestic dog. Based on behavioural and clinical signs, the period of pro-oestrus and oestrus takes 14–20 days (Van Heerden & Kuhn 1985, Van den Berghe *et al.* 2012). During pro-oestrus before mating, female proceptivity slowly increases and a strengthening in the bond with the dominant male can be seen (Van Heerden & Kuhn 1985, Creel *et al.* 1997, Van den Berghe *et al.* 2012). Similar to the domestic dog, increased oestrogen levels cause vulvar swelling and sanguineous vaginal discharge (Monfort *et al.* 1997, Van den Berghe *et al.* 2012), and mating occurs over a period of 3-7 days, when oestrogen declines and progesterone rises (Monfort *et al.* 1997). Recently, measurement of faecal progesterone metabolites collected individually from group-housed individuals demonstrated that females appear to cycle in the absence of males (Paris *et al.* 2008, Van der Weyde 2013).

Pregnancy lasts 69–72 days when counted from the last mating (Van Heerden & Kuhn 1985, Creel *et al.* 1997, Monfort *et al.* 1997). During this time, progesterone levels remain elevated as in the domestic dog, and drop coinciding with parturition (Monfort *et al.* 1997, Van den Berghe *et al.* 2012). Progesterone levels are also elevated in cycling non-pregnant females, indicating that spontaneous ovulation also occurs (Van der Weyde *et al.* 2012). The number of pairs of mammary glands vary between 6 and 8 (Van Heerden & Kuhn 1985), and litter sizes are large containing around 10–12 pups (Comizzoli *et al.* 2009, Van den Berghe *et al.* 2012). Although subdominant females are known to ovulate, allosuckling is rarely seen (Creel *et al.*

1997). Pups are weaned around 10 weeks of age although pups start to eat regurgitated food from 14 days onwards (Smithers 1983, Van den Berghe *et al.* 2012).

6.3. Timing of AI in domestic dogs

Because of the long mono-oestrus cycle, a detailed follow-up of the bitch should be performed during the peri-ovulatory period when scheduling for AI or natural mating. This is to ensure insemination at the right moment, i.e. during the fertile period of oestrus after ovulation and maturation of the oocytes (Thomassen & Farstad 2009). Moreover, as described above, there is considerable individual variation in the duration of pro-oestrus and oestrus, but also in the onset and duration of physical and behavioural signs and acceptance of the male (Moxon *et al.* 2012); making these parameters alone insufficient for timing of AI. Timing is particularly important when frozen semen is used, since its quality after freeze-thawing is less than for fresh sperm and has a short survival time (Peña & Linde-Forsberg 2000). For timing of ovulation and AI, progesterone measurement in the peripheral blood is the most widely used technique but is ideally coupled with other methods (e.g. vaginal cytology). These examinations should be performed on a regularly basis from the onset of heat until the moment of AI (1- to 3-day intervals depending on progression of peri-ovulatory events).

6.3.1. Analysis of blood hormones

The measurement of Luteinising hormone (LH) in the peripheral blood is a reliable and accurate method for detecting the LH surge and predicting timing of ovulation and AI. Baseline levels of LH in pro-oestrus are ≤1 ng/ml and these increase up to 3-40 ng/ml at the moment of the LH peak (Concannon 2011). Clinically it is quite difficult however to properly detect this LH surge, as multiple analysis per day are needed (Hase *et al.* 2000), LH analysis are not routinely available in every standard veterinary laboratory and might be quite expensive (Von Heimendahl & England 2010). In-house semi-quantitative test kits (displaying results as <1 ng/ml and >1ng/ml) have been developed using Rapid Immuno-Migration (Witness® LH Luteinising Hormone test). These tests have been very useful in timing AI, but daily analyses are still needed (Santos *et al.* 2012) and require multiple tests to be performed which can become quite expensive. Thus, to date, such kits are not routinely used during cycle follow-up.

Baseline levels of progesterone seen during pro-oestrus start increasing around the time of the LH surge and keep rising during oestrus, with each level reflecting specific events during the peri-ovulatory period (Fig. 1.3 and Table 1.2).

Table 1.2. *Important progesterone concentrations during the peri-ovulatory period in the domestic dog bitch (Von Heimendahl & England 2010).*

Event	***Progesterone level***
LH-surge	1.5-2.5 ng/ml (4.5-7.5 nmol/l)
Ovulation	5-8 ng/ml (15-24 nmol/l)
Fertile period	10-25 ng/ml (30-75 nmol/l)

Progesterone can be measured by radioimmunoassay (RIA), enzyme-linked immunosorbent assay (ELISA) or immunochemiluminescence assay. Assays are available in most veterinary diagnostic laboratories (Von Heimendahl & England 2010). Although serial evaluation is still needed, in most cases there is no need to do this daily, which is the main advantage of serum progesterone evaluation. Semi-quantitative assay kits are available displaying results corresponding to basal progesterone levels (0-1 ng/ml), intermediate levels at the moment of LH surge (1-2.5 ng/ml) and ovulation (2.5-8 ng/ml), or high progesterone levels (> 8 or 10 ng/ml; Payan-Carreira *et al.* 2011). These assays however, are generally less accurate than quantitative methods (Moxon *et al.* 2010), and in some cases other methods are needed for more accurate timing of AI.

6.3.2. Vaginal cytology

Under the influence of oestrogen present during pro-oestrus, there is an increase of mitotic divisions in the vaginal epithelium, leading to an increase in the number of cell layers and thereby a keratinization of the epithelial cell population in oestrus (cornification; Von Heimendahl & England 2010). By performing vaginal swabs, the phase of the reproductive cycle of the bitch can be determined (Fig. 1.4). This technique has also been performed in the AWD (Newell-Fugate *et al.* 2012).

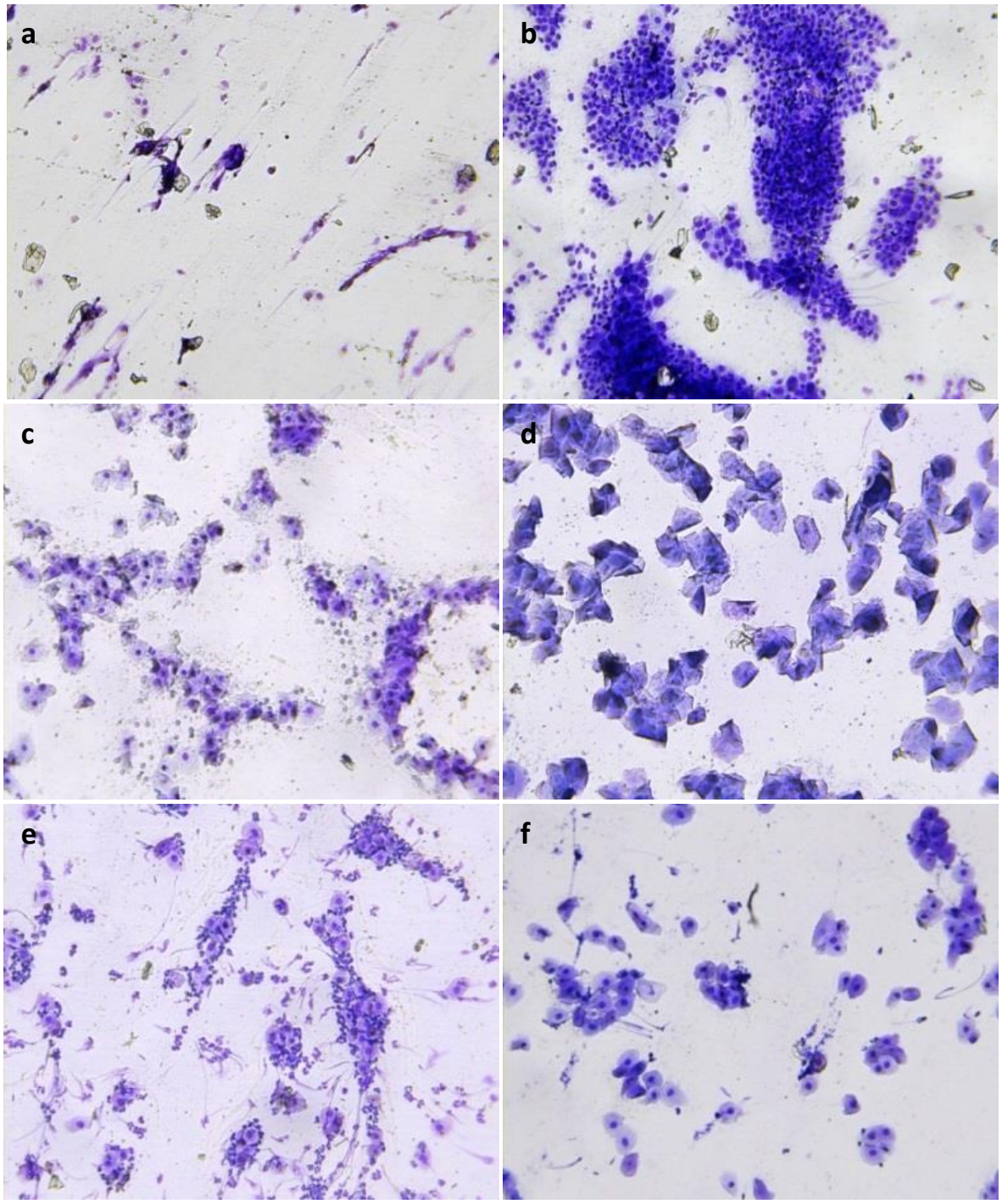


Figure 1.4. *Vaginal cytology (Diff-Quick staining, 250x) of a domestic dog during: (a) anoestrus, few cells are visible with the majority being small basal cells and isolated nuclei, mucus is present; (b) early pro-oestrus, increase in cellularity and presence of multiple mitotic cells; (c) mid-pro-oestrus, large amount of red blood cells with a shift of vaginal epithelium to larger, intermediary cells, some cells start to keratinize; (d) oestrus, presence of numerous (>80%) keratinized polygonal cells and decrease in number of red blood cells; (e) metoestrus (early dioestrus), abrupt shift from keratinized to intermediary epithelial cells, high cellularity and presence of numerous neutrophils and mucus; and (f) dioestrus, gradual decrease in cellularity, intermediary epithelial cells, mucus and some rare neutrophils.*

6.3.3. Vaginal endoscopy

The phase of the reproductive cycle can also be determined using vaginal endoscopy/vaginoscopy (Fig. 1.5). During anoestrus, the vaginal mucosa is pink with no oedema present. In pro-oestrus, due to rising oestrogen levels, an increase in oedema can be seen with the formation of rounded longitudinal mucosal folds. The mucosal wall shows a paler aspect due to the increase of cell layers coinciding with keratinization. At the time of the LH surge, there is a decrease in oestrogen levels together with an increase in progesterone (Fig. 1.3). This results in a reduction of tissue oedema resulting in a shrunken and wrinkled appearance (angulation) around the time of ovulation (Von Heimendahl & England 2010).

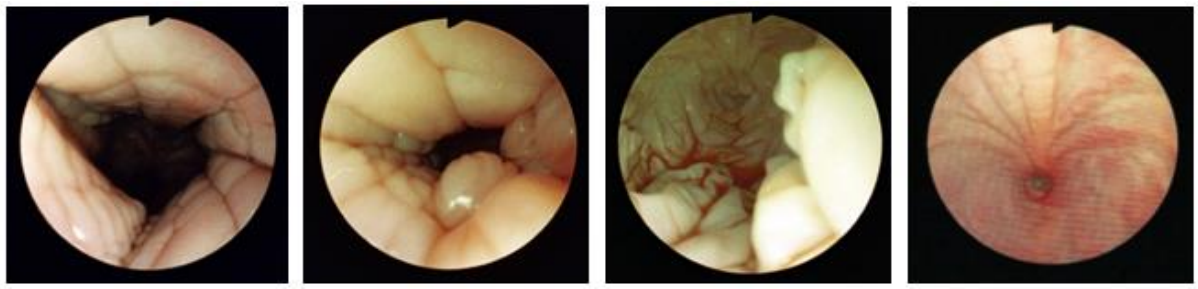


Figure 1.5. *Vaginal endoscopy of a domestic dog during (from left to right) early pro-oestrus, pro-oestrus, oestrus (angulation) and metoestrus (early dioestrus; Payan-Carreira et al. 2011).*

6.3.4. Transabdominal ovarian ultrasound

Performing ultrasound examination of the ovary in the domestic dog can be difficult but can be a highly valuable additional technique to time ovulation. Follicles are visible during pro-oestrus as small anechogenic structures, and their size increases to 6-9 mm in the pre-ovulatory period (Fontbonne & Malandain 2006). At the time of ovulation, follicles either completely disappear or become smaller and irregular (Fontbonne & Malandain 2006). However, since there is no obvious difference between pre-ovulatory follicles and early postovulatory corpora lutea, daily ultrasound examination is necessary (Wallace *et al.* 1992, Fontbonne & Malandain 2006). Moreover, some follicles don't collapse (Hayer *et al.* 1993) or ovulate (Wallace *et al.* 1992) and therefore remain visible after ovulation. Furthermore, as the ovary is surrounded by a bursa, it is less visible by ultrasound compared to other species. This is particularly the case for large breeds or obese bitches (Fontbonne & Malandain 2006).

Despite these challenges, daily ultrasound examination could predict ovulation in 91.7% of bitches (Fontbonne & Malandain 2006).

6.3.5. Alternative approaches

Crystallization patterns (ferning) in vaginal fluid from bitches in oestrus has been observed after the peak of plasma oestrogen (England & Allen 1989). Although the window of maximum ferning varied from 2 days before until 8 days after the LH surge, this technique combined with vaginal cytology increased the pregnancy rate to 92 %, compared to 78 % when mating was performed on fixed days after the onset of pro-oestrus (England 1992). In this study however, bitches in the treatment group were mated more frequently than bitches in the control group, probably contributing to the increase in pregnancy rate. Similar crystallization patterns have been observed in saliva from bitches in oestrus (Pardo-Carmona *et al.* 2010), but variation in the timing and amount of ferning was evident indicating that saliva samples may be ineffective for determining the exact timing for insemination.

In foxes, measurement of the electrical resistance of vaginal secretions is routinely used to determine the optimal time for AI. An increase in resistance can be seen during pro-oestrus, with highest conception rates achieved when AI is performed the second day after peak values in electrical resistance have been reached (Farstad *et al.* 1992). In the dog, electrical resistance of vaginal secretions also increases during pro-oestrus and generally remains elevated during ovulation. As in the fox, optimal timing of AI is thought to be at the moment of declining resistance (Gunzel *et al.* 1986).

Non-invasive measurements of reproductive hormones in excretions such as saliva, urine but particularly faeces have becoming a very important tool in wildlife research and management. The measurement of faecal steroid metabolites has already been validated for AWDs (Monfort *et al.* 1997) and adopted in reproductive research (Creel *et al.* 1997, Paris *et al.* 2008, Newell-Fugate *et al.* 2012, Van der Weyde 2013). In the domestic dog, research has investigated the measurement of faecal progesterone metabolites to estimate the timing of the LH surge and the concurrent progesterone rise in the peri-ovulatory period (Hay *et al.* 2000). Both faecal and serum progesterone were found to rise at the same time after ovulation (Hay *et al.* 2000). This means that levels of faecal progesterone metabolites could be used as a non-invasive, indirect method to estimate the time of the LH surge and

insemination in the domestic dog. Levels of faecal progesterone metabolites have also been useful to determine the time of insemination in wild canids such as the red wolf (Walker *et al.* 2002). Unfortunately, there are no reports about the measurement of progesterone in either saliva or urine of domestic dogs. In terms of the AWD, research is urgently needed to investigate the measurement of faecal progesterone combined with behavioural and clinical observations, as a non-invasive tool for timing of ovulation and artificial insemination.

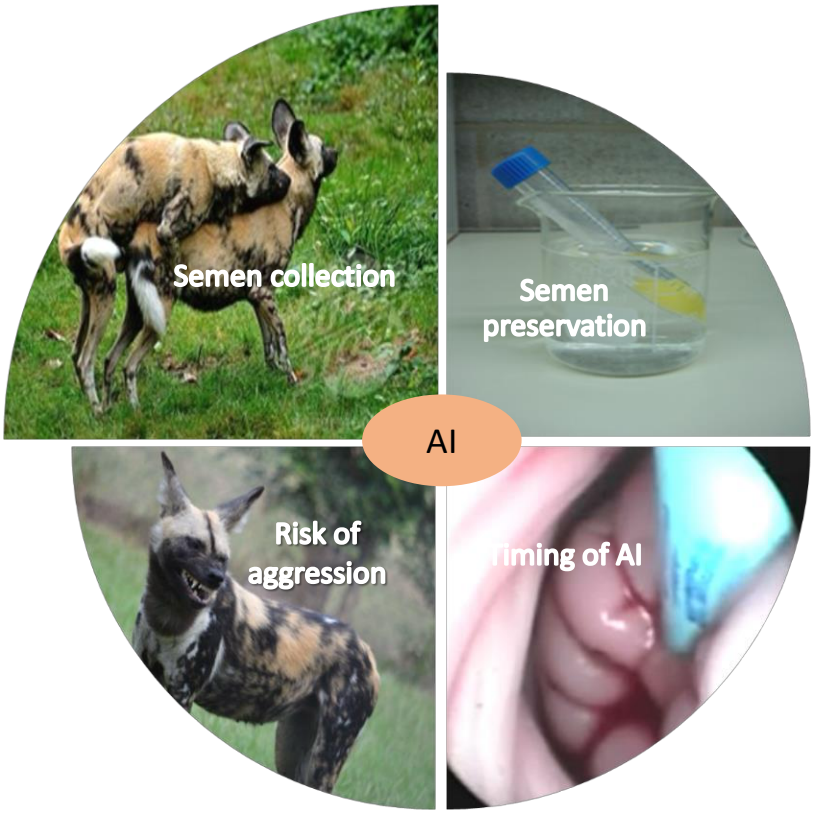
7. Aims of the thesis

Although the use of AI and semen banking can have an enormous impact on AWD breeding management and conservation, such techniques still need to be developed. Thus, the overall aim of this thesis is to develop assisted breeding and behavioural management techniques to enable the application of AI in this species. This will be achieved by addressing the following specific objectives based around the four questions proposed previously (Fig. 1.2.):

1. Clarify the effect of social rank on the fertility of AWD males, in order to identify from which males good quality semen can be collected for AI and semen banking (Chapter 2).
2. Optimise semen freezing in the AWD (Chapter 3).
3. Determine the mitigating effect of Dog Appeasing Pheromone on stress and intra-pack aggression after interventions (Chapter 4).
4. Develop a non-invasive method for timing of AI in AWD females (Chapter 5).

Chapter 2

The effect of social rank on male fertility



Van den Berghe F, Paris MCJ, Sarnyai Z, Briggs MB, Millar RP, Ganswindt A, Paris DBBP. (2018) Social rank does not affect fertility during the breeding season in male African wild dogs (*Lycaon pictus*). *Reproduction, Fertility and Development*, submitted.

Abstract

Sperm banking and artificial insemination could benefit conservation of endangered African wild dogs (AWDs; *Lycaon pictus*). However, it is not clear whether their strict dominance hierarchy causes subfertility in subordinate males that typically do not breed. Our study investigated the effect of social rank on male fertility parameters including hormones (faecal androgen - fAM and glucocorticoid - fGCM metabolite concentrations), prostate and testes volume, preputial gland size, semen collection success, and multiple measures of sperm quality. Samples were obtained from n=12 captive AWDs (4 US packs) in the pre-breeding season and n=24 captive AWDs (n=11 from 4 US packs; n=13 from 3 Namibian packs) in the breeding season. Male social hierarchy could be clearly determined by behavioural observations in all but 1 Namibian pack, and no difference in bodyweight was observed between social ranks. In the pre-breeding season, semen was successfully collected from all alpha but only half the subordinate males, with higher urine contamination associated with lower rank. Fertility parameters did not differ between social ranks, except for progressive motility and normal morphology of spermatozoa, which were lower in subordinates (progressive motility: 27.7 ± 16.8 *vs.* 0.0 ± 0.0%; normal morphology: 59.8 ± 13.0 *vs.* 21.4 ± 5.7% respectively; $P \leq 0.05$). In the breeding season, preputial gland size was positively associated with rank, but no difference was observed in fAM and fGCM concentrations, prostate or testes volume, semen collection success nor urine contamination between all social ranks. Moreover, sperm quality, which generally improved in the breeding season, did not differ between social ranks. In conclusion, reproductive suppression of subordinate males appears to be behaviourally mediated, given that AWD males of all social ranks produce semen of similar quality during the breeding season, making them suitable candidates for sperm banking.

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 & Creel 2002). This species, classified as endangered by the IUCN (Woodroffe & Sillero-Zubiri 2012), requires effective management of the captive and free-living population (Frantzen *et al.* 2001). Development of artificial insemination (AI) 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 & 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, indicating fertility (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 (H-P-G) 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 & Aitken 2005). 'Physiological castration' through higher stress levels in subordinate animals is 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, the level of fertility between dominant and subordinate AWD males warrants further investigation in order to assist 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 male fertility during the pre-breeding and breeding season in the AWD.

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 2.1).

As described in Chapter 4, 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 2.1); 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. Males were immobilised for health assessment and sample collection during the 2014 pre-breeding season (n=12 from 4 US packs: ABQ, BRK, BIN, TOP; May – early July 2014)

and breeding season (n=11 from 4 US packs: ABQ, BRK, BIN, OKC; August – September 2014; Table 2.1). 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 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 2.1). The PLA pack consisted of siblings from different litters, in total 13 males and 4 females of unknown age (Table 2.1). 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. 2.1). 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. 2.1) 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 2.1). 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. Males were immobilised for sample collection during the 2015/2016 breeding season (n=13 from 3 Namibian packs: BRU, PLA, SAN; November 2015 – March 2016; Table 2.1).

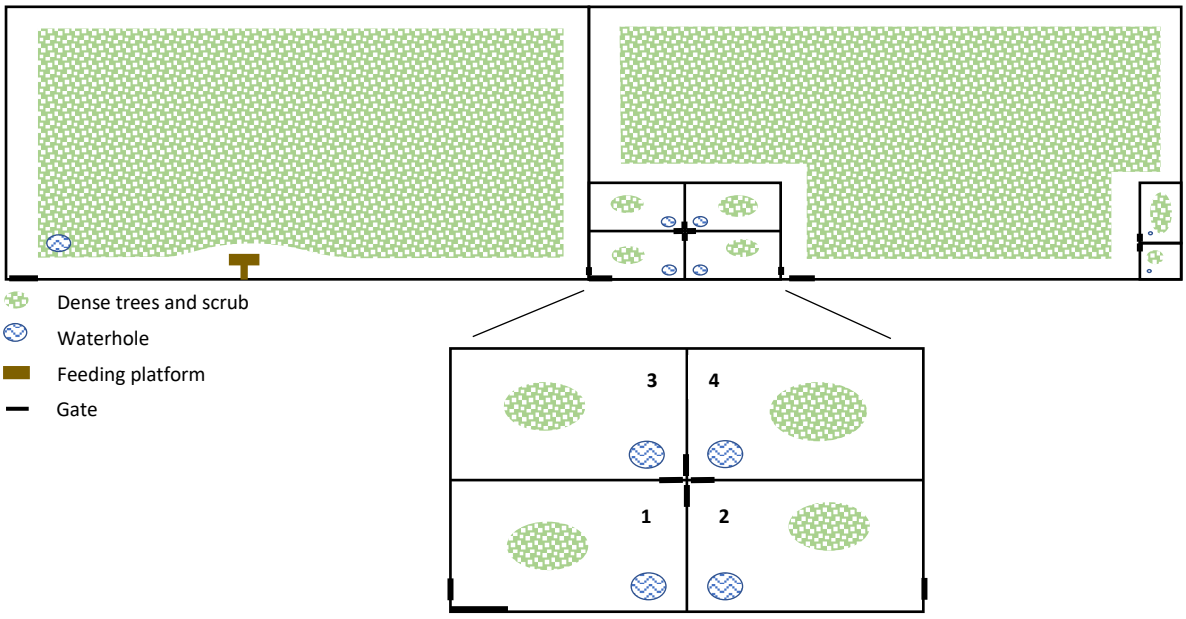


Figure 2.1. *Layout of the 14.4 ha Platform pack enclosure (left) and 4 adjacent 0.1 ha pens used for behavioural observations, faecal sample collection and capture.*

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 2.1). 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. 2.1) and filmed from a car parked in pen 1. Behaviour was recorded at times AWDs were most active in the individual settings; generally, 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 (Chapter 4). Males were then classified as alpha, beta, or gamma male within the pack based on the frequency of dominant *vs.* submissive behaviours respectively (Fig. 2.3 and Table 2.2; Chapter 4).

Table 2.1. *Pack composition, social rank, observation and sampling times of African wild dogs included in this study.*

						Total observation time (h)		Immobilisation date	
Pack	**ID - Name**	**Sex**	**Social rank***	**Age (y)**	**Relationship with conspecifics**	**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.

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 until the day of the immobilisation of males, using methods described in Chapter 4. Samples were oven-dried and transported to the University of Pretoria (South Africa) for steroid analysis (Chapter 4). 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 in Chapter 4. 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 analysis. All steroid extracts were measured for immunoreactive faecal glucocorticoid metabolite (fGCM) and faecal androgen metabolite (fAM) concentrations (validated in Chapter 4) using established enzyme-immunoassays (EIAs; Palme & Mostl 1994, Palme & 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

(Chapter 4). Anaesthetic protocols were as described in Chapter 3. 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. 2.1). 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 h. 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 2.1). 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. 2.2a-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 a 6 MHz transducer, except for the

PLA pack where a CTS-900V V1.39 ultrasound (SIUI, Guandong, China) with a 5 MHz 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). 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.



Figure 2.2. *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).*

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 Chapter 3. 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 Chapter 3. 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

Raw data was analysed for normal distribution and $\log_{10}$ transformed if necessary. Where transformation did not result in normal distribution, raw data was analysed by non-parametric tests. In the pre-breeding season, data was collected from all 3 males in each pack and differences in social rank were analysed by repeated measure ANOVA with 'rank' as repeated unit and 'pack' as the subject. Age differences between young (42-43 months) and old (81-93 months) animals were compared by independent sample t-test. Given that semen collection success was limited in subordinate animals at this timepoint, sperm data from beta and gamma males were grouped as 'subordinate' and compared to that from the alpha 'dominant' male using an independent sample t-test or a Mann-Whitney U test. Changes from the pre-breeding to breeding season were analysed by paired sample t-test or Sign test (motility rating) using only data from dogs sampled in both seasons. In the breeding season, an unequal number of alpha, beta and gamma males in each pack made it impossible to perform repeated measure ANOVA (except for US packs). Therefore, differences between social ranks were compared using one-way ANOVA or Kruskal-Wallis test. Differences in body weight, hormone concentrations, and prostate and testes volume between US and Namibian animals were compared by independent sample t-test. The relationships between body weight, age (males of unknown age excluded), hormone concentrations, and testis or prostate volume were tested using Pearson or Spearman correlation. Differences between social rank in sperm collection success, urine contamination, preputial gland development, and motility rating were evaluated by Chi-square to test for trends. $P \leq 0.05$ was considered significant and

all data is presented as mean ± SEM unless otherwise noted. Statistical analysis was performed with SPSS Statistics 23 (IBM® SPSS® Statistics 23, SPSS Inc., IBM, Armonk, New York, USA).

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. 2.3a). 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. 2.3a). 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. 2.3a). 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. 2.3a). 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 each other (Fig. 2.3b). 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. 2.3b). 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. 2.3b). No clear hierarchy was present in the PLA pack, with most males both giving and receiving dominant and submissive behaviours (Table 2.2). 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 2.2).

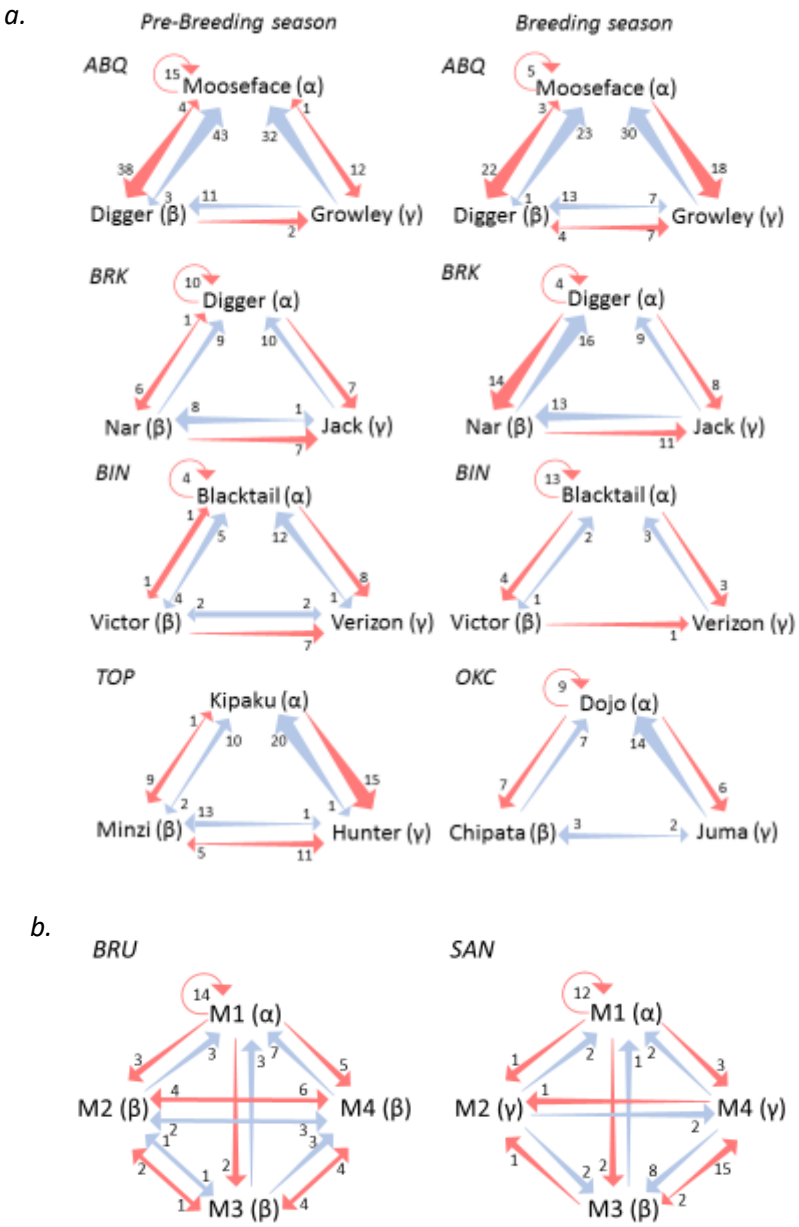


Figure 2.3. *Classification of social hierarchy based on the frequency of dominant (red arrows), submissive (blue arrows) and scent marking (round red arrows) behaviour in the (a) US packs during the pre-breeding and breeding season, and (b) BRU and SAN packs of Namibia during the breeding season. Arrow heads indicate direction of behaviour from actor to recipient. Numbers next to arrows indicate the frequency of a behaviour within the total observation time.*

Table 2.2. *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.

3.2. Effect of social rank on male fertility in the pre-breeding season

Mean body weight of all dogs (n=12, 4 packs) collected during the pre-breeding season was 30.8 ± 0.5 kg and did not differ between ranks (alpha 30.1 ± 1.2, n=4 *vs.* beta 31.2 ± 0.9, n=4 *vs.* gamma 31.2 ± 0.7 kg, n=4; $P = 0.691$). fAM and fGCM concentration was 0.46 ± 0.06 µg/g and 38.1 ± 2.8 ng/g DW respectively. Prostate and testes volume was 4.6 ± 0.6 cm^3 and 10.2 ± 1.3 cm^3 respectively and preputial gland score was 1.5 ± 0.4.

There was no difference in fAM or fGCM concentrations, or prostate and testes volume between males of different social rank (Fig. 2.4a), or between younger (42-43 months) and older (81-93 months) males (fAM: 0.58 ± 0.08 *vs.* 0.44 ± 0.08 µg/g; fGCM: 39.3 ± 5.4 *vs.* 33.5 ± 3.3 ng/g; prostate volume: 3.8 ± 0.6 *vs.* 5.2 ± 0.9 cm^3; $P > 0.05$). However, testes volume was lower for younger animals (5.5 ± 0.4 *vs.* 13.6 ± 0.8 cm^3; $P < 0.001$). fAM concentration was not correlated with prostate ($r = 0.025$, $P = 0.938$, n = 12) or testes volume (r = -0.400, $P = 0.197$, n = 12), and body weight was not correlated with prostate (r = 0.496, $P = 0.101$, n = 12) or testes volume (r = 0.405, $P = 0.192$, n = 12). Preputial gland development tended to be positively associated with dominance, but this was not significant (alpha 2.5 ± 0.5, n=4 *vs.* beta 1.25 ± 0.5, n=4 *vs.* gamma 0.75 ± 0.75, n=4; $X^2 = 3.546$, df = 1, $P = 0.060$).

Spermatozoa could be collected from 8 out of 12 dogs (67%), including all dominant males but only half of the beta and gamma males (Fig. 2.5a). 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 was negatively associated with rank (X^2 = 4.641, df = 1, *P* = 0.031; Fig. 2.5a). 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). Overall sperm quality was low with on average 17.3 ± 10.2% total motility, 12.8 ± 8.5% progressive motility, sperm motility index of 45.7 ± 29.2, and motility rating of 1.2 ± 0.6. The total number of ejaculated spermatozoa was 27.4 ± 11.5 x 10^6, with 40.6 ± 9.8% normal morphology, 63.1 ± 5.1% sperm viability, and 72.6 ± 5.2% acrosome integrity. As spermatozoa were only present in half of the beta and gamma males, these two groups were combined to enable statistical comparison with data from the alpha male (Table 2.3). Progressive motility and normal sperm morphology were significantly lower (and several other motility parameters near significance) in subordinate males (Table 2.3), but this can be attributed to higher rates of urine contamination at collection in these males. No significant differences could be seen in any other sperm quality parameters (Table 2.3).

Table 2.3. *Mean (± SEM) sperm quality between dominant and subordinate African wild dogs during the pre-breeding and breeding season.*

	Pre-breeding season		***Breeding season***		
Parameter	***Dominant (n=4)***	***Subordinate (n=4)***	***Alpha (n=6)***	***Beta (n=5)***	***Gamma (n=9)***
Total motility (%)	32.1 ± 18.4	2.5 ± 2.5	46.7 ± 12.3	46.0 ± 13.2	48.6 ± 11.4
Progressive motility (%)	27.7 ± 16.8[a]	0.0 ± 0.0[b]	29.4 ± 10.4	26.9 ± 14.5	33.2 ± 8.6
Sperm motility index (0-400)	88.9 ± 52.3	2.5 ± 2.5	107.9 ± 36.4	107.9 ± 46.8	118.5 ± 30.7
Motility rating (1-4)	2.3 ± 1.0	0.3 ± 0.3	1.6 ± 0.6	2.2 ± 0.7	2.1 ± 0.6
Total no. ejaculated sperm (x 10^6)	26.1 ± 20.6	28.8 ± 13.8	28.6 ± 14.4	5.4 ± 1.9	49.6 ± 16.5
Normal sperm morphology (%)	59.8 ± 13.0[a]	21.4 ± 5.7[b]	49.1 ± 11.9	44.0 ± 8.2	56.0 ± 7.6
Sperm viability (% alive)	70.3 ± 4.4	55.9 ± 8.1	73.1 ± 6.7	78.2 ± 4.7	73.1 ± 8.1
Sperm acrosome (% intact)	77.9 ± 7.4	67.3 ± 7.2	87.4 ± 3.0 (n=5)	80.3 ± 7.1	87.6 ± 4.8
DNA integrity (%)	-	-	99.8 ± 0.1 (n=3)	99.9 ± 0.1 (n=4)	99.6 ± 0.1

Values with different letters for each parameter are significantly different within a season (*P* ≤ 0.05).

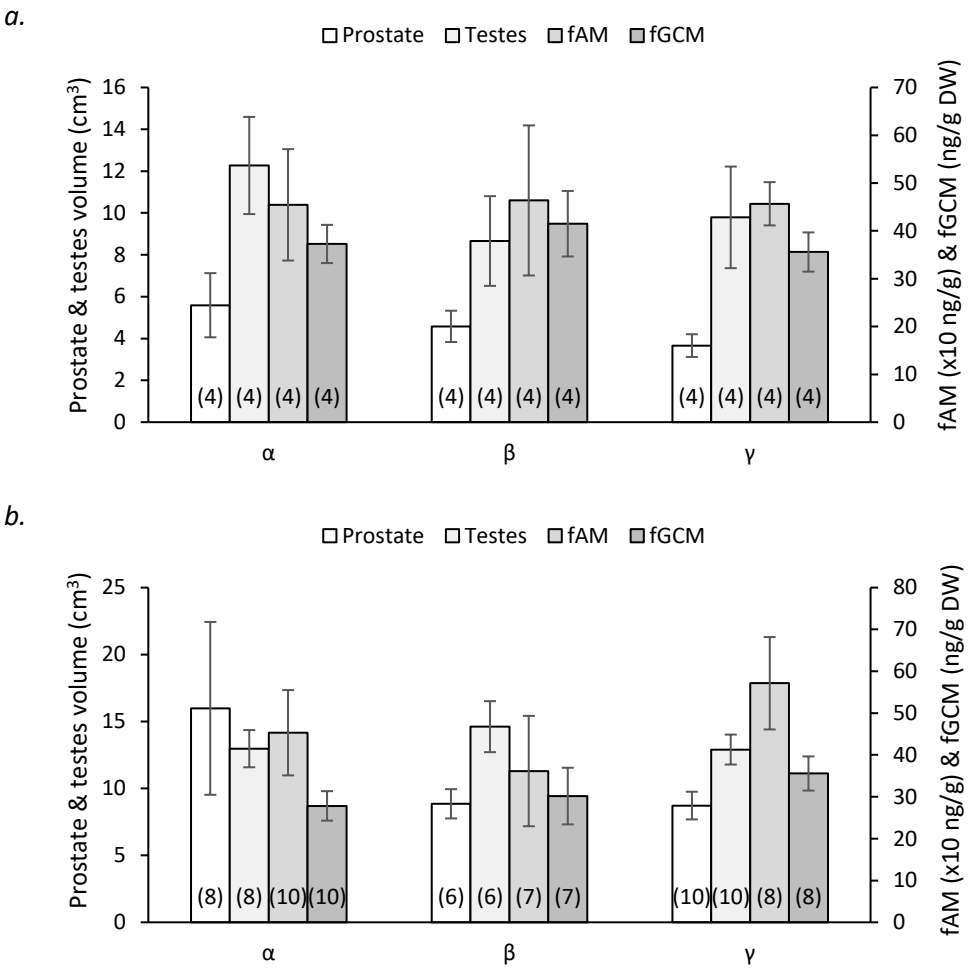


Figure 2.4. *Prostate and testes volume, and fAM and fGCM concentrations in African wild dogs grouped by social rank during the (a) pre-breeding and (b) breeding season. Numbers in parenthesis indicate sample size.*

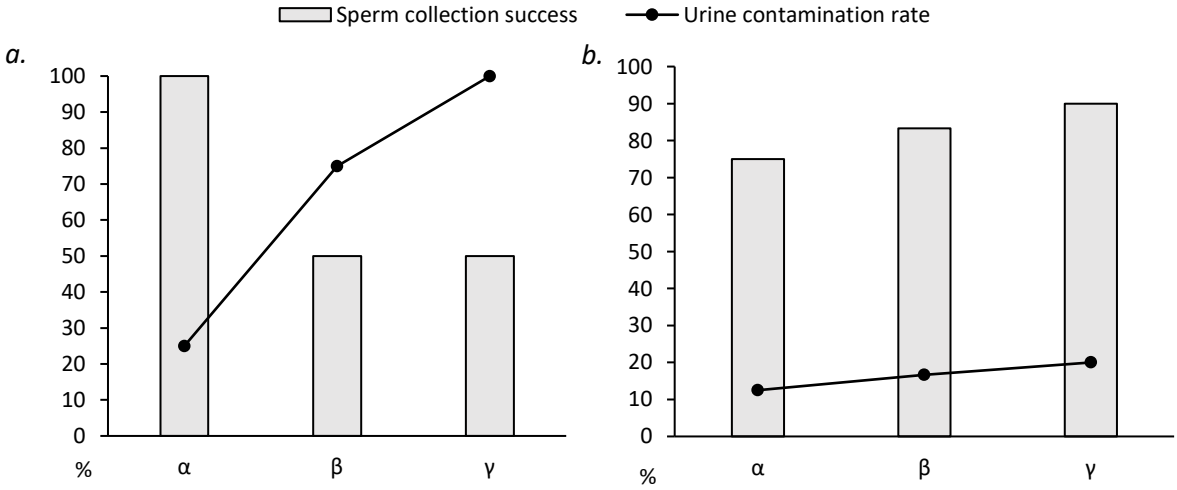


Figure 2.5. *Collection success and rate of urine contamination in sperm samples from African wild dogs grouped by social rank during the (a) pre-breeding and (b) breeding season.*

3.3. Effect of season on male fertility

A total of 8 dogs from 3 different packs were sampled twice and could be directly compared between both the pre-breeding and breeding season. Although the BRK alpha male was not sedated for sample collection in the breeding season, his faecal samples were still collected for hormonal analysis. fAM concentrations were higher in the breeding season in 6 out of the 9 AWDs but the overall increase was not significant (Table 2.4), while fGCM did not change between seasons. Prostate and testes volume increased in the breeding season (Table 2.4), which was most pronounced in gamma and beta males respectively (Fig. 2.6). The size of the preputial gland only increased in the breeding season in one male. Overall sperm quality was better in the breeding season with a significant increase in total motility, sperm motility index, total number of ejaculated spermatozoa and sperm viability (Table 2.4). Moreover, several other parameters (progressive motility and normal morphology) were near significant (Table 2.4).

Table 2.3. *Seasonal changes in mean (± SEM) reproductive parameters and sperm quality in African wild dogs (paired data).*

	Season		
Parameter	***Pre-breeding***	***Breeding***	***P-value***
Weight (kg; n=8)	31.7 ± 0.4	32.6 ± 0.4	0.069
fAM (µg/g; n=9)	0.49 ± 0.08	0.70 ± 0.10	0.110
fGCM (ng/g; n=9)	37.1 ± 3.7	40.6 ± 4.1	0.374
Prostate volume (cm^3; n=8)	4.6 ± 0.5^b	9.4 ± 1.5^a	0.012
Testes volume (cm^3; n=8)	11.8 ± 1.4^b	17.7 ± 1.0^a	0.012
Preputial gland size	1.8 ± 0.4	1.8 ± 0.4	
Total motility (%; n=5)	13.8 ± 11.2^b	62.4 ± 15.4^a	0.043
Sperm collection success (%; n=8)	75.0	87.5	
Progressive motility (%; n=5)	8.3 ± 8.1	41.4 ± 12.3	0.068
Sperm motility index (0-400; n=5)	30.7 ± 27.3^b	146.3 ± 44.8^a	0.043
Motility rating (1-4; n=5)	1.2 ± 0.7	2.2 ± 0.7	0.625
Total no. ejaculated sperm (x 10^6; n=5)	21.3 ± 12.2^b	60.7 ± 23.6^a	0.043
Normal sperm morphology (%; n=5)	41.0 ± 11.6	72.2 ± 6.0	0.080
Sperm viability (% alive; n=5)	55.6 ± 5.8^b	84.2 ± 5.8^a	0.031
Sperm acrosome (% intact; n=5)	73.7 ± 7.8	84.7 ± 7.7	0.223

Values with different letters for each parameter are significantly different ($P \leq 0.05$).

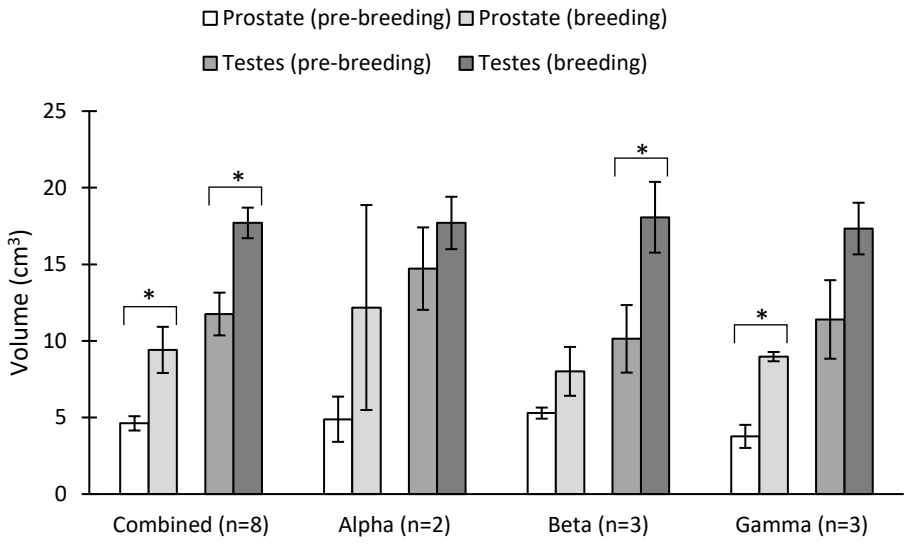


Figure 2.6. *Seasonal changes in prostate and testis volume in African wild dogs combined and grouped by social rank (paired data). *significantly different (P ≤ 0.05).*

3.4. Effect of social rank on male fertility in the breeding season

Mean body weight of all dogs (n=24, 7 packs) collected during the breeding season was 28.6 ± 0.9 kg, and did not differ between ranks for all (alpha 29.3 ± 1.5, n=8 *vs.* beta 29.3 ± 1.9, n=6 *vs.* gamma 27.7 ± 1.5 kg, n=10; $P = 0.668$), US (alpha 31.1 ± 0.6 *vs.* beta 32.5 ± 0.9 *vs.* gamma 30.9 ± 1.9 kg, n=3 in each group; $P = 0.397$), or Namibian AWDs (alpha 28.2 ± 2.3, n=5 *vs.* beta 23.6 ± 1.9, n=2 *vs.* gamma 24.9 ± 1.3 kg, n=6; $P = 0.298$). US AWDs were heavier than those in Namibia (31.7 ± 0.6, n=11 *vs.* 26.0 ± 1.1 kg, n=13; $P < 0.001$). fAM and fGCM concentration was 0.47 ± 0.06 µg/g and 31.0 ± 2.7 ng/g DW (n=25) respectively. Prostate and testes volume was 11.2 ± 2.2 cm^3 and 13.4 ± 0.8 cm^3 respectively, and overall preputial gland score was 1.9 ± 0.2 (n=24).

There was no difference in fAM and fGCM concentrations, or prostate and testes volume between males of different social rank (Fig. 4b; $P = 0.272$; $P = 0.537$; $P = 0.490$, and $P = 0.672$ respectively), however, preputial gland size was positively associated with dominance (alpha 2.4 ± 0.2, n=8 *vs.* beta 1.7 ± 0.3, n=6 *vs.* gamma 1.6 ± 0.3, n=10; $X^2 = 4.018$, df = 1, $P = 0.045$). AWDs in the US had higher fAM and fGCM concentrations, as well as testes volume, but not

prostate volume or preputial gland size, than those in Namibia (fAM: 0.62 ± 0.09 *vs.* 0.33 ± 0.08 µg/g; $P = 0.014$; fGCM: 40.7 ± 3.1 *vs.* 21.9 ± 2.3 ng/g; $P < 0.001$; testes volume: 15.8 ± 1.2 *vs.* 11.3 ± 0.6 cm^3; $P = 0.006$; prostate volume: 8.6 ± 1.2 *vs.* 13.4 ± 4.0 cm^3; $P = 0.235$; preputial gland size: 1.7 ± 0.3 *vs.* 2.0 ± 0.1; $P = 0.404$). However, fAM concentrations or testes volume did not differ by social rank in either population ($P > 0.05$). fGCM concentrations on the other hand were lower in beta compared to gamma males in the Namibia population (alpha 20.9 ± 3.8, n=6 *vs.* beta 14.2 ± 1.0, n=3 *vs.* gamma 29.2 ± 2.0, n=4; $P = 0.034$). In the US population, fGCM concentrations were higher in younger (35-45 months; 49.1 ± 4.9 ng/g DW; n=5) than older (83-95 months; 34.8 ± 1.9 ng/g DW; n=7; $P = 0.012$) males. However, there was no correlation between age and fAM concentrations ($r = 0.020$, $P = 0.950$, n = 13), fGCM concentrations ($r = -0.529$, $P = 0.063$, n = 13), prostate volume ($r = 0.502$, $P = 0.080$, n = 13) or testes volume ($r = 0.340$, $P = 0.255$, n = 13) among all AWDs of known age. There was no correlation between fAM concentrations and prostate ($r = -0.075$, $P = 0.740$, n = 22) or testes volume ($r = 0.294$, $P = 0.184$, n = 22). Body weight was not correlated with prostate ($r = -0.161$, $P = 0.451$, n = 24), but was positively correlated with testis volume ($r = 0.582$, $P = 0.003$, n = 24).

Spermatozoa could be collected from 20 out of 24 dogs (83.3%), with more than 75% success from males of all social ranks (Fig. 2.5b). Urine contamination of the sperm sample only occurred in 4 out of 24 males (17%) during the breeding season and, in contrast to the pre-breeding season, was not associated with rank ($X^2=0.172$, df=1, $p=0.678$; Fig. 2.5b). Overall sperm quality increased with 47.4 ± 6.7% total motility, 30.5 ± 5.8% progressive motility, sperm motility index of 112.7 ± 20.0 and motility rating of 2.0 ± 0.4 (n=20). The total number of ejaculated spermatozoa was 32.3 ± 9.2 x 10^6, with 50.9 ± 5.2% normal morphology, 74.4 ± 4.2% sperm viability (n=20), 85.6 ± 3.0% acrosome integrity (n=19) and 99.7 ± 0.1% sperm DNA integrity (n=16). No significant differences could be seen between social rank in any of the sperm quality parameters (Table 2.3).

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. We found that subordinate males are at higher risk of urine contamination of the semen sample and have lower sperm motility and normal morphology when collected in the pre-breeding season. However, in the breeding season, no differences in sperm quality could be seen between males of different social rank. This implies 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 H-P-G 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 short-term and longer-term studies conducted in semi-natural captivity (de Villiers *et al.* 1997), and the wild (Van der Weyde 2013). Moreover, a long-term study running over 2 years showed that 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), as well as the length of sample collection. However, using fGCM and sperm quality data, our results coupled with these previous studies, dismiss the role of elevated levels of the stress hormone cortisol as a putative mechanism for reproductive suppression in subordinate 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 H-P-G axis is functioning normally in all pack males. In addition, with the exception of preputial gland size, there was no significant difference in any other reproductive parameter between males of different social rank during the breeding season. Therefore, we conclude that reproductive suppression in males must be behaviourally mediated in 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 dominant males had higher sperm motility and normal morphology compared to subordinate animals. This is probably related to higher rates of urine contamination during sperm collection in the subordinates (7 out of 8), widely known to negatively affect sperm motility and cause secondary defects in sperm morphology (Chen *et al.* 1995, Kim & Kim 1998, Blanco *et al.* 2002, Santos *et al.* 2011, O'Brien *et al.* 2013). Although prostate size did not differ between social ranks, animals with urine contamination of the sperm samples tended to have smaller prostates. There is a higher risk of bladder stimulation, contraction and urine release during the electroejaculation procedure when prostate size is smaller, since the rectal probe (with 3-4 cm long electrodes) is normally positioned at the level of the prostate proximal to the bladder (Chapter 3). In this regard, prostate size generally increased in the breeding season and was associated with a much lower prevalence of urine contamination.

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 increased in 6 out of 9 animals during this transition but the overall increase was not significant. Similarly, androgen concentrations did not increase at this time in some studies (Creel *et al.* 1997, Johnston *et al.* 2007), while in others (Monfort *et al.* 1997, Newell-Fugate *et al.* 2012) fAM values were highest in December

(Southern hemisphere) or from July to September (Northern hemisphere), followed by a gradual decrease towards the non-breeding season. We also 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, which was most obvious in subordinate males. This was accompanied by an increase in sperm motility, number and viability, 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 in 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 the semen quality could be seen after collection using digital manipulation or electroejaculation (Ohl *et*

al. 1994, Christensen *et al.* 2011). However, different electroejaculation protocols or even different semen collection methods, need to be explored to try and improve semen quality and semen collection success. In addition, methods to avoid urine contamination of samples such as avoiding the use of alpha-2 adrenergic receptor agonists for immobilization when performing electroejaculation, coupled with draining and flushing the bladder prior to semen collection can help improve semen collection success (Johnston *et al.* 2007, Talukder & Hikasa 2009). In other carnivores, body weight, age and breeding history (Rijsselaere *et al.* 2007), as well as the level of inbreeding (Pukazhenthi *et al.* 2006, Lockyear *et al.* 2016) and diet (Santymire *et al.* 2015) are all related to sperm quality. In addition, low ejaculation frequency and ejaculation after a long period of sexual abstinence can result in low sperm quality at first sperm collection (Imrat *et al.* 2014).

In the US during the breeding season, fGCM concentrations were higher in younger (35-45 months) than older (83-95 months) adults. Subadults (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 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 this regard, we found captive US males had higher fGCM levels than Namibian males, held in larger more semi-natural enclosures. This has also been reported to occur in captive female AWDs in European zoos compared to free-ranging females (Van der Weyde 2013). Our observations could be due to a variety of factors, such as larger enclosure sizes, a natural climate and habitat, a larger and more natural pack composition, and different nutrition in the Namibian packs.

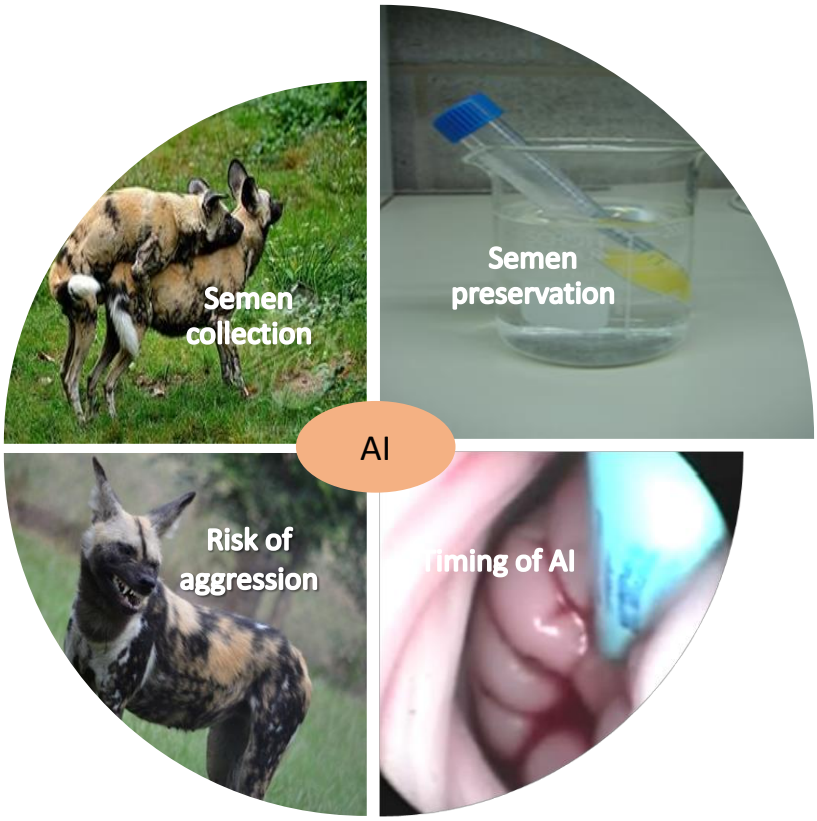
In our study, the breeding season was defined as the time mating occurs. In captive AWDs in the northern hemisphere, this is around August/September (late summer), with most births occurring in November (Van den Berghe *et al.* 2012). Therefore, AWDs in the US were collected in August and September for 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 highest male fertility 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, 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).

Chapter 3

Improved sperm freezing in the African wild dog



Van den Berghe F, Paris MCJ, Briggs MB, Farstad WK, Paris DBBP. (2018) A two-step dilution tris-egg yolk extender containing Equex STM significantly improves sperm cryopreservation in the African wild dog (*Lycaon pictus*). *Cryobiology*, 80: 18-25.

Abstract

Conservation management of endangered African wild dogs (AWD; *Lycaon pictus*) can benefit greatly from development of sperm freezing and artificial insemination. Previous freezing attempts yielded nearly 0% motile sperm within 2h of thawing. In this study, two canine freezing protocols were tested: Protocol 1: a one-step dilution in TRIS-20% egg yolk containing 8% glycerol; and Protocol 2: a two-step dilution in TRIS-20% egg yolk containing a final extender concentration of 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution. Semen was collected by electroejaculation from n=24 AWDs, of which 8 ejaculates of sufficient quality (4 good quality with initial sperm motility of 75.0 ± 4.4% and 4 poor quality; showing rapid decrease in sperm motility to 3.3 ± 3.3% prior to freezing) were frozen. For good quality samples, motility and sperm motility index persisted for up to 8h for Protocol 2, and was higher between 2-6h after thawing with a decrease from 4h of incubation. Motility dropped to nearly 0% after 2h incubation for Protocol 1. Viability was higher for Protocol 2 throughout the 8h of incubation, with a decrease after 6h, compared to 4h for Protocol 1. Acrosome integrity was higher for Protocol 2 throughout post-thaw incubation, with a decrease after 2h for both protocols. Protocols did not differ in normal sperm morphology or DNA integrity. Poor quality samples yielded similar results, except for acrosome integrity, which declined for Protocol 2. In conclusion, a two-step dilution in TRIS-egg yolk-glycerol extender containing Equex STM yields significantly improved post-thaw quality and longevity of AWD spermatozoa, making it suitable for sperm banking and artificial insemination initiatives.

1. Introduction

Over the last four decades, there has been a drastic 58% decrease in vertebrate wildlife numbers around the globe (WWF, 2016). The primary cause for this decline is human interference, particularly habitat loss and destruction (Gonzalez-Suarez & Revilla 2014, Martinez-Ramos *et al.* 2016, McCormick & Allan 2017). For many species, captive breeding has become extremely important to ensure their survival and to maintain their genetic diversity. However, the success of captive breeding programs is often limited by husbandry related issues such as species-specific enclosure requirements, nutritional deficiencies, and abnormal group structures that can impede natural breeding and/or reproductive health (Lasley *et al.* 1994, 2009). Assisted reproductive techniques are an important aid in overcoming some of the limitations inherent to the captive breeding of non-domestic animals (Lasley *et al.* 1994, 2009). Artificial insemination (AI) and sperm freezing are the most accessible and commonly used techniques and are already well-established tools for the breeding management of domestic animals (Durrant 2009, Mastromonaco *et al.* 2011, Jewgenow *et al.* 2017). Progressively, these techniques have been incorporated into the captive breeding programs of a wide range of wildlife species (2009). The major advantages of sperm freezing include: (i) securing genetic diversity; (ii) extending the fertility of a genetically important animal beyond its lifespan; and (iii) allow easy transport of semen across the globe (Rijsselaere *et al.* 2011, Van den Berghe *et al.* 2012).

The African wild dog (AWD, *Lycaon pictus*), once roaming most of sub-Saharan Africa, has disappeared from most of its original range with the current population now estimated at a mere 6,600 animals (Woodroffe & Sillero-Zubiri 2012). Sperm freezing, and development of AI techniques, can aid species management and conservation of the AWD (Van den Berghe *et al.* 2012). Sperm freezing in this species has been attempted previously by either diluting semen 1:3 in TEST (TES and Tris) buffer containing 7.5% glycerol and 15% egg yolk (EY) (Hermes *et al.* 2001); or by two-step dilution with the first dilution in Tris-citrate buffer containing 20% EY, and the second dilution after cooling to 5 °C in the same extender but containing 8% glycerol (Johnston *et al.* 2007). Initially, both protocols gave good immediate post-thaw sperm motility of up to 40% and 31.8 ± 5.8% respectively. However, within 2 h of thawing, sperm motility declined rapidly to nearly 0%. AI using spermatozoa with such a short motile lifespan is unlikely to lead to successful fertilization in the ampullae of the oviducts.

In domestic dogs, sperm motility and normal morphology are parameters typically used to determine the quality of frozen-thawed samples used for AI (Niżański 2006, Thomassen *et al.* 2006, Mason & Rous 2014). However, other sperm quality parameters such as viability, acrosome integrity and DNA integrity should be evaluated with equal importance. The sperm acrosome is crucial for zona pellucida penetration and fertilization (Bedford 1970), however it is often damaged during freezing, but such damage is not detected by a corresponding decline in motility (Oettlé 1986). Moreover, spermatozoa with damaged DNA do not necessarily show a decline in other sperm quality parameters, but such damage can significantly impair embryo development and cause pregnancy loss (Seli *et al.* 2004, Lewis & Aitken 2005, Paul *et al.* 2008, Peña *et al.* 2016).

There are species-, breed- and even individual differences in the resistance of the sperm cell to cold shock and the freeze-thaw process (Holt 2000, Waterhouse *et al.* 2006). However, when developing sperm freezing techniques for a new species, it is advisable to start with testing semen extenders and freezing protocols that give good results in closely related species (Comizzoli *et al.* 2012). The AWD belongs to the Canidae but is taxonomically quite distinct (the only species in the genus *Lycaon*) and relatively distantly related to the other canids (Wayne *et al.* 1997). However, the domestic dog can be used as an important model for the development of sperm freezing protocols. In the dog, the most commonly used laboratory-prepared extenders are still TRIS-citrate-fructose or -glucose based (Peña & Linde-Forsberg 2000, Thomassen *et al.* 2006, Varesi *et al.* 2014). EY is added to the semen extenders of most species, usually at a concentration of 20% for domestic dogs (Rota *et al.* 1997, Silva *et al.* 2002, Martins-Bessa *et al.* 2006). EY is a complex biological substance containing proteins, phospholipids, vitamins, glucose, antibacterial compounds and antioxidants (Farstad 2009); presumingly protecting the sperm plasma membrane against cold shock by interacting with the phospholipid bilayer to prevent or restore the loss of phospholipids (Watson 1995, Farstad 2009). The type of cryoprotectant added to the semen extender may vary for different species but for most, glycerol yields the best freezing outcomes; although toxic in high concentrations (Curry 2000, Holt 2000). Glycerol replaces intracellular water during dehydration (Holt 2000), reduces the extracellular freezing point by decreasing the electrolyte concentration, and integrates into the sperm plasma membrane changing its stability and permeability (Hammerstedt & Graham 1992, Holt 2000, Leahy & Gadella 2011).

In domestic dogs, optimal post-thaw semen quality has been achieved using glycerol concentrations between 2 - 8% (Olar *et al.* 1989, Peña *et al.* 1998, Rota *et al.* 1998).

The addition of sodium dodecyl sulphate (SDS), an anionic detergent, to EY-based extenders was beneficial to post-thaw sperm motility, viability, acrosome integrity and longevity in a wide range of species, including domestic dogs (Rota *et al.* 1997, Peña *et al.* 1998, Peña & Linde-Forsberg 2000, Tsutsui *et al.* 2000, Nizanski *et al.* 2001). The exact mechanism underlying this beneficial effect is not known, but SDS is believed to interact with molecules present in EY, stabilising the sperm membrane and increasing its permeability, and thereby avoiding osmotic shock (Rota *et al.* 1997, Bencharif *et al.* 2010). SDS has been added to extenders in its pure form (Peña *et al.* 1998), or as a component of Equex STM (Rota *et al.* 1997), Equex paste (Peña *et al.* 2003), or Orvus ES paste (Tsutsui *et al.* 2000, Nizanski *et al.* 2001). However, the beneficial effect of SDS appears to be influenced by additional factors in these additives. When using Equex STM in the domestic dog, an increased sperm longevity and viability was observed during post-thaw incubation, however these effects were absent when using Equex paste at the same dosage (Peña *et al.* 2003). Furthermore, Equex paste did not show any beneficial effects in generic grey and Mexican grey wolves (*Canis lupus* and *C.lupus baileyi;* Zindl *et al.* 2006). By contrast, post-thaw motility, plasma membrane integrity and acrosome integrity of spermatozoa are greatest when Equex STM was added at the end of the equilibration period (Peña & Linde-Forsberg 2000), making a two-step dilution important when using this additive. In addition, this two-step procedure has also been proposed to enable cooling and equilibration of spermatozoa in lower initial glycerol concentrations, thereby minimizing its toxic effect (Peña & Linde-Forsberg 2000).

The aim of this present study was to improve the longevity of AWD spermatozoa after thawing by testing two sperm freezing protocols which routinely yield high quality frozen-thawed spermatozoa in domestic dogs (Peña & Linde-Forsberg 2000, Thomassen *et al.* 2006): Protocol 1 - a one-step dilution in TRIS - 20% EY extender containing 8% glycerol, without subsequent thawing solution (one-step - thawing solution); and Protocol 2 - a two-step dilution in TRIS - 20% EY extender to a final concentration of 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution (two-step + thawing solution).

2. Materials and methods

2.1. Animals

Semen was collected by electroejaculation from a total of n=24 adult AWDs (≥2.8 years old; Table 3.1) from 4 US zoos: Albuquerque BioPark (ABQ pack; Albuquerque, NM), Brookfield Zoo (BRK pack; Chicago, IL), Binder Park Zoo (BIN pack; Battle Creek, MI), and Oklahoma City Zoo (OCZ pack; Oklahoma City, OK) during the 2014 breeding season (August-September); and from Namibia: Harnas Wildlife Foundation (BRU, PLA and SAN packs, Gobabis) during the 2015-2016 breeding season (November, January and March respectively). AWDs in the US were held in packs containing three males except for OCZ, which also contained a female. Animals were held in outside enclosures during the day with access to inside holding areas overnight, except at BIN in which animals were locked inside their holding area overnight. Animals in Namibia were held in large natural enclosures in mixed-sex packs. All animals had access to water *ad libitum* and in the US were individually-fed with ground horsemeat (Central Nebraska Packing Inc., NE, USA), occasionally replaced by bones, whole pig or goat carcass. In Namibia, animals were group-fed with donkey and horsemeat on the bone or intestines (local slaughter material), occasionally replaced by dog pellets (Hill's Pet Nutrition, Kansas, United States), or goat, sheep or wild game meat.

2.2. Anaesthesia

In the US, AWDs were immobilised with 1.2 - 4.0 mg/kg tiletamine/zolazepam hydrochloride (40 - 120 mg IM; Telazol®, Zoetis Inc., MI, USA) with or without 0 – 6.3 µg/kg medetomidine (0 - 0.4 mg IM; Medetomidine HCl®, ZooPharm, WY, USA), and maintained with Isoflurane (0.5-5% in 1 l/min O_2; IsoFlo®, Zoetis Inc., MI, USA; Isothesia®, Henry Schein Animal Health, OH, USA; or Isoflurane®, MWI Animal Health, ID, USA). In cases where medetomidine was administered, AWDs were reversed with atipamezole (0.01 - 0.1 mg/kg, IM; Antisedan®, Zoetis Inc., MI, USA) after intubation and/or at the end of anaesthesia. In Namibia, all animals were sedated with an initial dose of 3.9 - 7.5 mg/kg tiletamine/zolazepam hydrochloride (100 - 180 mg IM; Zoletil® 100; Virbac, Carros, France) with, where needed, the addition of another 20 - 40 mg IM to maintain the level of anaesthesia. In these animals, no medetomidine was administered.

Table 3.1. *Animal details and semen quality of African wild dogs (Lycaon pictus) included in this study.*

Pack	*ID - Name*	*Date of birth*	*Date of collection*	*Body weight (kg)*	*Semen volume (ml)*	*Total spermatozoa ejaculated (x 10^6)*	*Sperm motility (%)*	*Frozen*	*Comments/reason for not freezing*
ABQ	2393 - Mooseface	8 Nov 2006	8 Aug 2014	31.8	5.43[a]	58.3	83	Yes[d]	-
	2394 - Digger	8 Nov 2006	7 Aug 2014	32.0	0.00	-	-	No	No ejaculate
	2395 - Growlly	8 Nov 2006	7 Aug 2014	32.4	4.25[a]	142.4	74	Yes[d]	-
BRK	2494 - Nar	25 Nov 2010	21 Aug 2014	31.2	2.85 (urine)	3.8	1	No	Urine contamination, low motility
	2499 - Jack	25 Nov 2010	21 Aug 2014	34.2	4.40 (urine)	107.6	12	No	Urine contamination, low motility
BIN	2428 - Blacktail	24 Oct 2007	17 Sep 2014	31.6	1.70[a]	27.3	74	No[d]	Motility lost after centrifugation
	2383 - Victor	16 Oct 2006	18 Sep 2014	33.2	1.80[a]	71.9	80	Yes	-
	2427 - Verizon	24 Oct 2007	17 Sep 2014	34.3	0.43	10.8	36	No	Low motility
OCZ	T1 - Dojo	23 Nov 2011	30 Sep 2014	29.9	0.01	0.03	0	No	Low volume, no motility
	T3 - Chipata	23 Nov 2011	30 Sep 2014	31.3	0.08	0.8	55	No	Low volume, low motility
	T2 - Juma	23 Nov 2011	30 Sep 2014	27.7	0.02	0	-	No	No spermatozoa
BRU	M1 - Brutus	unknown	17 Nov 2015	35.3	0.41	1.3	50	No	Low motility
PLA	M1 - Zevon	unknown	14 Jan 2016	24.5	0.02	0.2	43	No	Low volume, low motility
	M6 - Styx	unknown	16 Jan 2016	27.7	1.58[a]	83.7	(75[b]) 31 (0[c])	Yes[e]	Rapid decrease in motility
	M8 - Harrison	unknown	16 Jan 2016	22.7	0.00	-	-	No	No ejaculate
	M10 - Garfunkel	unknown	15 Jan 2016	25.5	0.15	3.0	(80[b]) 59	Yes[e]	Rapid decrease in motility
	M11 - Lennon	unknown	15 Jan 2016	21.8	0.65	8.5	(90[b]) 79 (0[c])	Yes[e]	Rapid decrease in motility
	M2 - Marley	unknown	14 Jan 2016	24.0	4.36[a]	49.2	(90[b]) 76 (10[c])	No[e,f]	Rapid decrease in motility
	M3 - Zeppelin	unknown	14 Jan 2016	21.0	0.29	1.6	66	No[f]	-
	M4 - Dylan	unknown	14 Jan 2016	25.0	15.00 (urine)	14.6	0	No	Urine contamination, no motility
	M5 - Ozzy	unknown	15 Jan 2016	25.0	0.74	2.0	0	No	No motility
	M13 - Wilson	unknown	16 Jan 2016	24.0	2.55[a]	44.4	(90[b]) 66	Yes[e]	Rapid decrease in motility
SAN	M1	2011	20 Mar 2016	31.0	0.10	0	-	No	No spermatozoa
	M2	2011	19 Mar 2016	26.5	2.68[a]	13.0	63	Yes	-

[a] samples with large prostate fluid contribution

[b] estimated motility immediately after collection

[c] estimated motility immediately prior to extender dilution and refrigeration; motility values without superscript indicate values obtained from detailed sperm analysis

[d] samples centrifuged after sperm analysis; [e] samples had rapid decrease in motility immediately prior to extender dilution

[f] samples used to optimise freezing technique.

2.3. Semen collection

After positioning the AWD in dorsal or lateral recumbency, the penis was manually extruded from the prepuce and rinsed with sterile saline. The bladder was emptied using a 6 fr or 8 fr canine urinary catheter (Jørgen Kruuse A/S, Langeskov, Denmark) gently passed down the urethra, and flushed several times with sterile saline to avoid urine contamination. Semen was collected using a 20 or 25 mm diameter rectal probe (Beltron Instruments™, Bryan, TX, USA) with three raised longitudinally oriented 30 - 40 mm strip electrodes connected to a 20 Hz sine wave electroejaculator (CGS Products™ Pty. Ltd., Trafalgar, Victoria, Australia) (Paris *et al.* 2005). The probe was lubricated with KY Jelly™ (Johnson & Johnson, New Brunswick, NJ, USA) and inserted into the rectum with the electrodes oriented ventrally at the level of the prostate as estimated using ultrasound (6.5 - 17.5 cm deep, depending on animal). The electroejaculation protocol consisted of three series separated by rest periods of approximately 5 min. Each series included 30 stimulations of 4 sec, increasing the voltage after every 10 stimuli. During each 4-sec stimulation, the probe was gradually retracted towards the anus. Series 1 was conducted at 2, 3 and 4 V, series 2 at 3, 4 and 5 V, and series 3 at 3, 4 and 5 V or 4, 5 and 6 V depending on the reaction of the animal to stimulation. During stimulation, the penis was placed in a 50 ml or 15 ml pre-warmed plastic tube, which was replaced after each series to avoid urine contamination. Each tube was kept at 37°C until analysis, which began within 5 min after completion of the last series.

2.4. Semen evaluation

Each fraction was evaluated for volume, colour, presence of motile spermatozoa, and pH (if semen volume was sufficient), then all fractions were combined. To evaluate motility, 10 µl of sample was placed on a pre-warmed glass slide with cover-slip on a slide microscope fitted with a 37°C warm stage (LEC Instruments Pty. Ltd., Scoresby, Victoria, Australia). Where sperm concentration was high, a 10 µl aliquot was first diluted 1:1 v/v in phosphate buffered saline (PBS) at 37°C. Motility rating (1-4 scale) and sperm motility index (0-400 scale) 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) (Paris *et al.* 2005). The percentage of viable and morphologically normal versus abnormal spermatozoa were evaluated by eosin-nigrosine smear (≥ 100 cells, 1000 X magnification)

(Johnston *et al.* 2001). Abnormal spermatozoa were grouped by primary defects (abnormalities of the sperm head and intermediary piece) and secondary defects (tail abnormalities and cytoplasmic droplets). Sperm concentration and total number of spermatozoa ejaculated were calculated using a haemocytometer (BLAUBRAND® Neubauer improved bright-line, Brand GMBH, Wertheim, Germany) (2010).

Acrosome integrity was evaluated using Pisum Sativum Agglutinin (PSA) conjugated with FITC (Sigma-Aldrich, St-Louis, MO, USA) (Rijsselaere *et al.* 2004). Between 20 and 50 µl of semen was washed in PBS and centrifuged (720 x *g*, 5 min). The pellet was resuspended in 50 µl 95% ethyl alcohol (Sigma-Aldrich, St-Louis, MO, USA) and refrigerated at 4°C for at least 30 min. Approximately 20 µl semen was then smeared on a glass slide and air-dried. Glass slides were stored at 4°C for up to three days then stained in the dark with 20 µl 0.1% w/v PSA-FITC in PBS layered on top of the cells and incubated at 4°C for 15 min, then were rinsed in distilled water and air-dried. The acrosomes of 200 spermatozoa per male were evaluated at 1000 X magnification by fluorescence microscopy. Acrosome-intact spermatozoa displayed an intense green fluorescence over the entire acrosomal region, whereas acrosome-damaged spermatozoa had incomplete or minor fluorescence at the equatorial region (Fig. 3.1a).

Terminal deoxynucleotidyl transferase dUTP Nick End Labelling (TUNEL) was performed using the *In-Situ* Cell Death Detection Kit, Fluorescein™ (Roche Diagnostics, Basel, Switzerland). This test evaluates DNA integrity by fluorescently labelling free 3'OH termini associated with single and double stranded DNA breaks. Between 20 and 50 µl of semen was washed twice in 0.1% w/v polyvinylpyrrolidone (PVP; Sigma-Aldrich, St-Louis, MO, USA) in PBS by centrifugation (720 x *g*, 5 min), and the sperm pellet resuspended in PVP solution to a final concentration of 1 - 2 x 10^6 spermatozoa/ml. An aliquot of 10 µl was smeared onto a poly-L-lysine-coated micro slide (Sigma-Aldrich, St-Louis, MO, USA), air-dried then fixed with 20 µl 4% w/v paraformaldehyde in PBS (pH 7.4) for 30 min at room temperature and rinsed. Slides were then stored at 4°C, and subsequently permeabilised and stained within three days of collection. Spermatozoa were permeabilised with 50 µl 0.5% Triton X-100 in 0.1 % sodium citrate in PBS (Sigma-Aldrich, St-Louis, MO, USA) for 30 min at room temperature, rinsed with 500 µl of PBS, then incubated with 50 µl TUNEL reaction mixture (Labelling solution and Reaction enzyme solution combined) for 1 h at 37°C in the dark. After rinsing with another 500 µl of PBS, slides were incubated with 25 µl 1 µg/ml Hoechst 33342 in PBS (Sigma-Aldrich,

St-Louis, MO, USA) for 5 min at room temperature to counterstain sperm DNA. Each TUNEL assay included a negative (incubated as above with 50 µl Labelling solution only) and positive control slide. The positive control slide consisted of spermatozoa incubated for 1 h at 37°C with 50 µl 1000 U/ml DNase I (recombinant; Sigma-Aldrich, St-Louis, MO, USA) in incubation buffer (10 mM Tris-HCl, pH 7.4 containing 10 mM NaCl, 5 mM $MnCl_2$, 0.1 mM $CaCl_2$ and 25 mM KCl) to induce double-stranded DNA breaks. A minimum of 100 cells were evaluated per sample at 1000 X magnification by fluorescence microscopy. DNA fragmented spermatozoa showed an intense green fluorescence at the sperm head while DNA-intact sperm cells stained blue (Fig. 3.1b).

Ejaculates with ≥60% initial sperm motility were selected for freezing. Three US samples were centrifuged (720 *g*, 5 min) to increase sperm concentration prior to freezing, but this caused a decline in sperm motility in one sample, making it unsuitable for freezing (Table 3.1).

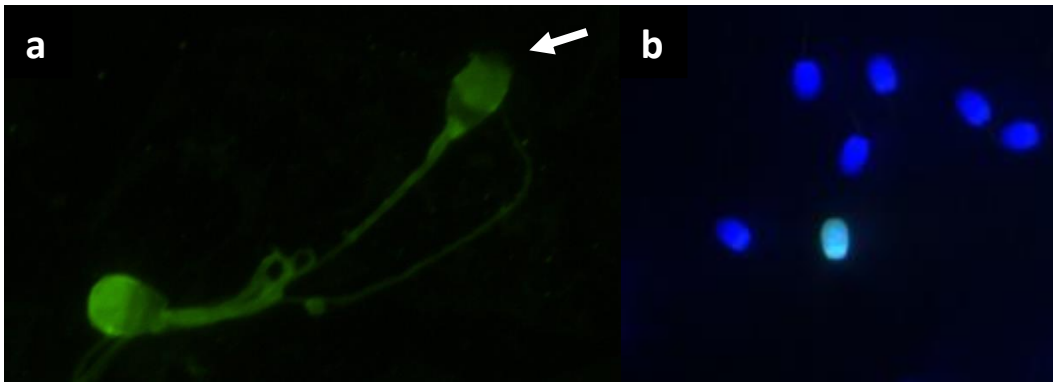


Figure 3.1. *(a) Acrosome intact (left) and incomplete (right) AWD sperm showing evidence of damaged membranes (arrow) stained with PSA-FITC; (b) DNA fragmented (green - FITC) and intact (blue - Hoechst 33342) AWD sperm evaluated by TUNEL assay.*

2.5. Sperm freezing

Each semen sample selected for freezing was split into two aliquots in 15 ml Falcon tubes and each aliquot was frozen using a different freezing protocol. For Protocol 1, semen was diluted 1:1 with a Tris-EY extender (0.25 M Tris-HCl; 0.08 M sodium citrate; 0.07 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate; 20% EY; and 8% glycerol), immersed in a 37°C water container and cooled over 2.5 h to 4°C. The sample was then loaded into 0.25 ml straws, suspended 4 cm over liquid nitrogen vapour for 10 min, then plunged into the liquid nitrogen. For each AWD, two straws were thawed in a 37°C water bath for 30 sec. Protocol 2 consisted

of an initial 2:1 v/v dilution (2 volumes of semen diluted in 1 volume of extender 1) with a Tris-EY extender containing a lower glycerol concentration (0.20 M Tris-HCl; 0.07 M sodium citrate; 0.04 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate; 20% EY; and 3% glycerol), which was cooled over 2.5 h to 4°C similar to Protocol 1. The same volume of a 4°C pre-cooled second extender was then added (3:1 dilution; 1 volume of extender 2 is added to the initial mixture), containing a higher glycerol concentration and Equex STM (0.20 M Tris-HCl; 0.07 M sodium citrate; 0.04 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate; 20% v/v EY; 7% v/v glycerol; and 1% v/v Equex STM), resulting in a final extender concentration (2 extenders combined) of 5% glycerol and 0.5% Equex STM, and so a concentration of 2.5% glycerol and 0.25% Equex STM after final semen dilution. Thawing of two straws per AWD was also performed in a 37°C water bath for 30 sec after which they were emptied 1:1 in a thawing solution (0.20 M Tris-HCl; 0.07 M sodium citrate; 0.04 M fructose; 0.6 g/l penicillin; 1 g/l dihydrostreptomycin sulphate). Sperm motility was evaluated at 5 min and every 2 h for 8 h after thawing. Viability, morphology and acrosome integrity was evaluated every 2 h for 6 h, and DNA integrity was evaluated immediately post-thaw. The 4 poor quality samples frozen from the PLA pack (Table 3.1) were only evaluated for motility, viability, morphology and acrosome integrity 5 min after thawing.

2.6. Statistical analysis

Percentage data were ARCSIN transformed, and sperm motility index data were $\log_{10}$ transformed. Paired sample t-tests or Sign test (motility rating) were used to evaluate differences between fresh and immediate post-thaw sperm quality for each freezing protocol, and to compare differences between protocols at each time point. Within each freezing protocol, changes in sperm quality over time during post-thaw incubation were evaluated using repeated measures ANOVA and post-hoc Least Significant Difference (LSD). $P \leq 0.05$ was considered significantly different. All data are presented as mean values ± SEM. Statistical analysis was performed with SPSS Statistics 23 (IBM® SPSS® Statistics 23, SPSS Inc., IBM, Armonk, New York, USA).

3. Results

Among the n=24 AWDs in which electroejaculation was attempted during the breeding season, n=17 produced urine-free semen of sufficient quantity to permit sperm analysis. Of these, the mean total spermatozoa ejaculated was 30.5 ± 9.7 x 10^6, motility was 55.0 ± 6.3%, viability was 76.6 ± 3.4%, acrosome integrity was 87.1 ± 2.8% (n=16), DNA integrity was 99.7 ± 0.1% (n=13), and normal sperm morphology was 53.8 ± 5.7%; with 23.4 ± 4.6% primary defects and 22.9 ± 3.3% secondary defects.

However, based on our selection criteria, initial sperm quality was sufficient for freezing in only 11 samples. One of these samples was excluded due to absent sperm motility after centrifugation; while 2 other samples were used in pilot trials to optimise the sperm freezing workflow in the field in Namibia (Table 3.1). Of the remaining 8 samples frozen, 4 exhibited a rapid decline in sperm motility immediately prior to extender dilution and refrigeration (PLA pack: M6, M10, M11 and M13; Table 3.1). As such, semen was divided into 4 good quality and 4 poor quality samples. Among the n=13 AWDs excluded from freezing, insufficient sperm quality was due to a variety of reasons including: low semen volume and/or low sperm motility (n=6), urine contamination of the sample (n=3), azoospermia (n=2), or aspermia (n=2; Table 3.1).

Total number of spermatozoa ejaculated was 71.4 ± 26.8 x 10^6 for good quality (n=4) and 34.9 ± 16.2 x 10^6 for poor quality samples (n=4) selected for freezing. Sperm motility, motility index and rating, viability, acrosome integrity, morphology and DNA integrity of fresh and frozen-thawed samples are presented in Table 3.2. Sperm motility was significantly lower for both freezing protocols immediately after thawing for good quality but not for poor quality samples due to their low pre-freeze value (Table 3.2). Sperm motility index and rating were only evaluated in good quality samples. No significant decrease in motility rating was found, while motility index decreased significantly for Protocol 2 immediate post-thaw. Viability declined significantly for both protocols after thawing in good and poor quality samples. However, post-thaw viability remained nearly 2-fold higher (nearly 60%) for Protocol 2 than Protocol 1 in both good (significant) and bad quality samples (Table 3.2). In good quality samples, acrosome integrity did not decline after freeze-thawing using Protocol 2; which was 3-fold significantly higher than Protocol 1 (Table 3.2). For poor quality samples, however, a

significant decline in acrosome integrity was only seen for Protocol 2, although this was most likely due to the highly variable results observed for Protocol 1 (n = 3; Table 3.2). The percentage of sperm with normal morphology, primary or secondary defects did not change after thawing using either protocol for good or poor quality samples (Table 3.2). DNA integrity was only measured in good quality samples but did not decline immediately after freeze-thawing using either protocol (Table 3.2).

Table 3.2. *Sperm quality (mean ± SEM) in fresh and thawed African wild dog (Lycaon pictus) semen frozen using Protocol 1 and 2 for good quality samples and poor quality samples (characterized by a rapid decrease in motility just prior to freezing).*

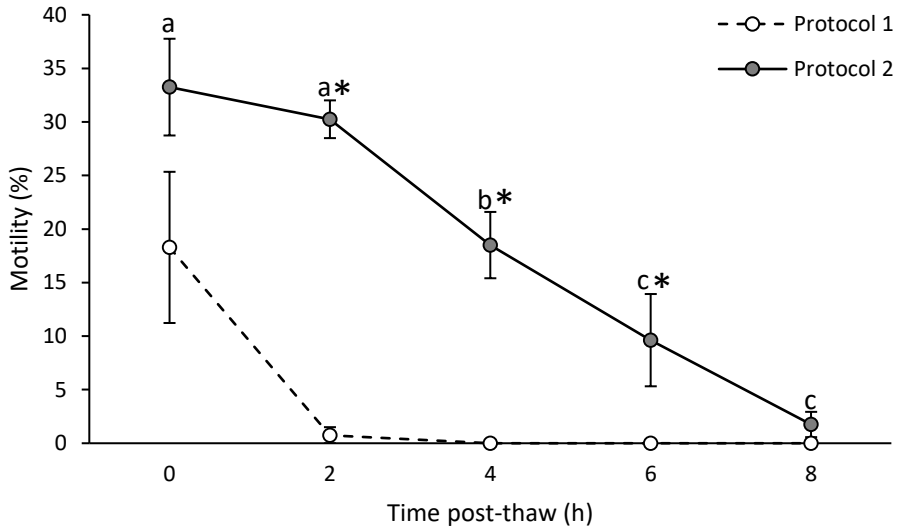
	Fresh	***Post-thaw Protocol 1***	***Post-thaw Protocol 2***
Good quality samples (n=4)			
Motility (%)	75.0 ± 4.4[a]	18.3 ± 7.1[b]	33.3 ± 4.5[b]
Motility rating (0-4)	3.3 ± 0.8	1.8 ± 0.8	1.8 ± 0.5
Sperm motility index (0-400)	194.0 ± 28.0[a]	31.5 ± 10.9[a]	59.4 ± 9.6[b]
Viability (%)	90.8 ± 1.3[a]	28.3 ± 9.6[c]	58.0 ± 10.1[b]
Acrosome integrity (%)	93.5 ± 2.0[a]	20.6 ± 6.2[b]	60.5 ± 10.8[a]
Normal morphology (%)	71.0 ± 6.8	42.8 ± 10.3	45.8 ± 10.8
Primary defects (%)	8.0 ± 2.9	11.0 ± 1.8	12.1 ± 1.7
Secondary defects (%)	19.5 ± 4.1	45.3 ± 9.3	41.5 ± 10.1
DNA integrity (%)	99.7 ± 0.3	99.5 ± 0.2	99.4 ± 0.2
Poor quality samples (n=4)			
Motility (%) (n=3)	3.3 ± 3.3	0.3 ± 0.3	1.7 ± 1.7
Viability (%)	89.5 ± 1.9[a]	29.0 ± 11.0[b]	55.8 ± 5.7[b]
Acrosome integrity (%) (n=3)	96.2 ± 1.6[a]	42.7 ± 22.7[a]	67.3 ± 5.7[b]
Normal morphology (%)	66.3 ± 6.2	67.5 ± 7.0	63.1 ± 6.0
Primary defects (%)	20.2 ± 3.5	24.0 ± 6.5	28.0 ± 5.2
Secondary defects (%)	13.6 ± 3.6	8.5 ± 3.7	9.0 ± 1.6

Different superscript (a-c) within a given row indicate significant difference between treatments.

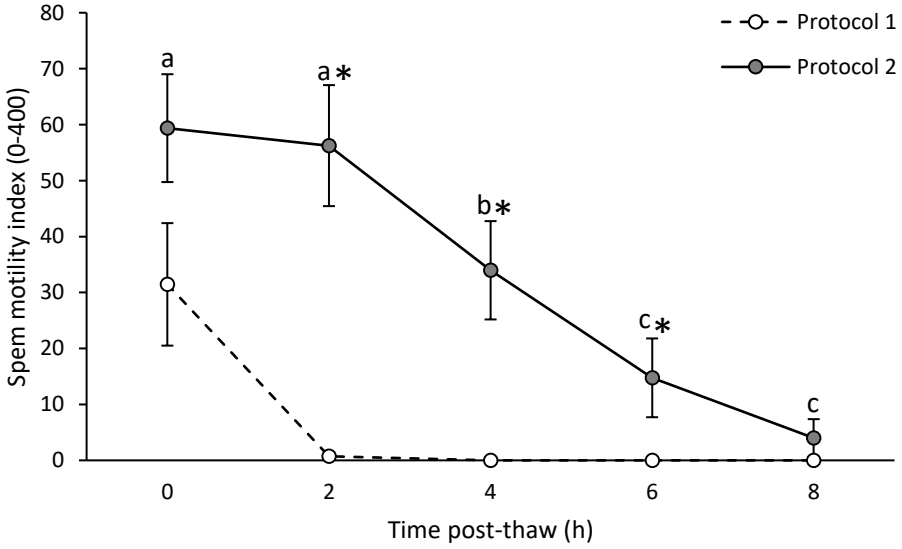
Within 2 h of post-thaw incubation, sperm motility dropped to less than 1% for Protocol 1 (Fig. 3.2a). However, a significant progressive decrease in sperm motility was only seen after 4 h of incubation for Protocol 2, with motility still present for up to 8 h (Fig. 3.2a). Moreover, motility for Protocol 2 remained significantly higher than Protocol 1 between 2 - 6 h after thawing. Similar results were observed for post-thaw sperm motility index (Fig. 3.2b). Sperm viability for Protocol 2 remained significantly higher (consistently 2 to 3.5-fold) than Protocol 1 throughout the post-thaw evaluation period (Fig. 3.2c). In addition, viability only declined significantly from immediate post-thaw levels after 6 h for Protocol 2, compared to 4 h for Protocol 1 (Fig. 3.2c). The integrity of the sperm acrosome for Protocol 2 essentially remained significantly higher (≥3-fold) than Protocol 1 throughout the post-thaw evaluation period (Fig.

3.2d). A small but significant decrease was seen after 2 h of incubation for both protocols (Fig. 3.2d). The proportion of spermatozoa with normal morphology or primary and secondary defects did not differ between freezing protocols throughout the post-thaw evaluation period (Fig. 3.2e). However, a small but significant decrease in normal morphology was observed after 2 h of incubation for Protocol 2; with a corresponding increase in secondary defects (Fig. 3.2e).

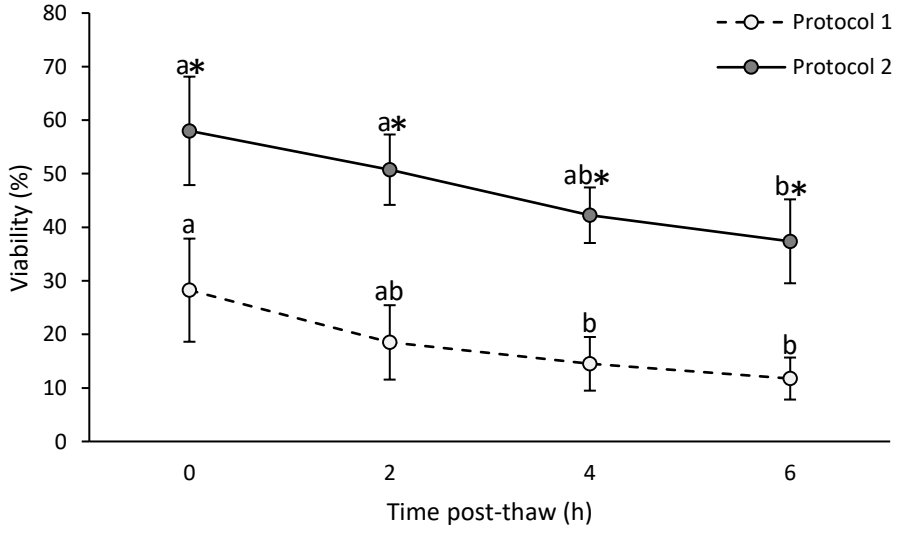
a.


Protocol 1
Protocol 2
Motility (%)
Time post-thaw (h)
a
a*
b*
c*
c

b.


Protocol 1
Protocol 2
Spem motility index (0-400)
Time post-thaw (h)
a
a*
b*
c*
c

c.


Protocol 1
Protocol 2
Viability (%)
Time post-thaw (h)
a*
a*
ab*
b*
a
ab
b
b

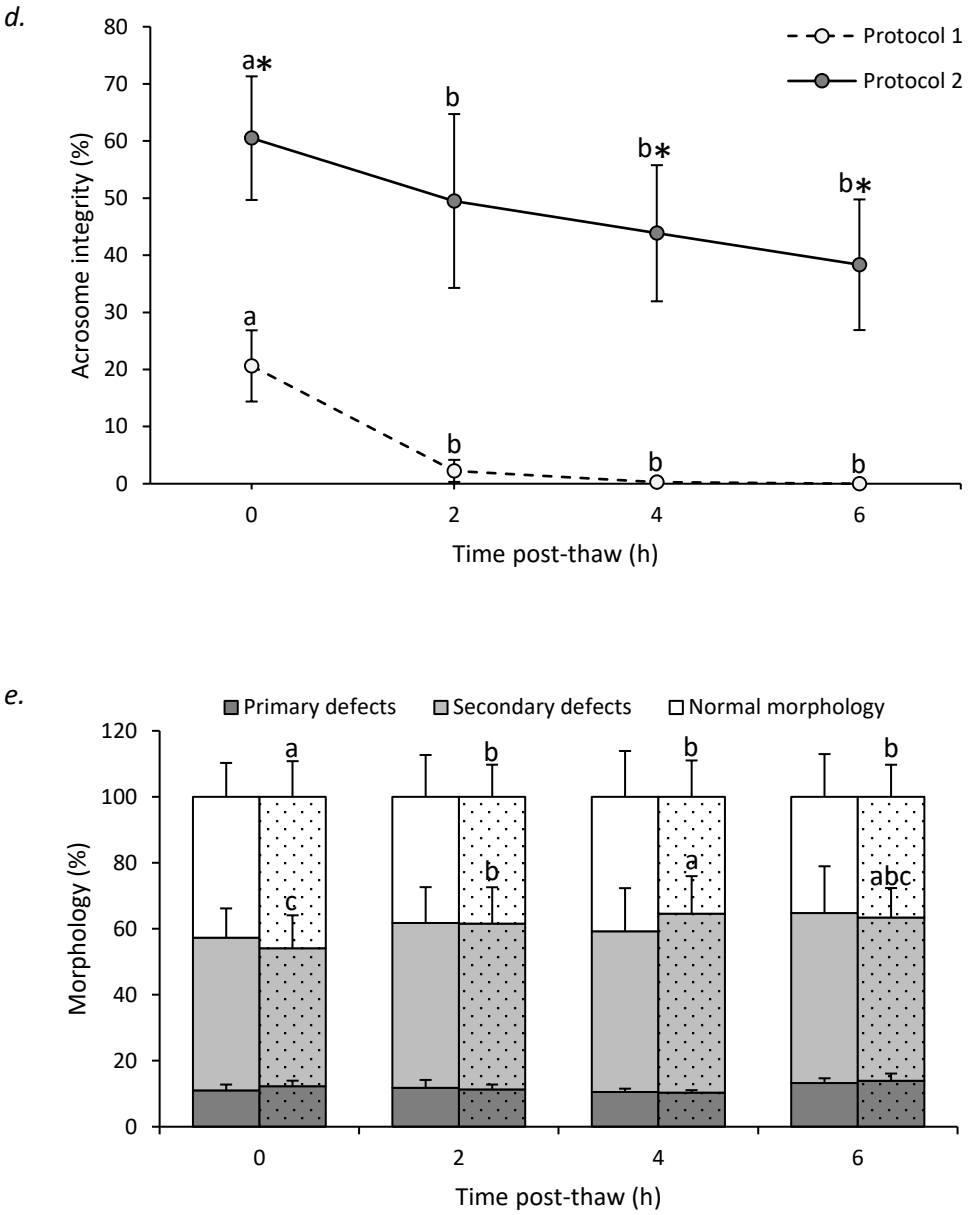


Figure 3.2. *Post-thaw (a) motility, (b) sperm motility index, (c) viability, (d) acrosome integrity and (e) morphology of African wild dog spermatozoa over a period of 6-8h after cryopreservation using Protocols 1 and 2.* indicates a significant difference between the 2 protocols at a specific time point. Different letters within each protocol indicate a significant difference between time points. In figure e, plain bars represent Protocol 1 results and dotted bars represent Protocol 2 results.*

4. Discussion

This study demonstrates that a sperm freezing protocol consisting of a two-step dilution in TRIS - 20% EY extender and final extender concentration of 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution, yields significantly improved post-thaw quality and longevity of AWD spermatozoa compared to previous studies (Hermes *et al.* 2001, Johnston *et al.* 2007). Importantly, sperm motility persists for up to 8 h and viability remains stable for more than 4 h after thawing. Moreover, DNA integrity and morphology of spermatozoa do not appear to be damaged by the freeze-thaw process. This protocol provides post-thaw spermatozoa of sufficient quality for use in AI, making it suitable for worldwide frozen transport and long-term gene banking for the first time in the AWD.

Protocol 1, using a one-step dilution with a TRIS - 20% EY extender containing 8% glycerol, yielded poor results similar to previous AWD sperm freezing studies. Sperm motility of 40% and 32%, respectively, was reported immediately post-thaw, but plummeted to nearly 0% within 2 h (Hermes *et al.* 2001, Johnston *et al.* 2007). By contrast for our Protocol 2, sperm motility was still >30% after 2 h and only started to decline significantly from 4 h, with motility present for up to 8 h; which represents a significant improvement. Although spermatozoa can be found at the top of the uterine horn within 1 min after natural mating in the domestic dog (Tsutsui 1989), it is unlikely that these spermatozoa are involved in fertilization. Sperm transport to the site of fertilization is known to occur in two phases in mammals (Overstreet & Cooper 1978a, 1978b), with spermatozoa responsible for fertilization reaching the oviduct during the sustained transport phase, several hours after insemination (Overstreet & Cooper 1978b). In addition, the timing when mature oocytes are ready for fertilization in the domestic dog is variable (60-108 h post ovulation; Rijsselaere *et al.* 2014). Fresh spermatozoa can survive in uterine crypts and the distal part of the uterotubal junction of the female reproductive tract for several days after natural mating or AI, enabling fertilization once the oocytes complete post-ovulatory maturation (Rijsselaere *et al.* 2014). Thus, the chance of pregnancy and larger litter sizes increases when using spermatozoa that can maintain motility for long periods.

In domestic dogs, most AI studies using frozen-thawed spermatozoa only evaluate sperm quality immediately post-thaw. A relatively large number of functional spermatozoa are

required for successful AI in the domestic dog, necessitating insemination using fresh sperm of good quality. Generally, fresh spermatozoa with motility above 70-75% are selected for freezing, and spermatozoa with a post-thaw motility of at least 40% result in whelping rates of 70% or higher (Linde-Forsberg *et al.* 1999, Thomassen *et al.* 2006). Immediate post-thaw motility of sperm in our study was just below 40%, however the maintenance of motility during post-thaw incubation was either similar or much better than that seen in domestic dog studies (Peña *et al.* 1998, Peña & Linde-Forsberg 2000, Silva *et al.* 2003).

Using a fluorescent staining technique, Johnston *et al.* (2007) observed 33% viable spermatozoa immediately after thawing, which decreased to 8% after 2 h of incubation. In our study, sperm viability was nearly 2-fold higher (58%) and only decreased 6 h after thawing for Protocol 2. In addition, post-thaw viability seen with Protocol 2 is similar to or better than results typically obtained in domestic dog sperm freezing studies (Rota *et al.* 1997, Peña & Linde-Forsberg 2000, Rijsselaere *et al.* 2004).

In our study, the integrity of the acrosome in good quality spermatozoa did not significantly degrade in response to the freeze-thawing process using Protocol 2, but was highly variable among samples over time. Bad quality samples gave a more variable result using Protocol 1. Protocol 2 yielded acrosome integrity 3-fold higher than Protocol 1 (60% *vs.* 20%), however it was still much lower than fresh and frozen-thawed AWD spermatozoa reported previously (98.5% and 95.6% respectively; Johnston *et al.* 2007); although the technique used was not described. We used a sensitive fluorescent staining technique to evaluate acrosomes, that may partly explain the difference in the level of acrosome defects detected between the two studies. In domestic dogs, post-thaw acrosome integrity ranges between 45% and 68% (Peña *et al.* 1998, Yildiz *et al.* 2000, Bencharif *et al.* 2010). The level of acrosome integrity observed using Protocol 2 falls within the higher end of this range; ensuring large numbers of fertilization-competent sperm are available post-thaw for AI.

To our knowledge, this is the first time DNA integrity has been evaluated in AWD spermatozoa. We found that DNA fragmentation is typically low, and that freeze-thawing using either protocol, does not appear to induce further damage. In domestic dog spermatozoa, DNA fragmentation increases slightly immediately post-thaw (Kim *et al.* 2010), however not in all studies (Koderle *et al.* 2009, Bencharif *et al.* 2010, Urbano *et al.* 2013, Varesi

et al. 2014). In all these domestic dog studies, post-thaw sperm DNA fragmentation index was relatively low (<5%), but different assay techniques were used (acridine orange, sperm chromatin dispersion test, and sperm chromatin structure assay), making it difficult to directly compare with our results. However, given that AWD spermatozoa have less than 1% DNA fragmentation as detected by TUNEL, it appears insensitive to the external stressors intrinsic to the freezing and thawing process. This provides some confidence that long term cryo-storage of spermatozoa from this species will not induce crucial DNA damage, that could otherwise lead to arrested early embryo development due to failed transcription of developmentally important but disrupted genes (Seli *et al.* 2004, Lewis & Aitken 2005, Paul *et al.* 2008, Peña *et al.* 2016).

Normal morphology, as well as primary and secondary sperm defects did not appear to change immediately after freeze-thawing using either protocol in our study. However, normal sperm morphology decreased slightly with a corresponding increase in secondary sperm defects (tail and cytoplasmic droplet) 2 h after thawing for Protocol 2. Hermes *et al.* (2001) reported an increase in tail defects from 20, 9 and 8% in the fresh sperm fractions to 59, 70 and 25% respectively after thawing. In other wild canids, an increase in tail abnormalities post-thaw was observed in the red wolf (*Canis rufus*; Goodrowe *et al.* 1998) and coyote (*Canis latrans*; Minter & DeLiberto 2005), but not in generic and Mexican grey wolves (Zindl *et al.* 2006), or arctic foxes (*Vulpes lagopus*; Stasiak *et al.* 2014). Tail defects caused during cooling, freezing and thawing could be due to cold shock or osmotic shock (Goodrowe *et al.* 1998), which means that a high increase in morphologically abnormal spermatozoa could indicate that the freezing extender and/or protocol is not optimal. Spermatozoa with an aberrant morphology can reduce pregnancy success, as luminal fluids and epithelial surfaces prevent their migration to higher parts of the female reproductive tract (Scott 2000).

As we used the same cooling, freezing and thawing rates for both our freezing protocols, the beneficial effect on sperm quality observed using Protocol 2 lies either in the different composition of this extender, or in the two-step dilution and/or thawing solution. The composition of each extender differed in our study with a lower Tris-HCl (0.20 M *vs.* 0.25 M), sodium citrate (0.07 M *vs.* 0.08 M), fructose (0.04 M *vs.* 0.07 M), and final glycerol concentration (5% *vs.* 8%), as well as the addition of Equex STM in Protocol 2 compared to Protocol 1. The function of the Tris-HCl-citrate is to buffer the semen extender to an optimal

pH. Fructose is generally added as an energy source to semen extenders, and can directly enhance sperm motility (Ponglowhapan *et al.* 2004). However, the fructose concentration used in our study was lower for the extender showing best post-thaw motility. Moreover, fructose concentrations as low as 0.01 M can maintain sperm motility above 70% for 4 days during refrigerated storage of canine spermatozoa (Ponglowhapan *et al.* 2004).

In the domestic dog, a two-step dilution has been proposed to enable cooling and equilibration with lower glycerol concentrations, minimizing its toxic effect (Peña & Linde-Forsberg 2000). However, several reports show no beneficial effects of adding glycerol later, indicating it is not toxic at the concentrations used for dog semen freezing (Peña *et al.* 1998, Peña & Linde-Forsberg 2000, Silva *et al.* 2003). It is possible however that AWD spermatozoa are more sensitive to the toxic effects of glycerol. Therefore, the lower concentration used in Protocol 2 could have caused part of the improvement observed in our study. A two-step dilution is also known to improve results when Equex STM is added in the second extender (Peña & Linde-Forsberg 2000). We hypothesize that the addition of Equex STM may largely explain the beneficial effect observed in Protocol 2. The combination of Equex STM with EY and glycerol appears to stabilize the sperm plasma membrane and optimize sperm cell dehydration and rehydration during freezing and thawing (Farstad 2009). In addition, the higher relative sperm viability, acrosome integrity and longevity we see with Protocol 2 after thawing is similar to the beneficial effects seen in domestic dogs when using Equex STM (Peña & Linde-Forsberg 2000). However, such a freezing protocol necessitates the use of a thawing solution (Rota *et al.* 1997, Peña & Linde-Forsberg 2000, Bencharif *et al.* 2010), which in this case was identical to the Protocol 2 extender minus glycerol, EY and Equex STM. The thawing solution allows dilution of potentially toxic glycerol and SDS (Curry 2000, Holt 2000), and could thereby increase sperm longevity. In domestic dogs, a 1:1 dilution improves post-thaw sperm motility, viability and acrosome integrity, however only after 5, 12 and 18 h of incubation respectively (Pena & Linde-Forsberg 2000). Collectively, this suggests that Equex STM is the main contributor to the beneficial effect observed in Protocol 2.

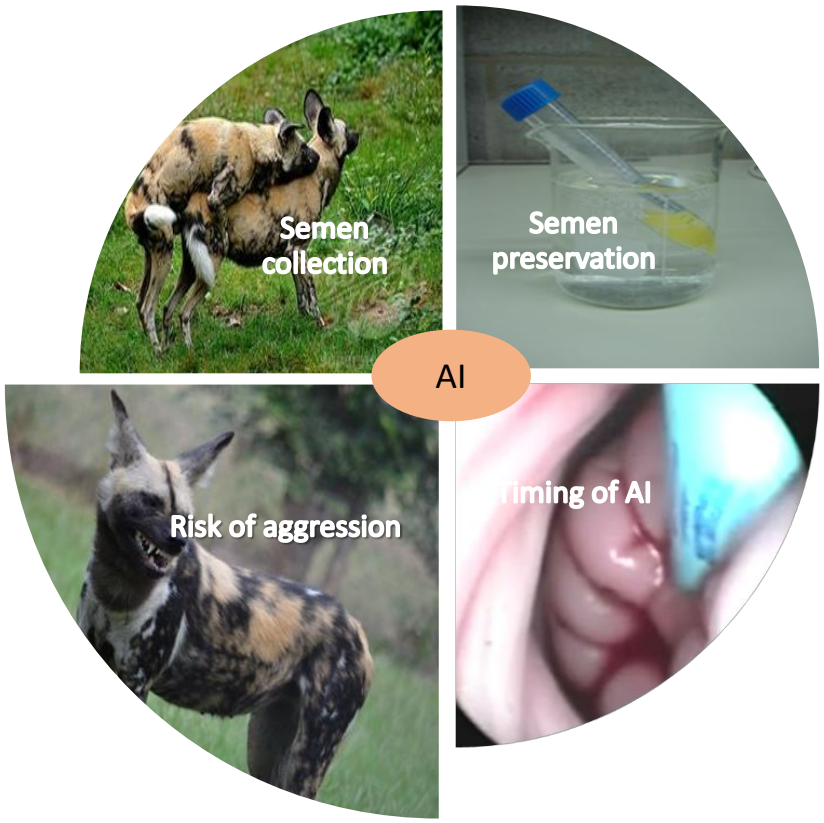
One important limitation to the successful cryopreservation of AWD spermatozoa encountered in this study was the failure to consistently collect good quality samples; an issue previously reported by other researchers (Johnston *et al.* 2007). Such inefficiencies must be considered when planning semen collections for gene banking in this species. Urine

contamination, absence of an ejaculate, absence of spermatozoa, low semen volume, and low sperm motility, all contributed to the reduced number of samples available for cryopreservation. Avoiding the using of alpha-2 adrenergic receptor agonists for immobilization, coupled with draining and flushing the bladder prior to electroejaculation, may go some way to avoid urine contamination of samples (Johnston *et al.* 2007, Talukder & Hikasa 2009). Moreover, compared to domestic dogs, the total number of spermatozoa ejaculated and sperm quality seen in AWDs in this study is very low, and similar to those reported during the breeding season by some researchers (Johnston *et al.* 2007), but not others (Newell-Fugate *et al.* 2012). Although sperm number is known to be lower after electroejaculation compared to manual collection in domestic dogs (Ohl *et al.* 1994), other sperm quality parameters do not differ (Ohl *et al.* 1994, Christensen *et al.* 2011). Thus, with the exception of sperm number, other sperm quality parameters observed in this study should reflect those inherent during natural mating and ejaculation in AWDs.

In conclusion, a two-step TRIS - EY freezing extender containing Equex STM and glycerol, coupled with a TRIS-citrate-fructose thawing solution, yields significantly improved post-thaw quality and longevity of African wild dog spermatozoa; greatly improving the chance of successful AI with frozen-thawed spermatozoa. With further refinement, this protocol should be suitable for (i) long-term sperm banking to maintain genetic diversity, (ii) transport of frozen gametes worldwide, and (iii) downstream use in AI for the management and conservation of this highly endangered species. However, AI trials are needed before this sperm freezing protocol can be considered successful for the management and conservation of the endangered African wild dog.

Chapter 4

Dog appeasing pheromones to manage temporary pack separation



Van den Berghe F, Paris MCJ, Sarnyai Z, Millar RP, Ganswindt A, Cozzi A, Pageat P, Paris DBBP. (2018) Dog Appeasing Pheromone prevents the androgen surge, and may reduce contact-dominance and active-submission behaviours after stressful interventions in African wild dogs (*Lycaon pictus*). *PloS One,* submitted.

Abstract

The endangered African wild dog (AWD; *Lycaon pictus*) is a highly social canid living in packs with a separate male and female hierarchy. Immobilisation, handling and translocations are acute stressors for AWDs, however such interventions are often needed for species management. In addition, new pack formation or temporary pack separation can lead to an increase in intra-pack aggression. The goal of this double-blinded placebo-controlled study was to evaluate whether dog appeasing pheromone (DAP) reduces behavioural stress and faecal glucocorticoid metabolite concentrations (fGCM) normally associated with pack separation, immobilisation and reintroduction (SIR), and to assess whether this reduces aggressive behaviours and faecal androgen metabolite concentrations (fAM). Four packs (n=11 males) were treated with DAP and 4 packs (n=12 males) were treated with a placebo solution, applied at the end of anaesthesia. Behavioural interactions as well as fGCM and fAM were determined from 3 days before until 4-6 days after SIR. No effect of DAP on fGCM was observed, however, fAM increased after SIR in placebo but not DAP treated animals. Moreover, on the day of reintroduction, DAP treated packs tended to show lower rates of contact-dominance and active-submission behaviour, but higher rates of non-contact dominance behaviour. As these effects could decrease the risk of agonistic interactions, DAP could be a useful tool to help manage new pack formations and temporary pack separation.

1. Introduction

The endangered African wild dog (AWD; *Lycaon pictus*) is a highly social canid living in packs of 5-15 adults and juveniles under free-roaming conditions (Creel & Creel 2002). AWDs show a complex social structure consisting of a separate male and female hierarchy, and a cooperative breeding system (Creel & Creel 2002). To maintain a viable population and current genetic diversity, a meta-population management plan was introduced in South Africa in 1998, involving the reintroduction of AWDs in small protected areas and their management through regular translocations between isolated subpopulations (Davies-Mostert *et al.* 2009). This management plan requires frequent formation of new packs, especially since reintroductions are more successful when captive bred AWDs are released together with wild-caught animals (Scheepers & Venzke 1995, Woodroffe & Ginsberg 1999). Furthermore, in the captive breeding programs of the European (EAZA) and American Associations of Zoo and Aquaria (AZA), juvenile or adult animals are often translocated between zoological institutions to form new breeding pairs or packs. In addition, animals in captivity sometimes require temporary separation from their existing pack, e.g. for medical treatment. The complex social system of AWDs creates considerable challenges to this type of management. New pack formation, or disruption of hierarchical bonds and pack instability caused by temporary pack separation, can lead to aggression between animals, and have frequently resulted in serious morbidity and even mortalities (Scheepers & Venzke 1995, Johnston *et al.* 2007, Vlamings 2011, Foster 2014, Quick 2014).

Immobilisation, handling and translocations are acute stressors for AWDs, causing increased glucocorticoid (stress hormone) output (de Villiers *et al.* 1995, Creel *et al.* 1997, de Villiers *et al.* 1997, Comizzoli *et al.* 2009). However, higher glucocorticoid levels have also been linked to increased aggression. In domestic dogs (*Canis familiaris*), stressed males show an increase in dominance behaviour and aggression (Beerda *et al.* 1999), and aggressive dogs show higher glucocorticoid concentrations than non-aggressive animals (Rosado *et al.* 2010). In captive AWDs, dominant males exerting active dominance characterised by high levels of aggression, have higher glucocorticoid levels than males exerting passive dominance and low levels of aggression (de Villiers *et al.* 1997). In free-living AWDs, dominant males show more agonistic aggressive behaviour than subdominant animals, and show higher baseline faecal glucocorticoid metabolite (fGCM) concentrations (Creel *et al.* 1997). Thus, aggression

potentially seen after temporary pack separation and during new pack formation could be caused by increased glucocorticoid concentrations.

The relationship between testosterone or its precursors and aggression has been well established in a variety of species (Mehta & Beer 2010, Wacker *et al.* 2016). For example, castration of domestic dogs often leads to a decrease in unwanted sexual behaviour, inter-male aggression, roaming, and urine marking (Maarschalkerweerd *et al.* 1997). In meerkats (*Suricata suricatta*), a social carnivore with a cooperative breeding system similar to the AWD, blockade of androgen receptors in subordinates results in a decrease in initiation of aggression and in dominant behaviour during play, but it enhances prosocial behaviour such as grooming (delBarco-Trillo *et al.* 2016). In AWDs, dominance is often mediated by aggression. In the breeding season, alpha (and beta) males show higher androgen levels than lower ranking males (Creel *et al.* 1997, Monfort *et al.* 1997, Johnston *et al.* 2007), as well as higher rates of fighting (Creel *et al.* 1997). Thus, a perceived stressor leading to an increase in glucocorticoid concentrations in AWDs, may cause intra-pack aggression, and is potentially associated with elevated androgen concentrations.

Appeasing pheromones could be a natural strategy to reduce stress in AWDs. Pheromones are mixtures of fatty acids that are secreted in the environment by an individual, perceived by a conspecific through inspiration into the vomeronasal organ, inducing both a physiological or behavioural response (Pageat & Gaultier 2003). In carnivores, pheromones are secreted by a series of glands in the facial area, on the feet, in the perianal, genital and mammary region, or secreted in the urine or faeces (Pageat & Gaultier 2003). In a wide range of mammals such as horses, cows, sheep, goats, dogs and cats, the structure and function of some pheromones have been identified and, for some, synthetic forms produced that prove useful to decrease stress or modify unwanted behaviour (Yonezawa *et al.* 2009, Mengoli *et al.* 2014, Pereira *et al.* 2016). Mammary appeasing pheromones (nipple-finding pheromones) are known to be secreted in the above-mentioned mammals. In dogs, they are secreted by sebaceous glands in the intermammary sulcus of lactating bitches from 3-4 days post-partum until 2-5 days post-weaning; giving a calming effect on puppies (Pageat & Gaultier 2003). The synthetic analogue of this pheromone, known as Dog Appeasing Pheromone (DAP; Adaptil^{TM}, CEVA), reduces stress and unwanted behaviour (e.g. house soiling, vocalisation during isolation, or pacing) during fear-inducing situations in adult dogs (Sheppard & Mills 2003, Tod *et al.* 2005,

Gandia Estelles & Mills 2006, Mills *et al.* 2006, Levine *et al.* 2007, Kim *et al.* 2010, Siracusa *et al.* 2010, Landsberg *et al.* 2015) and puppies (Taylor & Mills 2007, Denenberg & Landsberg 2008, Gaultier *et al.* 2008, Gaultier *et al.* 2009). Further, DAP has been shown to be as effective as clomipramine, a clinically used tricyclic antidepressant, in the treatment of separation anxiety in domestic dogs (Gaultier *et al.* 2005). Moreover, as pheromones are naturally occurring substances secreted by the target species, one of the major advantages of their use in behavioural management is the absence of toxicity or side effects (Gaultier *et al.* 2005). However, most studies performed in domestic dogs only use behaviour to evaluate the effect of DAP. As such, more research is needed to investigate the underlying physiological response to pheromone treatment.

A preliminary study indicates that AWDs might be capable of perceiving DAP (Vlamings 2011). DAP infused collars tended to reduce baseline fGCM concentrations in 2/3 of treated individuals. Moreover, the use of DAP collars and sprays before new pack formation tended to result in relatively low levels of aggression (Vlamings 2011). However, these preliminary trials lacked appropriate control groups and had small sample sizes. Thus, further testing is needed to determine the effectiveness of DAP for the behavioural management of AWDs. The goal of this study was to characterise the effect of minor medical intervention and temporary pack separation on behaviours and hormones associated with stress and aggression, as well as determine whether DAP can mitigate these deleterious effects. We hypothesised that after separation, immobilisation and re-introduction (SIR); an intervention compilation which should be stressful to AWDs, DAP-treated animals should show (a) lower fGCM concentrations; (b) lower levels of intra-pack aggression-associated behaviour and increased levels of submissive and affiliative behaviour; and (c) lower faecal androgen metabolite (fAM) concentrations than control animals.

2. Materials and methods

2.1. Experimental design, animal capture and release

This study was approved by the James Cook University Animal Ethics Committee and by the Institutional Animal Care and Use Committees (IACUC) of the participating institutions. The study included 5 AWD packs in the USA (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). Packs consisted either of 3 males (ABQ, TOP, BIN) or 3 males and 1 female (BRK, OKC). Males were scheduled for immobilisation for health assessment and sample collection (Chapter 2, Chapter 3) during the 2014 pre-breeding season (4 packs; ABQ, BRK, BIN, TOP; May – early July 2014) and breeding season (ABQ, BRK, BIN, OKC; August – September 2014; Table 4.1). The BRK pack female was euthanized (due to malignant tumour) after the pre-breeding season, reducing this pack to only 3 males for breeding season evaluation. All animals had access to water *ad libitum* and were individually fed with ground horse meat, occasionally replaced by bones, whole pig or goat carcass feeding. 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).

Table 4.1. *Pack composition, treatment schedule and mean daily observation times (h) per pack in the pre-breeding and breeding season*

		Pre-breeding season		Breeding season	
Zoo/Pack	**Pack composition**	**Treatment (4 groups/n=12)**	**Mean (min-max) observation time (h)**	**Treatment (4 groups/n=11)**	**Mean (min-max) observation time (h)**
ABQ	3 ♂	Placebo (n=3 ♂)	2:29 (1:53-3:13)	DAP (n=3 ♂)	3:10 (2:46-4:02)
BRK	3 ♂, 1 ♀ (in pre-breeding season)	Placebo (n=3 ♂)	3:15 (2:35-3:55)	DAP (n=2 ♂)	3:24 (3:09-3:34)
BIN	3 ♂	DAP (n=3 ♂)	3:21 (2:53-3:40)	Placebo (n=3 ♂)	2:59 (2:26-3:24)
TOP	3 ♂	DAP (n=3 ♂)	2:02 (1:31-2:29)	-	-
OKC	3 ♂, 1 ♀	-	-	Placebo (n=3 ♂)	4:43 (3:55-5:36)
DAP mean daily observation time				2:59 (1:31-4:02)	
Placebo mean daily observation time				3:21 (1:53-5:36)	
Combined mean daily observation time				3:10 (1:31-5:36)	

ABQ, Albuquerque BioPark Zoo; BRK, Brookfield Zoo; BIN, Binder Park Zoo; TOP, Topeka Zoo; OKC, Oklahoma City Zoo.

Health assessment and sample collection required the separation, immobilisation, and reintroduction after full recovery of individuals within the pack (SIR-procedure). AWDs were starved for at least 12 hours and separated into individual holding pens (with visual and olfactory contact with conspecifics) before immobilisation, which occurred over 1 or 2 consecutive days. Animals were either darted from a small distance or hand injected in a crush cage and subsequently transported to the zoo veterinary clinic for assessment. Anaesthesia was conducted as described in detail in Chapter 3. In short, AWDs were immobilised with tiletamine/zolazepam hydrochloride (30 - 180 mg IM; Telazol®, Zoetis Inc., MI, USA) with or without medetomidine (0 - 0.6 mg IM; Medetomidine HCl®, ZooPharm, WY, USA), and maintained with Isoflurane (0.5 - 5% in 1l/min O_2; IsoFlo®, Zoetis Inc., MI, USA; Isothesia®, Henry Schein Animal Health, OH, USA; or Isoflurane®, MWI Animal Health, ID, USA). In cases where medetomidine was administered, AWDs were reversed with atipamezole (0.01 - 0.1 mg/kg, IM; Antisedan®, Zoetis Inc., MI, USA) after intubation and/or at the end of anaesthesia.

After the procedure, 10 ml DAP or placebo spot-on solution (IRSEA, Apt, France) was applied to the skin and fur of each animal; 5 ml between the shoulders and 5 ml on the base of the tail. All animals immobilised from the same pack were treated with the same solution (Table 4.1). The placebo solution had the same composition as the DAP solution (transcutol gel), but without the pheromone. In a blinded experimental design, solutions were provided by IRSEA in 10 ml syringes marked ‘A’ or ‘B’. Details about which solution was DAP or placebo was only communicated at the end of the study, after samples were collected and analysed, data generated, and subsequent statistical analyses were completed. To control for putative seasonal effects, an equal number of packs were subjected to each treatment in both seasons (Table 4.1). To control for pack effects, 3 packs evaluated in both seasons were randomly assigned one treatment for each season (Table 4.1). In total, 4 packs (n=11 AWD males) were treated with DAP and 4 packs (n=12 AWD males) were treated with placebo solution (Table 4.1).

Animals recovered individually in their holding areas or in crates, with visual and olfactory contact with conspecifics until reintroduction. In most cases, immobilisation of the entire pack was complete within 1 day, with reintroductions performed the following morning by opening doors from individual holding areas towards the outside area and releasing the AWDs one by one within a few seconds of each other. For the OKC pack, males were first released together,

followed by the female 2 min later, once males had settled down. However, during the pre-breeding season for the BRK pack, reintroductions were performed within the holding area by opening gates between different cages. During the breeding season, the alpha male was not immobilised due to existing cardiac issues. Thus, only the 2 subdominant males were immobilised, and the alpha male was kept in the outside area during the day. All 3 males were re-introduced in their communal holding area overnight.

During the breeding season in the ABQ and BIN packs, 2 males, beta and gamma (ABQ) or alpha and beta (BIN) respectively, were sedated on day 1 and the remaining male the next day. In ABQ, the 2 subdominant males were re-introduced in the outside area on the morning the alpha male was sedated. The alpha male was isolated in his individual holding area until the following morning, with visual and olfactory contact with the 2 subdominant males which had access to the communal holding area. In BIN, all animals were isolated from each other in their holding areas over 2 days. Reintroduction was performed in the communal holding area during the evening of the second day.

2.2. Faecal sample collection

Faecal samples were collected daily from all animals while enclosures were cleaned, from 3 days before until 5-6 days after the immobilisation of males (Fig. 4.1). As enclosures were cleaned at the same time daily, all faeces were collected within 24 h and frozen immediately. Time of collection and moistness of the sample (fresh or dry) were noted. Individual-specific faeces were identified by adding non-toxic food-grade coloured plastic beads (Universal Polymers, Malaga, Australia) or glitter (local craft store, Battle Creek, MI) to an individual's food during normal feeding times. In AWDs, gut transit time averages 22.7 ± 0.9 h (Van der Weyde *et al.* 2015), so faecal samples collected were assumed to indicate cumulative hormone levels from the previous day. Immediately after collection, faecal samples were placed in sealable plastic Ziploc bags and stored at -20°C until transport to Hillsdale College (MI, USA) on dry ice. Faecal samples were dried in a conventional oven (National Appliance Company, Portland, Oregon, USA) at 60°C for 21.9 ± 0.4 hours, then stored at room temperature until transport to the Endocrine Research Laboratory, University of Pretoria, South Africa, for hormone analysis.

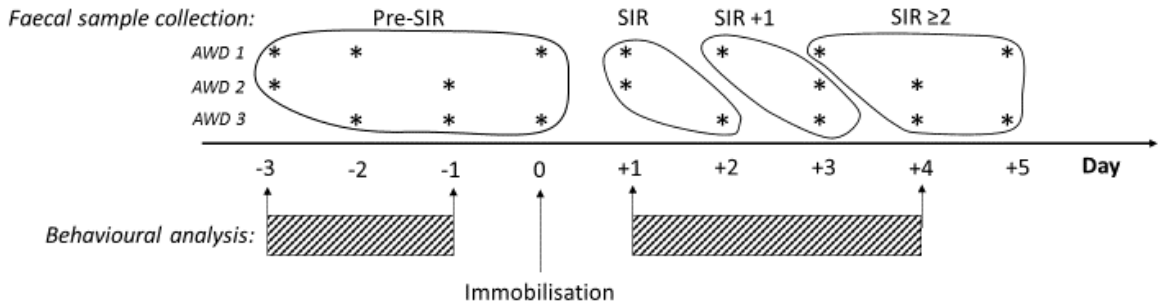


Figure 4.1. *Example of faecal sample (*) and behavioural data (hatched bars) collection from each African wild dog pack. Faecal samples, collected from 3 days before until 5 days after immobilisation, were grouped as pre-SIR: samples prior to and during immobilisation; SIR: first sample after procedure; SIR+1: second sample after procedure; and SIR≥2: all subsequent samples. Behavioural observations were performed for 3 days before to 4 days after immobilisation. Day 1 includes the reintroduction of the pack.*

2.3. Faecal steroid extraction and analysis

Dried faecal samples were pulverised and put through a strainer to isolate faecal powder. Per sample, 50 - 60 mg of faecal powder was extracted using 3 ml 80% ethanol in water. The suspension was vortexed for 10 min and subsequently centrifuged for 10 min at 1500 *g*. The supernatant was then decanted into micro-centrifuge tubes and stored at -20°C for further analysis (van der Goot *et al.* 2015).

Faecal steroid extracts were measured for immunoreactive fGCM and fAM concentrations using an enzyme-immunoassay (EIA) technique. A biotinylated cortisol-3-CMO assay coupled with bovine serum albumin (BSA) was used for determining fGCM concentrations (Palme & Mostl 1997). The sensitivity of the cortisol EIA was 1.2 ng/g dry faecal weight (DW). Intra- and inter-assay coefficients of variation of high and low quality controls were 4.8% and 5.6%, and 12.2% and 13.8% respectively. To evaluate the reliability of the cortisol EIA, an ACTH stimulation test was performed during a pilot study (Vlamings 2011). Two AWDs housed at the Ann van Dyk Cheetah Centre (De Wildt, South Africa) were injected with 25 IU IM synthetic ACTH (Synacthen®, Novartis RSA Pty Ltd, Kempton Park, South-Africa) using a pole syringe. Faecal samples were collected from 1 day before until 5 days after ACTH administration to monitor the rise and fall in fGCM levels caused by temporal adrenal stimulation. The same samples of 2 of these females were analysed for fAMs using EIA described below to exclude cross-reactivity to fGCMs. Moreover, as a biological validation, we compared the stress-

associated rise in fGCMs from the first faecal sample post SIR-procedure, to the lowest fGCM values obtained pre SIR-procedure from 10 randomly selected males.

A biotinylated 3β-androstanediol (5α-androstan-3β,17β-diol) T-3-CMO assay coupled with BSA was used to determine fAM concentrations in steroid extracts (Palme & Mostl 1994). The sensitivity of the testosterone EIA was 4.8 ng/g DW. Intra- and inter-assay coefficients of variation determined by measuring high and low quality controls were 5.0% and 5.1%, and 8.9% and 10.9% respectively. The testosterone EIA was validated for use in the AWD by comparing fAMs in 26 samples collected from 2 females (Vlamings 2011) with the male samples collected prior and during immobilisation (n=100 samples). Both cortisol and testosterone assays were performed according to Ganswindt *et al.* (2002).

2.4. Effect of time and temperature on steroid metabolite concentration post-defecation

We conducted a pilot study to evaluate the effect time and temperature play in degradation of immunoreactive fAMs and fGCMs by microorganisms due to delayed sample collection. Nineteen faecal samples collected from 8 males during a different study (Chapter 2) were thawed, pooled (total weight 645 g), thoroughly mixed and divided into 54 equal subsamples. Half of these were refrigerated at 4ºC and the other half were left in the sun; with fridge temperature and minimum/maximum outside temperatures monitored. Three technical replicates of each subsample were stored frozen at 0 h, 1 h, 2 h, 4 h, 8 h, 16 h, 24 h, 48 h and 72 h until processing as described above.

2.5. Behavioural observations

Behaviour was recorded using a Sony HDR-SR10 digital video camera from the public viewing area or zookeeper section from 3 days before until 4 days after immobilisation (Fig. 4.1). Recordings started between 07:00-09:30 am (depending on zoo and day of the week), when AWDs were usually most active. The BIN and BRK packs were also observed during high activity between approximately 02:00-04:30 pm and 03:30-05:30 pm respectively. In cases were focal individuals were not visible during an observation session, the recorded time was excluded from the total daily observation time (see Table 4.1).

Behavioural analysis was performed blind using a modified ethogram from Vlamings (2011; Table 4.2). The actor(s) and recipient(s) were classified in order to analyse social interactions between 2 or more individuals, then grouped as either severe aggression, ritualised aggression, and dominant (also including urine marking), submissive, affiliative and play behaviour (Table 4.2; Fig. 4.2). Dominant interactions were subdivided into behaviours where the actor makes physical contact with the recipient (contact dominant behaviour; e.g. mount, scruff-oriented approach, or inguinal inspection), behaviours without physical contact (non-contact dominant behaviour; e.g. fixating, approach in high posture), and marking behaviour (Table 4.2). Submissive interactions were subdivided into behaviours where the subdominant actor initiates unprovoked submission (active submissive behaviour), and behaviour where submission is provoked by an action from a more dominant actor (passive submissive behaviour; Table 4.2). All social interactions in the context of food competition were excluded, as was play behaviour due to very low frequency. Hourly rates of each behaviour type were then calculated for each observation day per pack.

2.6. Statistical analysis

For the sample storage experiment, fAM and fGCM concentrations were standardised to relative concentrations, setting the mean hormone value at t=0 to 1.0 for each subset. To correct for individual variation in baseline levels, steroid hormone concentrations were also standardised when evaluating the effect of DAP treatment; with individual mean pre-SIR-procedure fGCM and fAM concentrations set at 1.0, and were then grouped for each treatment as values from (a) all pre-SIR-procedure samples (pre-SIR); (b) the first faecal sample after the SIR-procedure, reflecting hormone levels associated with the procedure (SIR); (c) the first following sample (SIR+1); and (d) all subsequent samples (SIR≥2; Fig. 4.1). All fGCM and fAM data was tested for normality using the Shapiro-Wilk test and histograms and data $\log_{10}$ transformed where not normally distributed. For EIA validation, differences in fGCM concentrations before and after the SIR-procedure, and differences in fAM concentrations between male and female samples, were analysed with paired sample or independent t-test respectively. For the sample storage experiment and the effect of DAP treatment, differences in relative fGCM and fAM concentrations between treatments at any time point were determined using t-test, and changes in fAM and fGCM concentrations over time were determined using one-way ANOVA and post-hoc Bonferroni. Per pack, hourly rates

were calculated for each behaviour type for each day and standardized relative to the mean pre-SIR rates (Fig. 4.1). Day 1 (Fig. 4.1) included the reintroduction of the pack and the first day of observation. As we were unable to video record the reintroductions for the BRK pack, this pack was excluded for day 1. Differences between treatments in relative hourly rates on each day, as well as differences in hourly rates between day 1 and pre-SIR baseline levels were evaluated using Mann-Whitney U test. Changes over time were evaluated using Friedman test combined with post-hoc Wilcoxon signed-rank test. $P \leq 0.05$ was considered significant. Statistical analysis was performed with SPSS Statistics 23 (IBM® SPSS® Statistics 23, SPSS Inc., IBM, Armonk, New York, USA).



Figure 4.2. *Social behaviours of captive African wild dogs (AWDs). (a) AWD approaches the scruff of a conspecific in high posture (ears forward and high tail; contact dominant behaviour), who reacts by rolling on his side (present body; passive submission). (b) 2 AWDs walk parallel touching each other's muzzle, exhibiting the characteristic facial grin accompanied by a high-pitched giggle vocalisation (greeting; affiliative behaviour). (c) AWD on the right initiates muzzle contact with a conspecific from a low posture (low posture snout contact; active submission). (d) AWD on the right approaches a conspecific with a bone in a high posture while looking at him (food approach; non-contact dominant behaviour), who reacts by retreating in a low posture (food retreat; passive submission). (e) AWD on the right snaps towards a conspecific (snap; ritualised aggression), who reacts by retreating (shrinks back; passive submission). (f) AWD male scent marking (mark; dominant behaviour).*

Table 4.2. *African wild dog (Lycaon pictus) ethogram showing social interactions used for behavioural analysis. Modified from Vlamings (2011).*

Behaviour	*Description*
Severe aggressive behaviour	
Assault*	A brusque approach at full speed, often followed by an aggressive physical contact.
Severe biting*	The actor has closed jaws and teeth having a strong hold of any part of the actor's body e.g. legs, tail, throat or head. The bite is with full strength (uninhibited).
Ritualised aggressive behaviour	
Chase away	The actor walks or runs in pursuit to elicit a flee response from the recipient.
Push Down	The actor presses down the recipient by an inhibited bite in the neck.
Embrace	The actor embraces the neck of the recipient from the front, the recipient can stand on four or two hind legs.
Snout bite	The actor seizes the snout of the recipient between its jaws from the side or from above and holds it gently for a short while.
Scruff bite	Scruff orientated inhibited bite.
Snap	The actor lunges with a bite movement towards the recipient, without making contact and often with an audible sound of the jaws, when they come together.
Ritualized approach	A restrained gallop in high posture towards the recipient, not resulting in physical contact.
Dominant behaviour	
Aggressive vocalisation[a]	Growling.
Scruff orientated approach[b]	The actor approaches the scruff of the recipient without biting.
Stalk approach[a]	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[a]	The actor stops an interaction between two interactants by approach, stand in between or threat towards one of the recipients respectively.
Fixating[a]	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[b]	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[b]	The actor initiates an inguinal contact and investigates the genitals of the recipient while the latter remains passive.
Point[a]	The actor directs, with an abrupt movement of his head or a short jump, towards the recipient.
Mount[b]	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[b]	The actor stands across a lying recipient.
Approach in high posture[a]	Moving towards the recipient in a high posture, while looking at him.
High posture snout[b]	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[b]	The actor licks the nose, lips and mandibular region of the recipient while being in a high posture.
Submissive behaviour	
Escape/flight[c]	The actor runs away from the recipient, often seen during conflicts.
Retreat[c]	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[c]	The actor jumps back from the recipient, after being approached by him.
Avoid[c]	The actor stands aside for the recipient, after being approached by him.
Active submission[d]	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[c]	The actor pushes himself down in front of the recipient.
Head turning[c]	The actor turns his head and avoid eye contact with the recipient, exposing the neck region towards the recipient.
Low posture standing[c,d]	Stand in a low position, with the ears pulled back.
Approach in low posture[c,d]	The actor moves towards the recipient in a low posture while looking at him.
Low posture snout contact[c,d]	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a low posture.
Submissive vocalisation[c,d]	Twittering, whimpering, yelping, whining vocalisations.
Present body[c]	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[c,d]	The actor licks the nose, lips and mandibular region of the recipient while being in a low posture.
Affiliative behaviour	
Close contact*	The actor stands or lies within one body length from the recipient. The recipient may be standing, sitting or lying.
Approach in neutral posture	The actor moves towards the recipient in a neutral posture while looking at him.
Neutral posture snout	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a neutral posture.
Neutral posture face lick	The actor licks the nose, lips and mandibular region of the recipient while being in a neutral posture.
Pass under head	The actor passes from a lateral side close under the head of the recipient, usually in a somewhat crouching manner; often a short nose-chin contact with the recipient is evident.
Head under	The actor pushes with his head towards the ventro-lateral side of the recipient, occasionally lifting the recipient's back quarters from the ground with his head.
Fur sniff/licking	Self-explanatory
Paw/head on	The actor places a paw or its head on the back of the recipient.
Grin	Only clear facial expression shown by AWDs. The mouth corners remain retracted and the mouth may be slightly open so that the teeth become visible. The behaviour occurs mostly in combination with 'giggle' and has a friendly nature.
Giggle	A vocalisation characterised by a high tone level and a 'staccato' rhythm. The sound is made often in combination with the behaviour 'Grin'.
Greeting	The actor stands or walks in parallel with the recipient, tries to contact his muzzle, and performs a complex of behaviours including food-solicit or inspection behaviour, the facial expression 'grin' and the vocalisation 'giggle'.
Parallel walk or run	Two animals walking side by side in the same direction.
Regurgitation	Expulsion of undigested food from the mouth, pharynx, or oesophagus.
Rub on	The actor establishes intensive latero-lateral contact with the recipient. This may occur while both animals are in motion.
Play behaviour	
Play solicit*	The actor initiates a play interaction with the recipient by solicit behaviour such as nose pushing or tugging the recipients fur with an inhibited bite.
Fur bite*	The actor tugs the recipient fur by an inhibited bite.
Play fighting*	Playful non-competitive fighting in which attacker and defender exchanges roles and no winner or loser emerges. Interactions rarely include behaviours which can inflict injury.
Play chase*	The actor follows in a fast pursuit the recipient, who tries to escape by abruptly changing the direction. The roles of follow and escape may change.
Play wrestle*	This behaviour involves all play situations, in which the actor shows inhibited bite movements towards the recipient, while he keeps the recipient in constant eye-contact.
Play sniff*	Two or more pack members are engaged in extensive sniffing at an object or some particle on the ground, while their heads are in direct contact.
Social play*	All other forms of play.

[a]Behaviours included in non-contact dominant behaviour; [b]Behaviours included in contact dominant behaviour; [c]Behaviours included in passive submission behaviour; [d]Behaviours included in active submission behaviour; *Behaviours not observed (severe aggressive behaviour, hoo call) or not included in analysis (play behaviour, food associated behaviour, behaviours that are a state e.g. close contact lying).

3. Results

3.1. EIA validation and steroid degradation experiment

ACTH injection resulted in detection of a greater than 2.7-fold increase in fGCM levels by 8 h, which declined to baseline 35 h later. Cross-reactivity to fGCMs by the fAM assay was excluded since no rise in fAM levels were detected over the same period (Fig. 4.3a & b). Moreover, mean fGCM levels after acute stress associated with the SIR procedure was significantly higher than pre-SIR baseline levels (62.1 ± 8.6 *vs.* 36.1 ± 2.7 ng/g DW; n=10; t=-2.527, df=9, *P*=0.032). Furthermore, the fAM assay detected sex-specific differences in androgen levels with 10-fold higher fAM levels in male than female samples (611.9 ± 41.1 *vs.* 62.9 ± 2.3 ng/g DW; t=26.233, df=123, *P*<0.001). Collectively these results provide experimental and biological validation that the cortisol-3-CMO and T-3-CMO assays are suitable for analyzing fGCM and fAM levels respectively in AWDs.

During the steroid degradation experiment, refrigeration temperature remained at 3-4°C except at 16 h and 72 h when it decreased to 1°C and 2°C respectively. External ambient summer temperatures ranged between 11°C at night to over 50°C (beyond the range of the thermometer) in direct sun during the day. Relative fGCM and fAM levels did not change over time in refrigerated or ambiently stored samples (df=8, F=0.947, *P*=0.504; df=8, F=0.420, *P*=0.894; df=8, F=2.489, *P*=0.052; and df=8, F=1.445, *P*=0.245 respectively), however at 24 h and 8 h respectively, refrigerated samples showed significantly higher concentrations (t=4.023, df=4, *P*=0.016; t=3.551, df=4, *P*=0.024; Fig. 4.3c & d). This indicates that fAM and fGCM do not appear to degrade for at least 24 h in ambient conditions and thus, should be similar to concentrations found in freshly collected and stored samples.

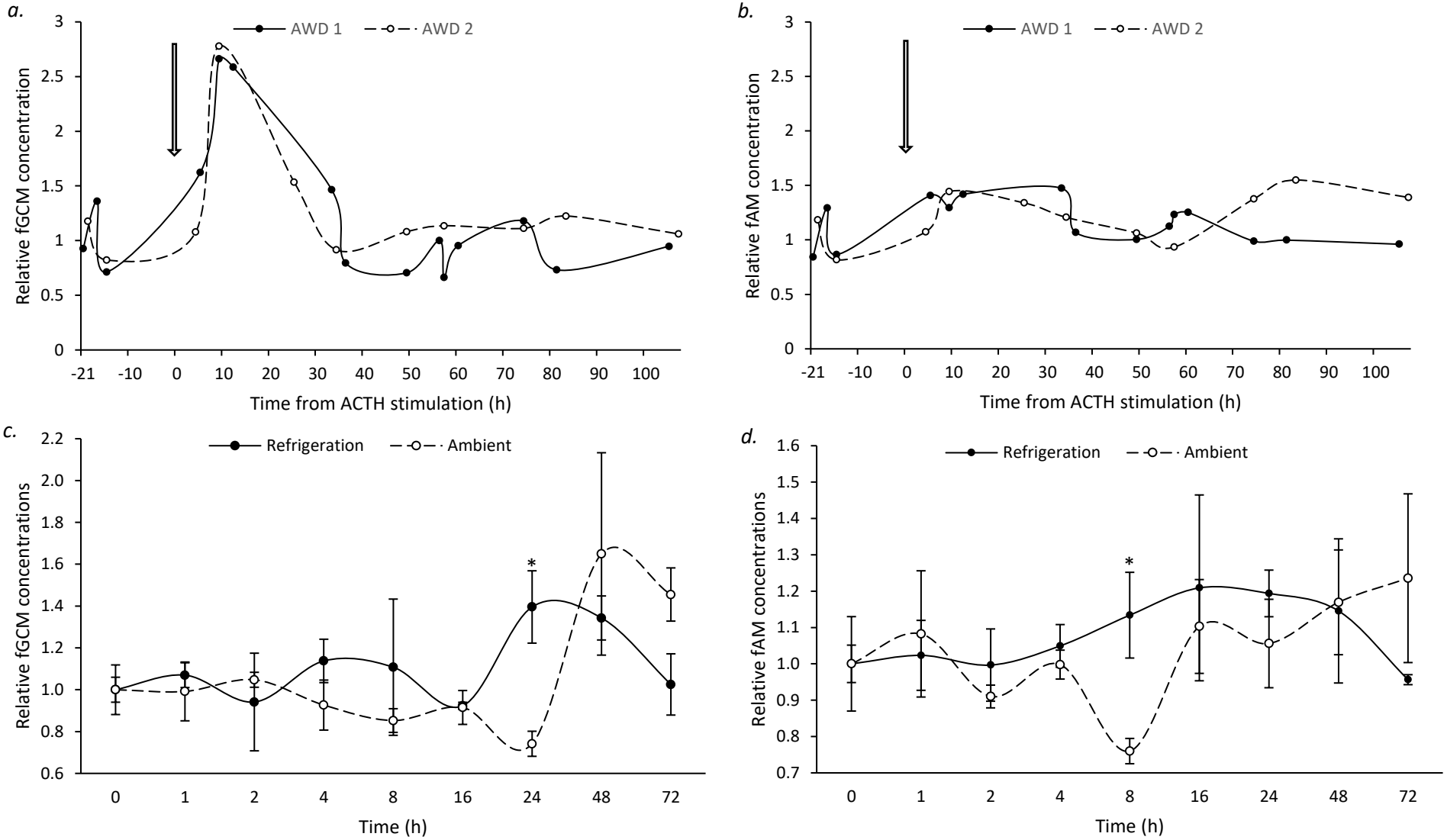


Figure 4.3. *(a) Relative fGCM and (b) fAM profiles from n=2 African wild dog females injected with ACTH (arrow); (c) Mean (± SEM) relative hormone profiles of fGCM and (d) fAM concentrations measured over 72h after storage of fecal samples at refrigerated (solid line) or ambient (dashed line) temperatures; * significant differences between treatments.*

3.2. fGCM and fAM hormone concentrations

Three untreated animals that were not immobilised during the SIR procedure (BRK female in pre-breeding season; BRK male and OKC female in breeding season; Table 4.1), did not show a rise in fGCM concentrations (Fig. 4.4a). Moreover, relative fGCM levels at SIR and SIR≥2 in these animals, were significantly lower than those in placebo treated animals (0.4 ± 0.1 *vs.* 1.9 ± 0.3, t=-4.251, df=13 *P*=0.001; and 0.8 ± 0.2 *vs.* 1.1 ± 0.1, t=-2.255, df=35, *P*=0.030); indicating that immobilisation is responsible for the rise in fGCM (Fig. 4.4b). fAM levels in the single non-immobilised male, had a 3.2-fold increase at SIR+1 compared to baseline; indicating reintroduction of conspecifics is responsible for the rise in fAM (Fig. 4.4a).

Among immobilised animals, a significant rise in fGCM of more than 1.5-fold occurred at SIR in both placebo and DAP treated AWDs (df=3, F=12.306, *P*<0.001; df=3, F=6.831, *P*<0.001 respectively), with levels decreasing at SIR+1. There was no significant difference in fGCM between treatments (t=-0.422, df=21, *P*=0.677; t=-0.516, df=16, *P*=0.613; and t=0.991, df=53, *P*=0.326 at SIR, SIR+1, and SIR≥2 respectively; Fig. 4.4b). Compared to pre-SIR levels, placebo treated animals had between 1.2- to 2.2-fold higher fAM throughout the post-immobilisation period, with significantly higher levels observed at SIR+1 (df=3, F=3.839, *P*=0.012; Fig. 4.4c). By contrast, this rise did not occur in DAP treated animals (df=3, F=0.775, *P*=0.512), with fAM levels significantly lower than placebo treated animals at SIR+1 and SIR≥2 (t=-1.712, df=21, *P*=0.097; t=-2.623, df=16, *P*=0.018; and t=-2.464, df=53, *P*=0.013 at SIR, SIR+1, and SIR≥2 respectively; Fig. 4.4c).

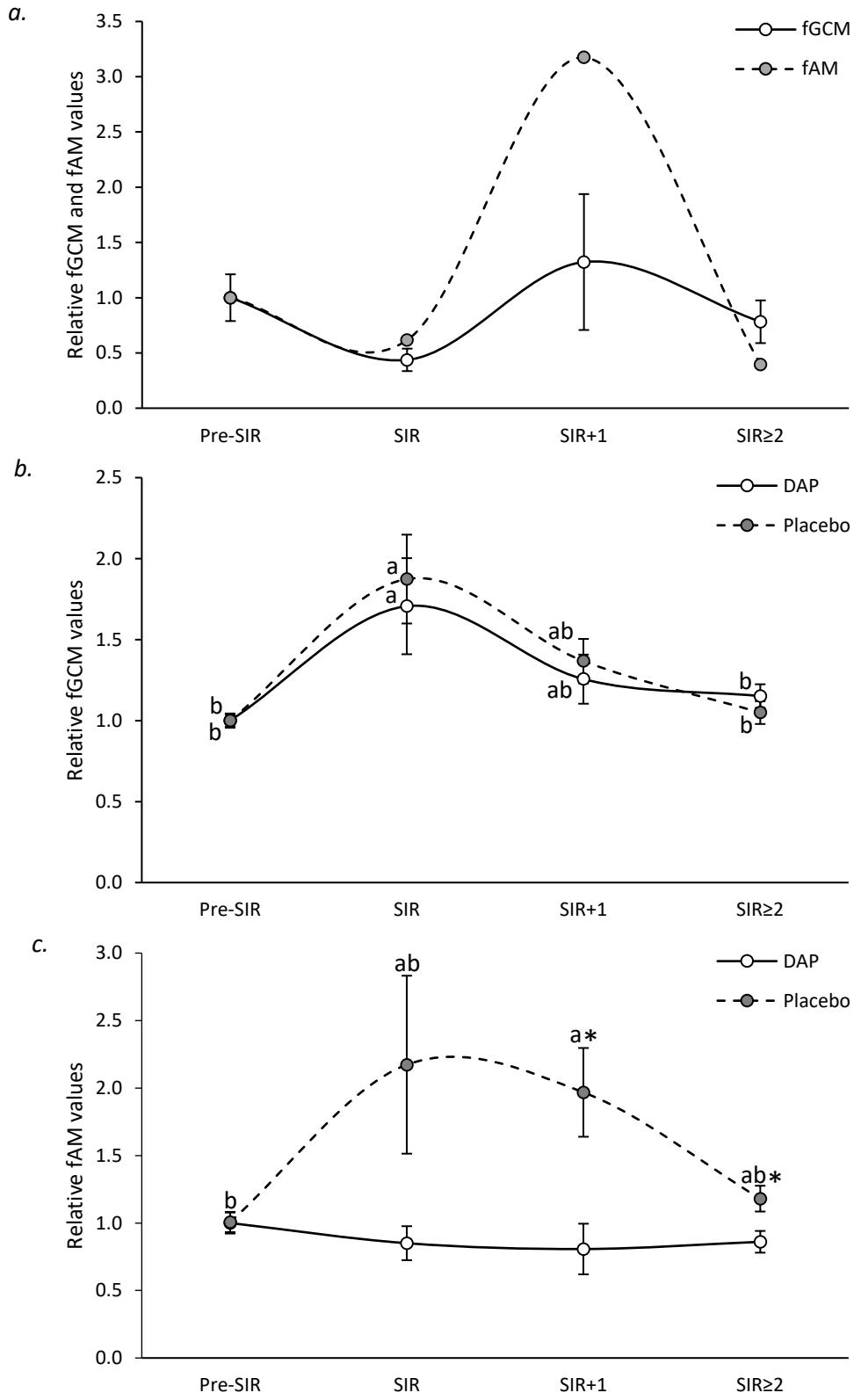


Figure 4.4. *Mean (± SEM) relative hormone profiles before (pre-SIR) and after (SIR, SIR+1, SIR≥2) separation, immobilisation and reintroduction for (a) fGCM (n=2 African wild dog females and 1 male; solid line) and fAM (n=1 male; dashed line) in non-immobilised animals; (b) fGCM and (c) fAM in n=11 DAP (solid line) or n=12 placebo (dashed line) treated males; different letters indicate significant differences during the SIR procedure within each treatment; * significant differences between treatments.*

3.3. Behaviour

Mean daily observation time did not differ between placebo and DAP treated packs (Mann-Whitney U, *P*=0.431; Table 4.1). For all packs combined, baseline rates of behaviour each hour pre-SIR were 2.2 ± 0.2 combined dominant behaviour; consisting of 0.5 ± 0.1 contact dominant, 0.8 ± 0.2 non-contact dominant and 0.9 ± 0.2 marking behaviours. Rates for combined submissive behaviour were 2.9 ± 0.5; consisting of 1.4 ± 0.3 active submissive and 1.4 ± 0.3 passive submissive behaviours. Affiliative behaviour was the most frequently observed at 4.2 ± 0.5 events per hour. Severe aggression was absent in all packs throughout the study period (including reintroduction), and ritualised aggression was low at 0.2 ± 0.1 events per hour.

Relative hourly rates of dominant behaviour in DAP and placebo treated groups are shown in Figure 4.5. In placebo treated packs, total and contact dominant behaviour changed significantly over time (Friedman test: χ^2=12.571, df=6, *P* =0.050 and χ^2=13.304, df=6, *P* =0.038 respectively; Fig. 4.5a & b), but the specific days could not be clarified by post hoc Wilcoxon signed-rank test. However, relative hourly rates of total, contact and non-contact dominant behaviour were significantly higher on the day of release (+1) than before intervention (Mann-Whitney U: *P*=0.009, *P*=0.010; and *P*=0.009 respectively, Fig. 4.5a-c). In DAP treated packs, no dominant behaviour changed significantly over time (Fig. 4.5), but non-contact dominant behaviour significantly increased on the day of release compared to baseline (Mann-Whitney U: *P*=0.021; Fig. 4.5c). No changes could be seen in marking behaviour for either treatment group (Fig. 4.5d). Although no significant differences were observed in any dominant behaviours between placebo and DAP treated packs, placebo groups tended to exhibit 2.2-fold higher contact dominant behaviour (Fig. 4.5b), while DAP groups tended to exhibit 2.2-fold higher non-contact dominant behaviour (Fig. 4.5c).

Relative hourly rates of total or passive submissive behaviour did not change significantly over time in either treatment group (Fig. 4.6a & b). However, changes in active submission over time were near-significant for the placebo group (χ^2=11.122, df=6, *P*=0.085), but not DAP group (χ^2=7.714, df=6, *P*=0.260; Fig. 4.6c). However, total, active and passive submission was significantly higher on the day of release than before intervention for both treatments (Mann-Whitney U: *P*<0.05; Fig. 4.6). Although no significant differences were observed in any

submissive behaviours between placebo and DAP treated packs, placebo groups tended to exhibit 2.2-fold higher active submissive behaviour (Fig. 4.6c).

With the exception of a spike in ritualised aggression observed on Day 3 in the ABQ pack (caused by a delayed restoration of normal hierarchy due to a transient shift), relative hourly rates of both affiliative and aggressive behaviour did not change significantly over time nor between placebo and DAP treated packs (Fig. 4.7).

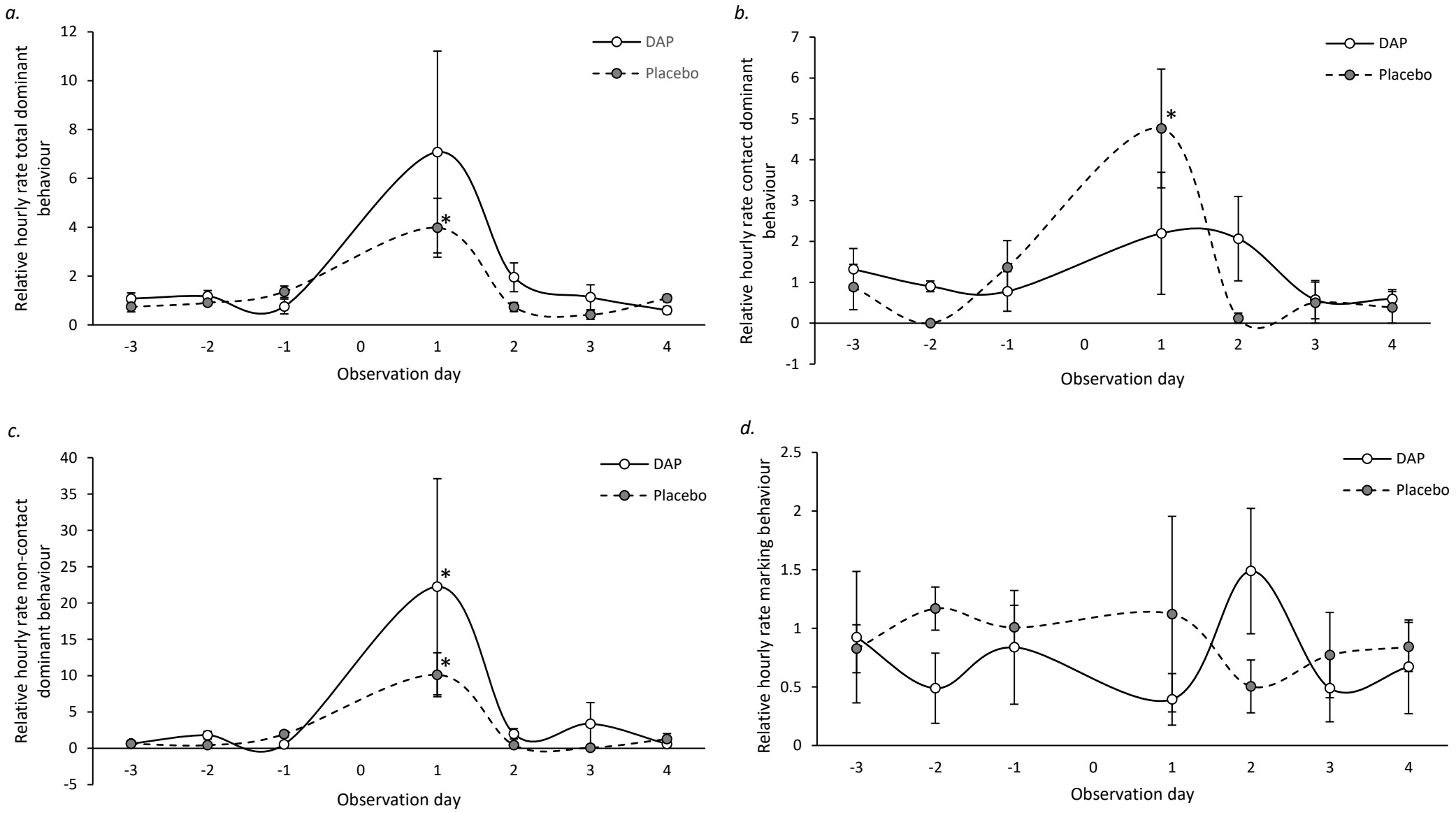


Figure 4.5. *Mean (± SEM) relative hourly rates of (a) total, (b) contact and (c) non-contact dominance behaviour, and (d) marking in DAP (solid line) and placebo (dashed line) treated packs throughout the observation period; * significantly different to respective baseline levels.*

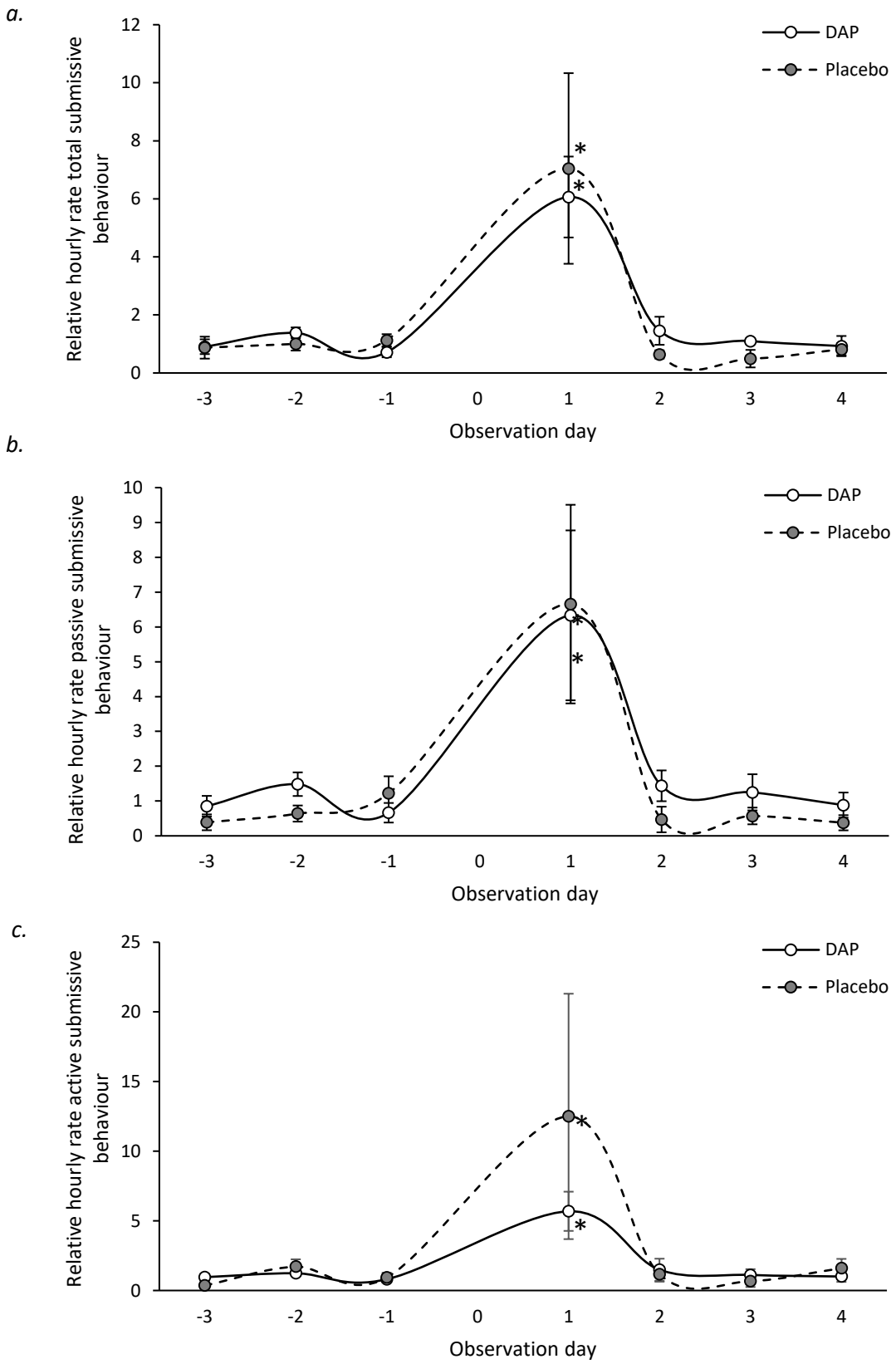


Figure 4.6. *Mean (± SEM) relative hourly rates of (a) total, (b) passive and (c) active submissive behaviour in DAP (solid line) and placebo (dashed line) treated packs throughout the observation period; * significantly different to respective baseline levels.*

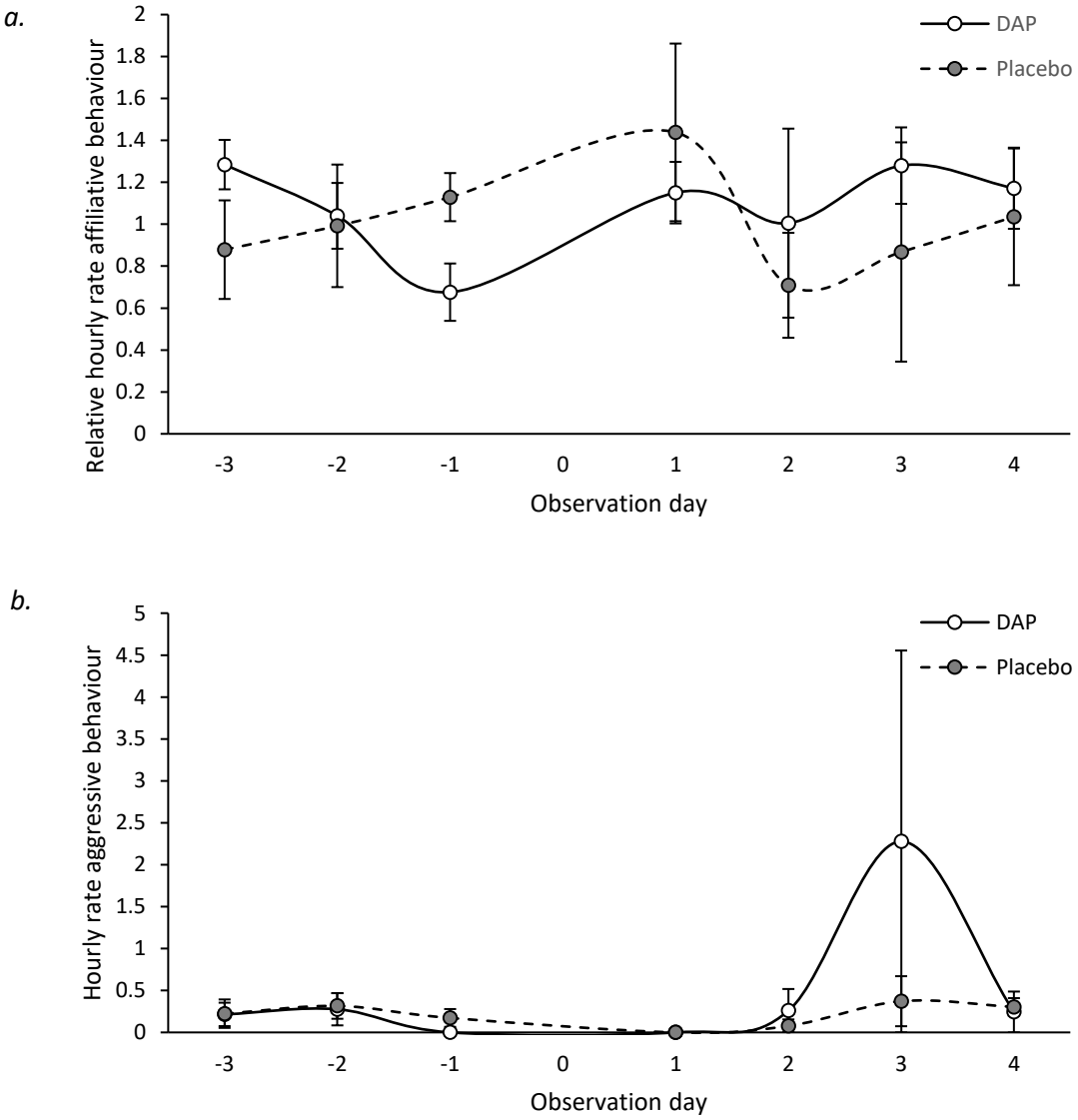


Figure 4.7. *Mean (± SEM) relative hourly rates of (a) affiliative and (b) aggressive behaviour in DAP (solid line) and placebo (dashed line) treated packs throughout the observation period.*

4. Discussion

Dog appeasing pheromone offers tremendous potential for the management of behaviour in wild canids. Given its role in alleviating stress and anxiety-related behaviours in domestic dogs (Sheppard & Mills 2003, Tod *et al.* 2005, Gandia Estelles & Mills 2006, Mills *et al.* 2006, Levine *et al.* 2007, Kim *et al.* 2010, Landsberg *et al.* 2015), DAP was presumed to act via the hypothalamic-pituitary-adrenal (H-P-A) axis to suppress cortisol production. Interestingly, our double-blinded placebo-controlled study demonstrates that DAP does not mitigate the faecal glucocorticoid surge associated with stressful interventions. By contrast, DAP prevents the rise in faecal androgens seen in placebo treated animals after separation, immobilisation and reintroduction procedures. Moreover, on the day of reintroduction, DAP treated packs tend to show lower rates of contact-dominance and active-submission behaviour, but higher rates of non-contact dominance behaviour. These results instead suggest that DAP could reduce hormones & behaviours leading to aggression in captive AWDs.

To our knowledge, this is the first time androgens have been measured during a pack reintroduction event in AWDs; with a significant increase in fAMs observed in placebo treated animals. Increased testosterone is associated with competitive interaction (Zilioli & Bird 2017) and elevates the risk of agonistic interactions between individuals. Although not significant, placebo treated animals experiencing the testosterone surge, tended to show higher rates of contact dominant and active submission behaviour. In addition, total and contact dominant behaviour was significantly higher compared to baseline levels in placebo but not in DAP treated packs. The increase in faecal androgen was seen in most placebo treated animals irrespective of their dominance status; with dominant behaviours exerted by more dominant males and submissive behaviours exerted by subdominants. Recent studies in humans show however that the relationship between testosterone and agonistic behaviour is not always straightforward. Testosterone can also enhance prosocial behaviours if these promote increasing social status (Dreher *et al.* 2016). In addition, increased testosterone enhances dominant behaviour in men with high social status but causes strategic submission in socially lower-ranked individuals (Inoue *et al.* 2017). A similar effect in the placebo-treated AWD packs in this study could explain the tendency for subdominants to show higher active submission, while also showing higher testosterone levels.

Given that faecal samples collected at SIR reflect fAM concentrations accumulated across the entire separation, immobilisation and reintroduction procedure, it could be argued that the higher levels in placebo treated animals might not necessarily be caused by the reintroduction itself. In our study, treatment was only applied at the end of anaesthesia, to reduce stress related to the reintroduction. DAP therefore, could not suppress any putative increase in fAMs induced by capture or anaesthesia. However, studies in other species show that testosterone actually decreases during capture (Lincoln 1978). Moreover, we saw a similar rise in fAMs in the AWD male that was not sedated; suggesting that the increase in fAMs in the placebo group is caused by the reintroduction event.

No differences in fGCM levels could be seen between DAP and placebo treated animals, with both showing a significant peak in response to the SIR-procedure. Faecal samples collected at SIR reflect stress hormones accumulated across the entire separation, immobilisation and reintroduction procedure. As such, due to the time of treatment, DAP could not suppress any cortisol produced by stressful events associated with capture and immobilisation. Serial blood samples drawn from AWDs after chemical immobilisation show a rise in cortisol concentrations for up to 40 min post-darting (de Villiers *et al.* 1997). Moreover, despite being subjected to similar levels of stress caused by pack separation and reintroduction, the 3 non-sedated animals in our study failed to show a similar fGCM peak; suggesting that the increase in fGCM in both placebo and DAP treated animals is caused by the immobilisation event prior to the application of DAP. This explains the lack of difference between the 2 treatment groups. However, given the mechanism of pheromone action, it is unlikely that DAP would be effective in mitigating the cortisol rise in an unconscious animal. More research investigating the effect of DAP during stressful interventions not necessitating anaesthesia, such as new pack formations, could further clarify its effect on fGCM levels and social interactions.

Most domestic dog studies use behaviour to evaluate the effectiveness of DAP; which has been shown to illicit positive behaviour in adult dogs subjected to a variety of fear- and stress-inducing situations (Sheppard & Mills 2003, Tod *et al.* 2005, Gandia Estelles & Mills 2006, Mills *et al.* 2006, Levine *et al.* 2007, Kim *et al.* 2010, Landsberg *et al.* 2015). However, such studies have not examined the physiological/hormonal response to the pheromone, so its exact underlying mechanism of action is still unknown. The vomeronasal organ transduces pheromonal signals to the amygdala and hypothalamus (Pageat & Gaultier 2003), but the

neurological pathways involved in signal transduction are not understood. Prolactin decreases in placebo, but less in DAP treated domestic dogs after surgery, but no effect was observed in any other parameter, including salivary cortisol (Siracusa *et al.* 2010).

As little is known about the underlying mechanism of action of DAP, it is unclear how it suppressed fAMs after intervention in our study. It is possible that DAP may act directly on the hypothalamic-pituitary-gonadal (H-P-G) axis. However, the effect could be mediated by elevated prolactin concentrations in DAP treated animals (Siracusa *et al.* 2010), known to decrease testosterone secretion by inhibiting GnRH neurons (Gill-Sharma 2009). Moreover, prolactin increases in domestic dogs during positive, affiliative interactions with humans (Odendaal & Meintjes 2003). Thus, it is possible that DAP may upregulate the prolactin pathway, thereby triggering positive behavioural experiences, while simultaneously suppressing testosterone-mediated aggressive or dominant behaviours. Further studies are necessary in domestic dogs to determine these mechanisms.

A major limitation when working with any endangered species with such complex behaviour like the AWD, is access to sufficiently large numbers of packs/animals to evaluate behaviour. In our study, we had to exclude behavioural analysis at the time of reintroduction in one pack for both the DAP and placebo treatment; leaving only 3 packs in each group to evaluate the behavioural effect of DAP on the day of reintroduction. There were however, significant changes on the day of release compared to baseline levels, and trends towards different behaviours between DAP and placebo treated packs were evident. We believe that increasing the sample size will reveal significant differences in behaviour between the 2 treatments.

Although appeasing pheromones are thought to be species-specific, they have the same core chemical composition in different species. Three fatty acids (oleic acid, palmitic acid, and linoleic acid) appear in the same ratio across all species. Other fatty acids then follow in a species-specific ratio which are, for the domestic dog, identified as myristic acid, lauric acid, pentadecanoic acid, and stearic acid (Pageat & Gaultier 2003). Given the similarity of these core fatty acids, it is possible that a highly concentrated species-specific pheromone could have an effect on a related species (Gaultier *et al.* 2005, Vlamings 2011); which has been confirmed in this study. However, it is possible an even more prominent effect on behaviour could be seen in response to an AWD-specific appeasing pheromone, which is yet to be

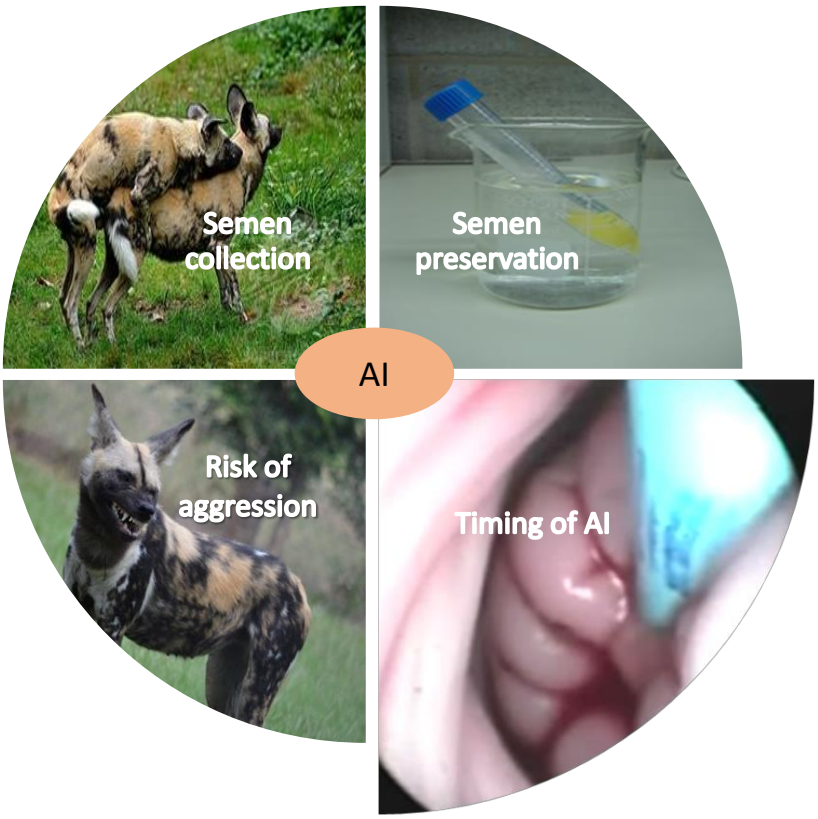
developed. Thus, future parallel studies should focus on isolation and testing of such wild dog-specific pheromones.

In our study, little to no aggression occurred during reintroduction events involving both DAP or placebo treated packs. Aggression after temporary pack separation is quite common in captive AWDs (Scheepers & Venzke 1995, Johnston *et al.* 2007, Vlamings 2011, Foster 2014, Quick 2014), and is the reason why many zoological institutions are reluctant to immobilise animals in order to participate in conservation research or even in annual health check-ups (Johnston *et al.* 2007). However, this and previous studies (Johnston *et al.* 2007), show that when separation and reintroduction is well managed, it can be performed without major issues. We advocate that DAP can play an important role to reduce these risks further. Reintroductions in this study were typically characterised by a peak in dominant and submissive behaviour at reintroduction. Affiliative behaviour remained close to baseline levels, and aggression was almost completely absent during the study period. One of the factors thought to improve our success during reintroduction was the isolation of all pack members during intervention, thereby avoiding the formation of different social bonds between individuals. By contrast, 2 subdominant males were reintroduced to each other in the ABQ pack during the breeding season without the dominant male, who was released the next day. This caused a hierarchy shift of the dominant male to the lowest rank without any sign of aggression. Three days after reintroduction, the original hierarchy was re-established, resulting in an episode of ritualised aggression from the alpha and beta male toward the original gamma male. To avoid similar situations, we advise the separation of all AWDs where group size and infrastructure permits it, combined with the simultaneous reintroduction of all members of the pack.

In conclusion, in our study, DAP prevented the faecal androgen but not faecal cortisol surge in AWDs caused by stressful interventions. This was associated with a trend for lower rates of contact dominance and active submission. These effects could decrease the risk of agonistic interactions and suggest that DAP may be a useful tool to help manage new pack formations and temporary pack separation. However, more research is needed using increased sample size or AWD-specific appeasing pheromones to conclusively elucidate the behavioural effects of DAP and its underlying mechanism of action.

Chapter 5

Timing of artificial insemination



Van den Berghe F, Paris DBBP, Sarnyai Z, Ganswindt A, Paris MCJ. (2018) Periovulatory changes in behaviour and faecal hormones but not vaginal cytology or vaginoscopy could provide predictive tools to determine the fertile period in female African wild dogs (*Lycaon pictus*). *Theriogenology*, in preparation.

Abstract

The development of artificial insemination (AI) can significantly aid conservation of the endangered African wild dog (AWD; *Lycaon pictus*). However, one major limitation of AI in wild canids is determining the female's fertile period. Methods to time AI routinely used in domestic dogs are impossible to perform in the AWD without immobilisation. The aim of this study therefore, was to determine whether certain behaviours coupled with faecal oestrogen (fEM) and progesterone (fPM) metabolite concentrations, could be used as non-invasive parameters to time the fertile period in female AWDs. Three AWD alpha females (Harnas Wildlife Foundation, Gobabis, Namibia) were observed during the 2015-2016 breeding season over a period of 21 - 32 days and detailed behavioural analysis performed from 3-6 days before the first until 2-6 days after the last observed copulation. Faecal samples were collected opportunistically and analysed for oestrogen, progesterone and glucocorticoid metabolite levels. Females were immobilised 2 or 3 times during the periovulatory period to evaluate vulvar condition and blood steroid hormone levels, as well as conduct vaginal cytology, vaginoscopy, and ovarian ultrasound. Late oestrus (the fertile period) could easily be distinguished from pro-oestrus, early oestrus and early dioestrus using behaviour, with a 2- to 5-fold significantly higher rate of male to female affiliative behaviour, alpha male initiating behaviour, sexual follow (of the alpha female by the alpha male), ride-up and copulation ($P \leq 0.05$). All sexual behaviours, as well as male-female resting patterns dropped significantly or ceased completely the day after last mating. One female was anovulatory as shown by the absence of increasing fPM or blood progesterone during oestrus/dioestrus. The two other females showed a 2.5- to 3-fold increase in fPM in late oestrus compared to baseline levels in pro-oestrus ($P \leq 0.05$). In addition, elevated fEM levels that rose in pro-oestrus, declined to baseline by late oestrus. Vaginal cytology and vaginoscopy were not suitable to distinguish late oestrus from early oestrus or pro-oestrus as cell populations and vaginal lining did not noticeably change and differed markedly (absence of 100% keratinisation or angulation) from that observed in domestic dogs. Ovaries were difficult to visualise by ultrasound and no distinction between follicles and corpora lutea could be made, making this technique unsuitable to determine the time of ovulation. Blood progesterone profiles were similar to fPM, but absolute values were lower than in domestic dogs. In conclusion, distinct behaviours of increased frequency during late oestrus, together with rising fPM and declining fEM profiles, appear to be suitable parameters to determine the fertile period in AWD females.

Such parameters could be used to time AI attempts. However, without frequent invasive sampling, blood steroid hormone concentrations, vaginal cytology, vaginoscopy and ultrasound images need to be interpreted with great care.

1. Introduction

African wild dogs (AWD; *Lycaon pictus*) are endangered with current population numbers estimated at 6,600 animals, fragmented over several subpopulations mostly in Southern and Eastern Africa (Woodroffe & Sillero-Zubiri 2012). Habitat fragmentation, human conflict and diseases are the main causes of this decline and continue to put the population under pressure (Woodroffe & Sillero-Zubiri 2012). Assisted breeding techniques have gained importance in wildlife conservation over the last years. Sperm freezing and artificial insemination (AI) can significantly aid conservation by providing a genetic backup of important animals and improve transport/distribution of genetics (Van den Berghe *et al.* 2012). In captive AWDs, adult animals are often translocated between institutions and introduced to each other to constitute a new breeding group. These introductions often lead to aggression between animals and this sometimes causes mortality, while AI could minimise these problems (Van den Berghe *et al.* 2012).

Before AI can be developed in a wildlife species, it is crucial to not only gain extensive knowledge about sperm quality and freezing, but also about the reproductive anatomy, physiology and the oestrus cycle of the female. This will permit the development of a suitable AI technique, performed at the appropriate time relative to ovulation (Thomassen & Farstad 2009). Reproductive physiology can differ widely between similar species and information cannot always be easily transferred (Paris *et al.* 2007). One of the major limitations of performing AI in wild canid species is determining the moment the female has her fertile period. Luckily, most canids studied to date have similar reproductive cycles (Asa & Valdespino 1998) including a mono-oestrous cycle with a long pro-oestrus and oestrus (together classified as heat), a pregnant or non-pregnant (pseudopregnant) period of dioestrus, and an obligatory period of anoestrus (Asa & Valdespino 1998, Concannon 2009, Van den Berghe *et al.* 2012).

In domestic dogs, pro-oestrus is characterized by follicular growth and rising oestrogen levels, responsible for vulvar swelling and haemorrhagic discharge originating from the uterus (Concannon 2011). Generally, the female attracts male dogs but is not yet receptive to mating. Pro-oestrus generally takes about 8 days but can vary significantly (3 days to 3 weeks) between females and between cycles. Oestrus starts with the LH (luteinising hormone) surge and extends for a variable period of typically 8 days during which time the female is generally receptive to mating. Progesterone starts increasing prior to the LH surge and continues to rise

throughout oestrus with maximum levels observed during the dioestrus phase (England 2010). By contrast, oestrogen levels decrease during oestrus resulting in a diminished swelling and discharge from the vulva at this time (Concannon 2011). Typically in domestic dogs, oocytes are ovulated in metaphase I approximately 48-60h after the LH surge, but need to mature to metaphase II for a further 48-60h in the distal oviduct before fertilization can occur (England 2010). Therefore, the most fertile period in domestic dogs, where AI is typically performed, occurs in late oestrus.

The reproductive cycle in AWD females appears similar to that of domestic dogs. Based on behavioural and clinical signs, the combined period of pro-oestrus and oestrus lasts 14 to 20 days (Van Heerden & Kuhn 1985). Pro-oestrus is characterised by an increase in (faecal and urinary) oestrogen metabolite levels, an increase in female proceptivity, vulvar lip swelling, sanguineous vaginal discharge, and a strengthening of the bond with the dominant male (Van Heerden & Kuhn 1985, Creel *et al.* 1997, Monfort *et al.* 1997). In oestrus, intense sexual follow of the female by the dominant male, and intense urine scent marking has been observed (Van Heerden & Kuhn 1985). Copulation occurs over a period of 3 to 7 days, when faecal and urinary oestrogen metabolite concentrations decline, and progesterone metabolite levels rise (Monfort *et al.* 1997). However, any other details about reproductive physiology around the peri-ovulatory period in female AWD are still missing.

Accurate estimation of the fertile period is crucial when performing AI, and even more so when using frozen semen, since its quality after freeze-thawing is generally poor, showing a decreased longevity (Peña & Linde-Forsberg 2000). To estimate the fertile period in domestic dogs, progesterone measurement in the peripheral blood is the most widely used technique. Progesterone is at baseline levels during pro-oestrus (< 1 ng/ml) and steadily increases during oestrus. Therefore, it provides a good indication of the timing of periovulatory events, enabling the estimation of the LH-surge (1.5-2.5 ng/ml), ovulation (5-8 ng/ml), and the fertile period (10-25 ng/ml; Von Heimendahl & England 2010). However, some individual variation exists, therefore, the use of additional tools is advised. Vaginal cytology and endoscopy can both help determine the phase of the reproductive cycle as hormonal changes cause significant changes in the vaginal epithelium and mucosal folds (Von Heimendahl & England 2010). In addition, transabdominal ovarian ultrasound can be a highly valuable supplementary technique to time ovulation, since follicles either completely disappear or become smaller and

irregular at that time (Fontbonne & Malandain 2006). However, these examinations should be performed on a regular basis throughout pro-oestrus and oestrus until the moment of AI. Unfortunately, such frequent and invasive evaluations are not possible without immobilisation in wild canids, therefore precluding their utility as diagnostic tools for determining the timing of ovulation in AWDs.

Non-invasive measurement of reproductive hormones in faeces could be a valuable alternative to time AI. This technique has already been validated for AWDs (Monfort *et al.* 1997) and adopted in reproductive research (Creel *et al.* 1997, Paris *et al.* 2008, Newell-Fugate *et al.* 2012, Van der Weyde 2013). Moreover, in domestic dogs, it was found that faecal and serum progesterone rise simultaneously after ovulation, indicating that the measurement of faecal progesterone metabolites can be used as a non-invasive, indirect method to estimate the time of the LH surge and AI (Hay *et al.* 2000). A similar result has been observed in the red wolf (*Canis rufus*), where it was suggested that AI be performed 5 to 6 days after the initial rise in faecal progestogen levels (Walker *et al.* 2002). For the AWD however, research into non-invasive tools to time AI is currently missing.

The aim of this study, therefore, was to determine whether certain behaviours, coupled with faecal oestrogen and progesterone metabolite levels, could be used as routine non-invasive parameters to determine the fertile period for optimal timing of AI in female AWDs. More specifically, we endeavoured to validate these non-invasive parameters against observed mating, clinical signs of oestrus, serum oestrogen and progesterone concentrations, vaginoscopy, vaginal cytology, and ultrasound measurements.

2. Materials and methods

2.1. Animals, husbandry and behavioural observations

This study included n=3 alpha females from 3 different packs housed at Harnas Wildlife Foundation, Gobabis, Namibia (Brutus pack, BRU F1 ♀; Platform pack, PLA F1 ♀; San pack, SAN F1 ♀; Chapter 2; Table 5.1). As described in Chapter 2, packs were of mixed-sex and held in large enclosures (0.7 ha, 14.4 ha, and 3.6 ha respectively) of natural habitat consisting of dense trees, scrub and an artificial waterhole. However, the PLA pack was moved into 4 smaller adjacent enclosures (0.1 ha each; Fig. 2.1) for the period of study (observation period; Table 5.1) to facilitate observations, faecal sample collection, and capture as described in Chapter 2. Pack composition is shown in Table 5.1. Animals were group-fed daily with donkey and 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. Water was available *ad libitum*.

Table 5.1. *Pack composition, age, observation and analysis time, and reproductive history of the n=3 alpha females sampled in this study.*

Pack	Composition, age	Observation period	Daily observation time (h; mean ± SEM)	Behavioural analysis period	Daily behavioural analysis time (h; mean ± SEM)	Reproductive history α ♀
BRU	**Total 4 ♂, 3 ♀** α ♂ & ♀: est. > 7 y (unknown age) Offspring 3 ♂, 2 ♀: 1.8 y	32 days (15 Sep - 16 Oct 2015)	4.1 ± 0.3	23 days (23 Sep - 15 Oct 2015)	1.6 ± 0.1	≥ 1 litter
PLA	**Total 13 ♂, 4 ♀** All siblings different litters: est. 3-5 y (unknown age)	28 days (7 Jan - 3 Feb 2016)	3.1 ± 0.3	17 days (18 Jan - 3 Feb 2016)	2.3 ± 0.1	unknown
SAN	**Total 4 ♂, 6 ♀** Siblings 2 ♂, 3 ♀ incl. α pair: 5 y Offspring 2 ♂, 3 ♀: 1.7 y	21 days (12 Mar - 1 Apr 2016)	2.3 ± 0.3	15 days (16 Mar - 30 Mar 2016)	1.8 ± 0.1	≥ 1 litter

The alpha female within each pack was observed over a period of 21 - 32 days (observation period) for signs of heat during the 2015-2016 breeding season (defined as the time mating occurs; Table 5.1). During this time their behaviour was video recorded for several hours each day (daily observation time; Table 5.1). As indicated in Table 5.1. and Chapter 2, The PLA and SAN pack females were observed to mate in January and March respectively, which falls within the expected breeding season. By contrast, the BRU pack female was observed to mate around October. Recording was performed within enclosures from the top of a car, which was moved when necessary to maintain visual contact with the alpha female. Recorded videos were

analysed from 3-6 days before the first until 2-6 days after the last observed copulation (behavioural analysis period; Table 5.1). The entire captured footage was scanned for each day to check for the presence of copulation, then detailed behavioural analysis was confined to a subset of times each alpha pair was most sexually active. This was generally in the afternoon after feeding time for the BRU pack, and in the morning after feeding for the PLA and SAN packs. In cases where an alpha female was not visible, this period was also excluded from the daily observation time, resulting in a daily behavioural analysis time indicated in Table 5.1. Behaviour was not observed on the day of immobilisation and sampling of alpha females (this study) or males (Chapter 2). This amounted to 5, 4 and 3 days for the BRU, PLA and SAN pack (Fig. 5.3: days -4, -1, +6, +9, +13; Fig. 5.4: days +1, +4, +7, +12; Fig. 5.5: days -2, -1 and +8 respectively).

Sexual behaviour was defined according to the ethogram in Table 5.2. Other behaviours were also noted, as defined in detail in Chapter 4 (Table 4.2) and included non-sexual follow of the alpha female by the alpha male, marking behaviour (male and female combined), affiliative, dominant and submissive behaviour between the alpha male and female, and all interactions between the alpha female and other pack members. In addition, resting relations (static lying, standing or eating) were also noted including the duration, and grouped as close contact between alpha female and male exclusively; close contact between the alpha female and other pack members with or without the alpha male; and the alpha female alone. Hourly rates were calculated for each day for each behaviour. The PLA pack males did not show a clear stable hierarchy, with different males exhibiting sexual behaviour with the alpha female on different observation days. These males were considered ‘alpha male’ on those specific days (M6, M8 and to a lesser extent M11; Table 2.1 and 2.2).

Table 5.2. *African wild dog (Lycaon pictus) ethogram characterising sexual behaviour used in behavioural analysis. Modified from Vlamings (2011).*

Behaviour	***Description***
Sexual follow	The male follows the female closely, with his neck straight and nose in a sniffing attitude.
Ride-up	The male mounts the female; this is accompanied by pelvic thrusting but there is no intromission.
Copulation	The act of mating, resulting in a copulatory tie.
Present, this includes:	
Present reactive	The female directs her anogenital region to the male (sometimes averts her tail) as a reaction to the advances of the male.
Present active	Same as above, but characterized by an active backwards approach, pushing towards the male.
Mate guarding	All behaviours where the male prevents other pack members from approaching the female (intervention by approach, chase away).
Initiating behaviour from male, this includes:	
Genital lick	Self-explanatory.
Head under	The actor pushes with his head towards the ventro-lateral side of the recipient, occasionally lifting, with his head, the recipient's back quarters from the ground.
Pass under head	The actor passes from a lateral side close under the head of the recipient, usually in a somewhat crouching manner; often a short nose-chin contact with the recipient is evident.
Push side/scruff	The actor pushes with his nose on the side or the scruff of the recipient.
Tail position	The actor stands behind the recipient with his head directed towards his ano-genital region.
Fur licking	Self-explanatory.

2.2. Faecal sample collection and steroid hormone analysis

As described in Chapter 2, faecal samples were collected opportunistically within a few minutes after the n=3 alpha AWD females were seen defecating during the observation period. Faecal samples were also collected from n=7 subdominant (anoestrus) females. Samples were sealed in plastic bags and kept in a cooler box on ice until the end of observation, then frozen at -20°C. Samples were transported to the University of Namibia (Windhoek, Namibia) on dry ice, oven dried, pulverised, and then ethanol extracted. Dried extracts (1 ml, 45°C) were transported at room temperature to the University of Pretoria (Pretoria, South Africa) where they were reconstituted in 1 ml ethanol and analysed. Faecal steroid extracts were measured for immunoreactive faecal glucocorticoid (fGCM), progesterone (fPM), and oestrogen (fEM) metabolite concentrations using enzyme-immunoassays (EIAs). As described in Chapter 4, a biotinylated cortisol-3-CMO assay coupled with bovine serum albumin (BSA) was used for determining fGCM concentrations (Palme & Mostl 1997). The sensitivity of the EIA was 1.2 ng/g dry faecal weight (DW). Intra- and inter-assay coefficients of variation of high- and low-quality controls were 4.8% and 5.6%, and 12.2% and 13.8% respectively. For analysis of fPM concentrations, a P4-Ak6 assay was used with pregnanolone (5β-pregnane-3α-ol-20-one) coupled with BSA as antibody and biotinylated allopregnanolone (5α-pregnane-3β-ol-20-one) as label (Schwarzenberger *et al.* 1996). The

sensitivity was 20 ng/g DW and intra- and inter-assay coefficients of variation (CV) were 4.0% and 6.6%, and 5.5% and 10.1% respectively. Finally, fEM concentrations were determined using a 17β-oestradiol assay (Palme & Mostl 1994). Sensitivity was 0.5 ng/g DW and intra- and inter-assay CV were 4.8% and 6.7%, and 11.4% and 14.2% respectively.

2.3. Immobilisation and sample collection

To more precisely link behaviour and faecal hormone levels with physiological events associated with oestrus and ovulation, each of the 3 alpha females were immobilised on 2 or 3 occasions at different stages in and around the oestrus period. For each female, oestrus was considered to begin from the first day in which copulation was observed (day 0) to the last day of copulation (day +11, day +10, and day +4 for BRU F1 ♀, PLA F1 ♀, and SAN F1 ♀ respectively). To collect physiological measurements spanning late pro-oestrus, oestrus, and early dioestrus, BRU F1 ♀ was immobilised on days -1, +6, and +9; PLA F1 ♀ on days +1, +4 and +7; and SAN F1 ♀ on days +4 and +8 (Table 5.5). In addition, one lower ranking PLA pack female not displaying signs of oestrus, was also immobilised (PLA F2 ♀; anoestrus control), 2 days after the oestrus period of PLA F1 ♀ ceased. Detailed behavioural analysis was not performed for this female. Immobilisation mainly occurred in the morning using a combination of 5.3-12.2 mg/kg ketamine (Anesketin®, Albrecht GmbH, Aulendorf, Germany) and 1.4-2.3 mg/kg xylazinehydrochloride (Rompun® TS, Bayer Vital GmbH, Leverkusen, Germany) administered IM using a CO_2 dart gun, after which the animal was transported to the clinic for sample collection. After sampling, anaesthesia was reversed with 0.1-0.2 mg/kg IM atipamezole hydrochloride (Revertor®, CP-Pharma, Burgdorf, Germany) and AWDs monitored throughout the day until fully recovered. To facilitate reintroduction into the pack, 10 ml of dog appeasing pheromone (DAP) was applied between the shoulders and on the base of the tail at the end of anaesthesia (Chapter 4). Females were reintroduced to the pack in the evening the same day, except for the last immobilisation of BRU F1 ♀ which occurred in the afternoon, resulting in her reintroduction the following morning.

At the veterinary clinic, each female first underwent a complete physical examination. Mammary glands were inspected and palpated to exclude the presence of mammary tumours. The vulva was inspected for lesions, swelling and mucosal colour. Vulvar thickness (W; Fig. 5.1a) and height (H; Fig. 5.1b) was measured using callipers (Kinchrome Australia Pty Ltd,

Scoresby, VIC, Australia) and vulvar size was calculated as W x H. A swab was taken from the cranial vagina and the colour of it was noted, after which it was gently rolled onto several glass slides. Slides were stained with Diff-Quick (*Kyro-Quick stain*; Kyron Laboratories, Benrose, South Africa) and a modified Harris-Shorr stain for vaginal cytology. For the Harris-Shorr stain, slides were immersed in 95% alcohol for 5 min, then 10 passages in both 70% and 50% alcohol before rinsing in distilled water. Slides were then stained in Harris (Hematoxylin solution, Harris modified, Sigma-Aldrich, St-Louis, MO, USA) for 3 min at room temperature, rinsed in distilled water then submerged in ammoniac water until cells showed a blue aspect. After rinsing in distilled water, slides were submerged in 70% and 95% alcohol for 10 seconds each, stained with Shorr (Merck Millipore, Billerica, MA, USA) for 3 min and then rinsed in 95% alcohol. Diff-Quick-stained slides were first examined for cellularity at 100 X magnification under brightfield microscopy. Thereafter, individual epithelial cells (> 200 cells) were analysed at 400 X magnification and, according to their nucleus/cytoplasm ratio and form, classified as small, medium, large or superficial/keratinised cells (Fig. 5.2). The difference between superficial and keratinised cells could not be distinguished using Diff-Quick stain, as both have a polygonal aspect (Fig. 5.2d). Therefore, the percentage of keratinised cells was calculated using Harris-Shorr stain, in which keratinised cells stain red-orange while superficial non-keratinised and all other cells stain blue-green (Fig. 5.2e).



Figure 5.1. *Measurement of vulva thickness (a) and height (b) using callipers in female African wild dogs.*

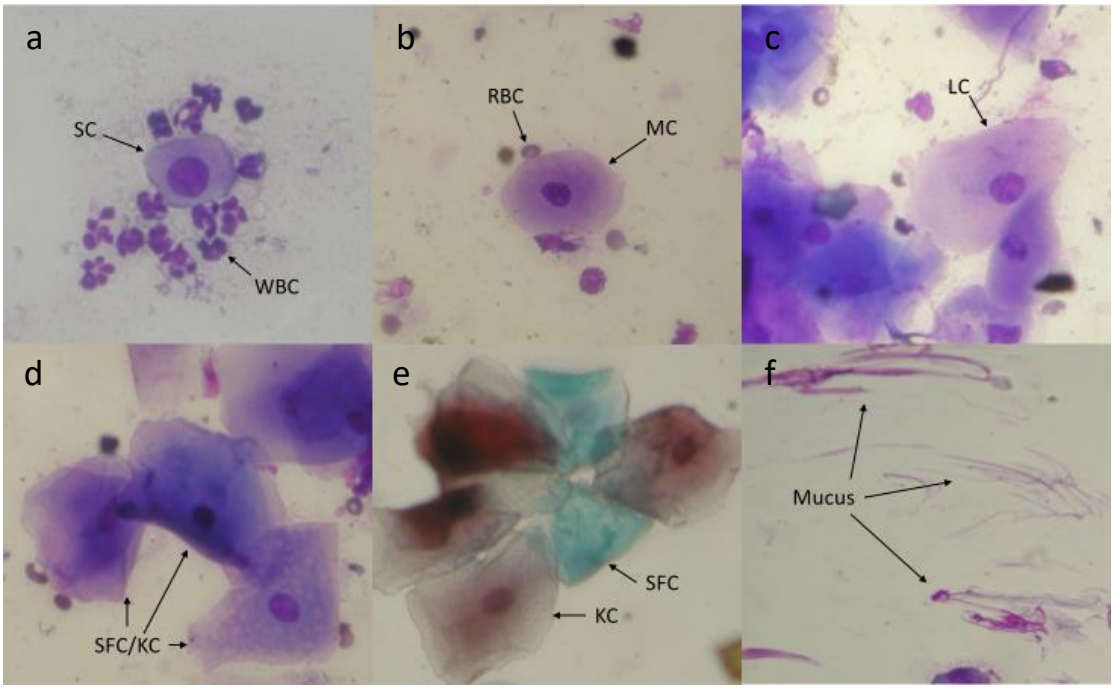


Figure 5.2. *Classification of African wild dog vaginal epithelial cells stained with Diff-Quick (a-d, f) or Harris-Shorr (e). (a) SC, small epithelial cell (rounded, parabasal and small intermediary cells with large nucleus/cytoplasm ratio); (b) MC, medium epithelial cell (rounded, medium nucleus/cytoplasm ratio); (c) LC, large epithelial cell (rounded, very low nucleus/cytoplasm ratio); (d) SFC, superficial epithelial cell (polygonal and very large, nucleus often pyknotic or absent); (e) KC, keratinised cell (polygonal and very large, nucleus often pyknotic or absent); (f) mucus secretion. 400 X magnification; WBC, white blood cell (neutrophil); RBC, red blood cell*.

Deep vaginal endoscopy was performed using a Single Channel Ureteroscope A2940 (Olympus, Shinjuku, Tokyo, Japan), and colour of the vaginal mucosa, presence and colour (haemorrhagic, serosanguinous, serologic) of uterine secretions, and amount of vaginal oedema were noted. The latter was scored as 0 – no oedema; 1 – presence of primary folds (oedema +++); 2 – appearance of secondary folds (oedema ++); 3 – secondary folds (oedema +); 4 – start angulation/crenulation (oedema ±); 5 – angulation (oedema -). Transabdominal ovarian and uterine ultrasound was performed using an Ibex Portable ultrasound (EI Medical Imaging, Loveland, CO, USA) with a 6 MHz transducer. Ten ml of blood was collected from the cephalic or saphenous veins into dry blood tubes, allowed to stand for 30 min before centrifugation at 3,000 *g* for 15 min. Serum was frozen at -18°C in 1.5 ml plastic tubes. Although some serum samples accidently thawed during storage due to freezer failure (just above freezing temperature, < 12 h; Table 5.5), this event should not have significantly affected hormone levels (Reimers *et al.* 1983, Reimers *et al.* 1991, Tahir *et al.* 2013). Samples were transported to the University of Namibia (Windhoek, Namibia) on dry ice where they were thawed. Subsequently, 0.5 ml was diluted in 2.5 ml diethyl ether and vortexed for 5 min.

The solution was then kept at room temperature for 5 min after which it was frozen in a dry ice/ethanol bath. This enabled easy separation of the top ether layer containing the steroid hormones. Approximately 1.5-2 ml of ether was air-dried by letting tubes stand in a fume hood for 5-6 h. Dried extracts were transported at room temperature to the University of Pretoria (Pretoria, South Africa), where they were reconstituted in assay buffer and analysed for oestrogens and progestogens using the EIAs described above. Sensitivity was 0.64 ng/ml and 16 pg/ml serum for the progesterone and oestrogen EIAs respectively.

2.4. Statistical analysis

Based on observed copulation and the intensity of other sexual behaviours, the period of behavioural analysis could be separated into pro-oestrus (prior to first copulation), early oestrus (first half of copulation period), late oestrus (second half of copulation period), and dioestrus phases (after last copulation) for each of the 3 alpha females. Hourly rates of a range of sexual and non-sexual behaviours, and resting patterns (expressed as fraction of total resting time) for the 3 females were obtained each day and grouped by reproductive phase, with data tested for normality by Shapiro-Wilk test and histograms. Differences in hourly rates of these behaviours between phases were evaluated using Kruskal-Wallis and post-hoc Mann-Whitney U tests with Bonferroni adjustment; except for male-female and solitary female resting patterns (normally distributed), which were analysed by one-way ANOVA and post-hoc Tukey test. Faecal steroid hormone concentrations during the different reproductive phases were compared by one-way ANOVA with post-hoc Tukey test for BRU F1 ♀ and PLA ♀ F1. $P \leq 0.05$ was significant. Statistical analysis was performed with SPSS Statistics 23 (IBM® SPSS® Statistics 23, SPSS Inc., IBM, Armonk, New York, USA).

3. Results

3.1. Behaviour

In the BRU pack, sexual behaviour including copulation attempts (ride-up) started on day -2, however copulation was only seen on days +0, +8, +10 and +11 (Fig. 5.3a and b). For the PLA pack there was no ride-up prior to the first day of observed copulation, which occurred on days 0, +2, +6, +8, +9, and +10 (Fig. 5.4a and b). The oestrus period in the SAN pack alpha female only lasted for 5 days, with first ride-up coinciding with the first day of observed copulation, which occurred on days +0, +2, +3 and +4 (Fig. 5.5a and b). However, observations could not be performed on days -1 and -2 in the SAN pack due to immobilisation of males on those days for a separate study (Chapter 2). As such, it is possible that the onset of early oestrus in this female may have begun on day -1 or -2. All 3 alpha females showed a clear biphasic increase in most sexual behaviours and a monophasic increase in female-male resting behaviour, that typically extended from a few days before oestrus and ended abruptly after the last day of copulation (Fig 5.3, 5.4 and 5.5). The hourly rate of several of these behaviours was typically higher during the second phase of oestrus (classified as late oestrus).

The period of behavioural analysis was subdivided into 4 distinct reproductive phases : (1) pro-oestrus: days -6 to -1 for BRU F1 ♀, days -3 to -1 for PLA F1 ♀ and days -5 to -3 for SAN F1 ♀; (2) early oestrus: days +0 to +6 for BRU F1 ♀, days +0 to +5 for PLA F1 ♀, and days 0 to +2 for SAN F1 ♀; (3) late oestrus: days +7 to +11 for BRU F1 ♀, days +6 to +10 for PLA F1 ♀, and days +3 to +4 for SAN F1 ♀; and (4) dioestrus: days +12 to +16 for BRU F1 ♀, days +11 to +13 for PLA F1 ♀, and days +5 to +9 for SAN F1 ♀ (Fig. 5.3, 5.4 & 5.5).

When data was analysed by reproductive phase, there was no difference in any sexual or non-sexual behaviours nor resting patterns from pro-oestrus to early oestrus ($P > 0.05$; Table 5.3). Hourly rates of sexual follow, ride-up, copulation and initiating behaviour increased over 3-fold, and alpha male to female affiliative behaviour increased over 2-fold from early to late oestrus ($P \leq 0.05$), with sexual follow and initiating behaviour occurring at particularly high frequency (Table 5.3). With the exception of sexual follow, these behaviours were higher during late oestrus than any other reproductive phase ($P \leq 0.05$), and all dropped abruptly or ceased completely on the day after last copulation (Fig. 5.3, Fig. 5.4, Fig. 5.5, Table 5.3); clearly indicating the first day of dioestrus. While hourly rates of alpha female - male resting patterns

tended to increase and solitary female resting patterns tended to decrease during late oestrus, hourly rates of these patterns as well as present and marking behaviour only differed at dioestrus ($P \leq 0.05$). Mate guarding was higher during the entire oestrus period but tended to be highest in late oestrus (Table 5.3).

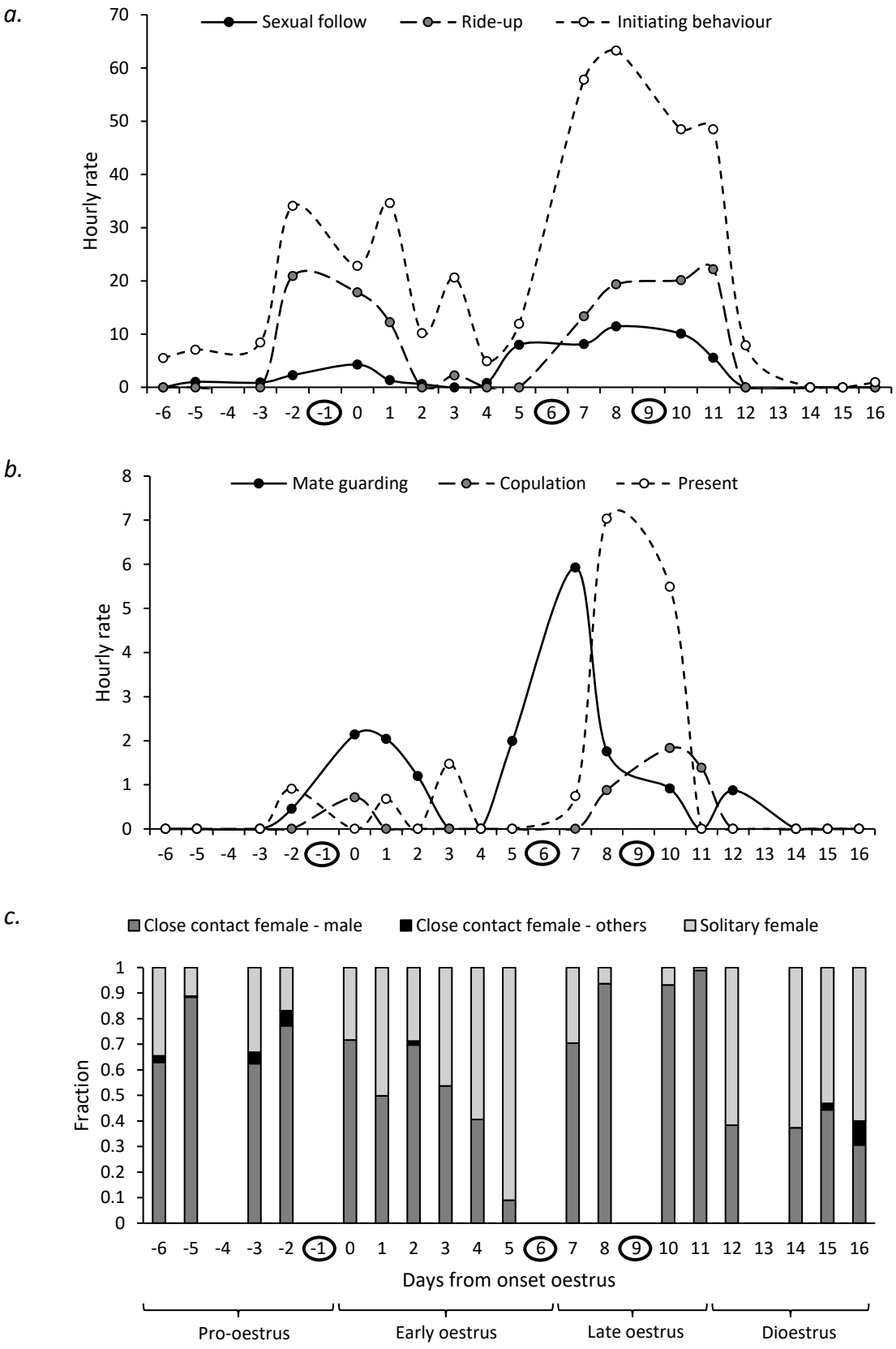


Figure 5.3. *(a, b) Hourly rates of sexual behaviour and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of oestrus for the alpha African wild dog female BRU F1 ♀. Circles indicate days of immobilisation.*

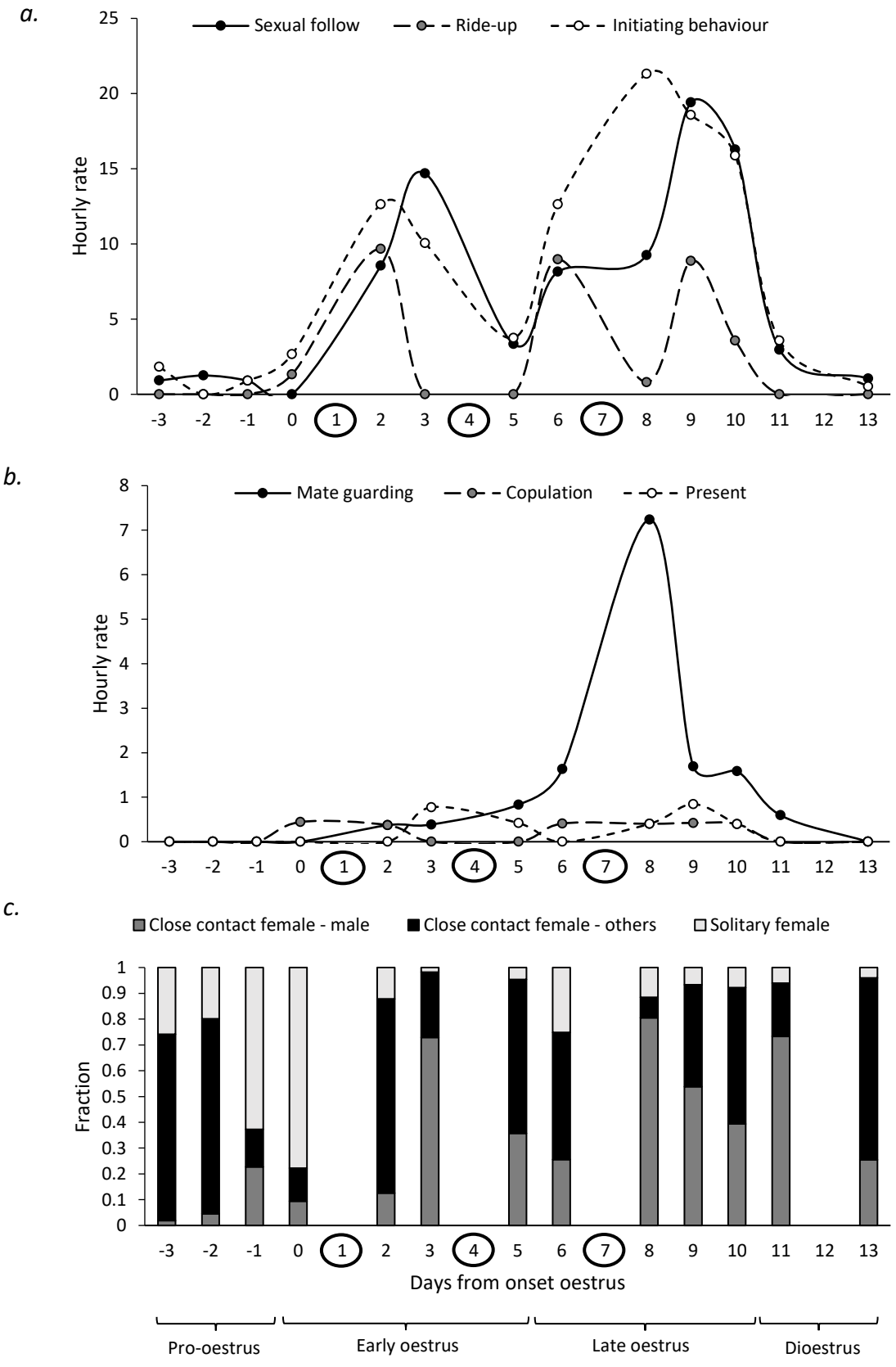


Figure 5.4. *(a, b) Hourly rates of sexual behaviour and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of oestrus for the alpha African wild dog female PLA F1 ♀. Circles indicate days of immobilisation.*

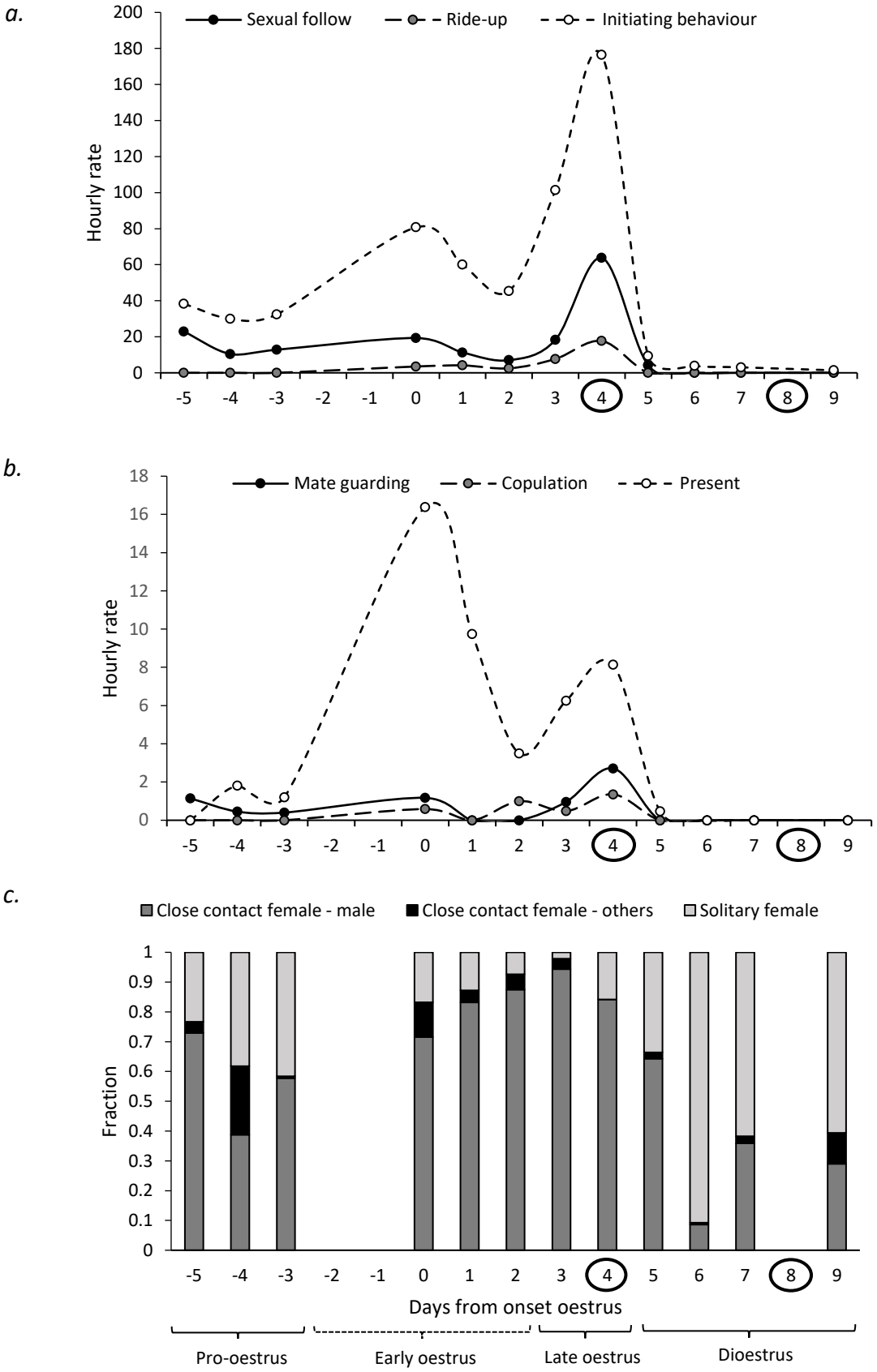


Figure 5.5. *(a, b) Hourly rates of sexual behaviour and (c) resting relationships (expressed as fraction of total resting time) relative to the onset of oestrus for the alpha African wild dog female SAN F1 ♀. Circles indicate days of immobilisation. Dashed bracket, putative early oestrus period.*

Table 5.3. *Mean (± SEM) hourly rates of sexual and non-sexual behaviour and fractional resting patterns in n=3 African wild dog alpha females during pro-oestrus, early oestrus, late oestrus and dioestrus.*

	Pro-oestrus	***Early oestrus***	***Late oestrus***	***Dioestrus***	***P-value****
Sexual behaviour					
Sexual follow	5.3 ± 2.4^{ab}	4.9 ± 1.4^{b}	17.0 ± 5.4^{a}	0.8 ± 0.5^{b}	<0.001
Mate guarding	0.2 ± 0.1^{b}	0.8 ± 0.2^{ab}	2.4 ± 0.7^{a}	0.1 ± 0.1^{b}	0.001
Ride-up	2.1 ± 2.1^{bc}	3.9 ± 1.6^{b}	12.2 ± 2.3^{a}	0.0 ± 0.0^{c}	<0.001
Copulation	0.0 ± 0.0^{b}	0.2 ± 0.1^{b}	0.8 ± 0.2^{a}	0.0 ± 0.0^{b}	<0.001
Present	0.4 ± 0.2^{ab}	0.3 ± 0.1^{ab}	2.9 ± 1.1^{a}	0.0 ± 0.0^{b}	0.011
Initiating behaviour	15.8 ± 5.0^{bc}	11.7 ± 2.4^{b}	56.4 ± 15.9^{a}	3.1 ± 1.0^{c}	<0.001
Non-sexual behaviour					
Non-sexual follow of f1 by m1	3.7 ± 1.0	4.3 ± 1.0	8.7 ± 1.8	4.3 ± 2.1	0.037
Marking behaviour	3.1 ± 0.9^{ab}	5.6 ± 1.4^{ab}	5.3 ± 0.8^{a}	1.6 ± 0.8^{b}	0.013
Interactions f1 - others	2.7 ± 0.5	1.9 ± 0.5	2.5 ± 1.1	3.4 ± 0.9	0.480
Affiliative behaviour m1 - f1	1.0 ± 0.3^{b}	3.6 ± 0.9^{b}	8.4 ± 1.5^{a}	1.4 ± 0.5^{b}	<0.001
Dominant behaviour f1 to m1	1.0 ± 0.6	0.4 ± 0.2	2.2 ± 0.8	1.3 ± 0.8	0.135
Submissive behaviour f1 to m1	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0	0.609
Dominant behaviour m1 to f1	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	0.623
Submissive behaviour m1 to f1	0.2 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.2	0.211
Resting pattern					
Close contact female - male	0.49 ± 0.10^{ab}	0.48 ± 0.08^{ab}	0.73 ± 0.08^{a}	0.39 ± 0.06^{b}	0.031
Close contact female - others	0.20 ± 0.09	0.16 ± 0.07	0.15 ± 0.07	0.12 ± 0.07	0.512
Solitary female	0.31 ± 0.05^{ab}	0.36 ± 0.08^{ab}	0.11 ± 0.03^{a}	0.49 ± 0.09^{b}	0.005

Values with different letters for a particular behaviour indicate a significant difference between reproductive phases. m1, alpha male; f1, alpha female. ****P*-value of Kruskal-Wallis/one-way ANOVA analysis

3.2. Faecal steroid hormone metabolites

There were differences in the faecal steroid hormone metabolite profiles of the 3 alpha females. For BRU F1 ♀, fPM levels were at baseline (8.1 ± 1.1 µg/g DW) until day -6 then rose over 2-fold by day -1 and continued to rise during oestrus (24.9 ± 3.4 µg/g DW; Fig. 5.6a); with levels in early and late oestrus significantly higher compared to pro-oestrus (Table 5.4). Baseline levels of fEM could be observed from days -14 to -10 (0.18 ± 0.10 µg/g DW) and a distinct peak on day -7 (1.28 µg/g DW), after which levels decreased again over early oestrus (0.54 ± 0.08 µg/g DW) and declined to baseline during late oestrus (0.15 ± 0.06 µg/g DW; Fig. 5.6a). However, no significant differences could be seen in fEM levels between the different reproductive phases (Table 5.4). fGCM levels appeared to fluctuate but rose significantly during the oestrus/dioestrus phases ($P \leq 0.05$), although post hoc tests could not discriminate which phases were different from each other (Fig. 5.6a; Table 5.4). Moreover, there were no distinctive peaks visible on the days after immobilisation, suggesting an absence of immobilisation-related stress (Fig. 5.6a).

For PLA F1 ♀, fPM levels did not rise from baseline levels during the oestrus period (Fig. 5.6b; Table 5.4), and were similar to levels observed in anoestrus females (Table 5.4), possibly indicating an anovulatory cycle in this female. Although fEM levels on day -10 were slightly higher compared to the other pro-oestrus samples of this female, a distinct peak in fEM levels of the same magnitude as observed for BRU F1 ♀ was absent. Faecal samples could not be collected on days -9 to -4, but a small spike (0.35 µg/g DW) in fEM could be seen at day -2 (Fig. 5.6b). Levels of fEM did not change across the reproductive phases (Table 5.4), providing further evidence to support anovulation in this female. No significant differences in fGCM concentrations could be seen between the different reproductive phases (Table 5.4).

Very few faecal samples could be collected for SAN F1 ♀ because of her preference to defecate in the very dense habitat of the SAN pack enclosure, thereby making statistical comparison by reproductive phase impossible (Table 5.4). However, the steroid hormone patterns obtained showed similar trends to that observed for BRU F1 ♀. Baseline fPM levels were seen on days -8 to -6 (11.3 ± 2.5 µg/g DW), after which they rose on day -5 and continued to rise into dioestrus at day +8 (63.3 µg/g DW; Fig. 5.6c). As for BRU F1 ♀, levels in late oestrus were approximately 2.5-fold higher than baseline. Baseline fEM levels (day -8 to -6; 0.20 ± 0.14 µg/g DW) rose to a peak on day -5 (1.93 µg/g DW) and dropped back to baseline levels in late oestrus on day +4 (0.09 µg/g DW). In addition, fGCM concentrations did not appear to change across the different phases (Table 5.4; Fig. 5.6c).

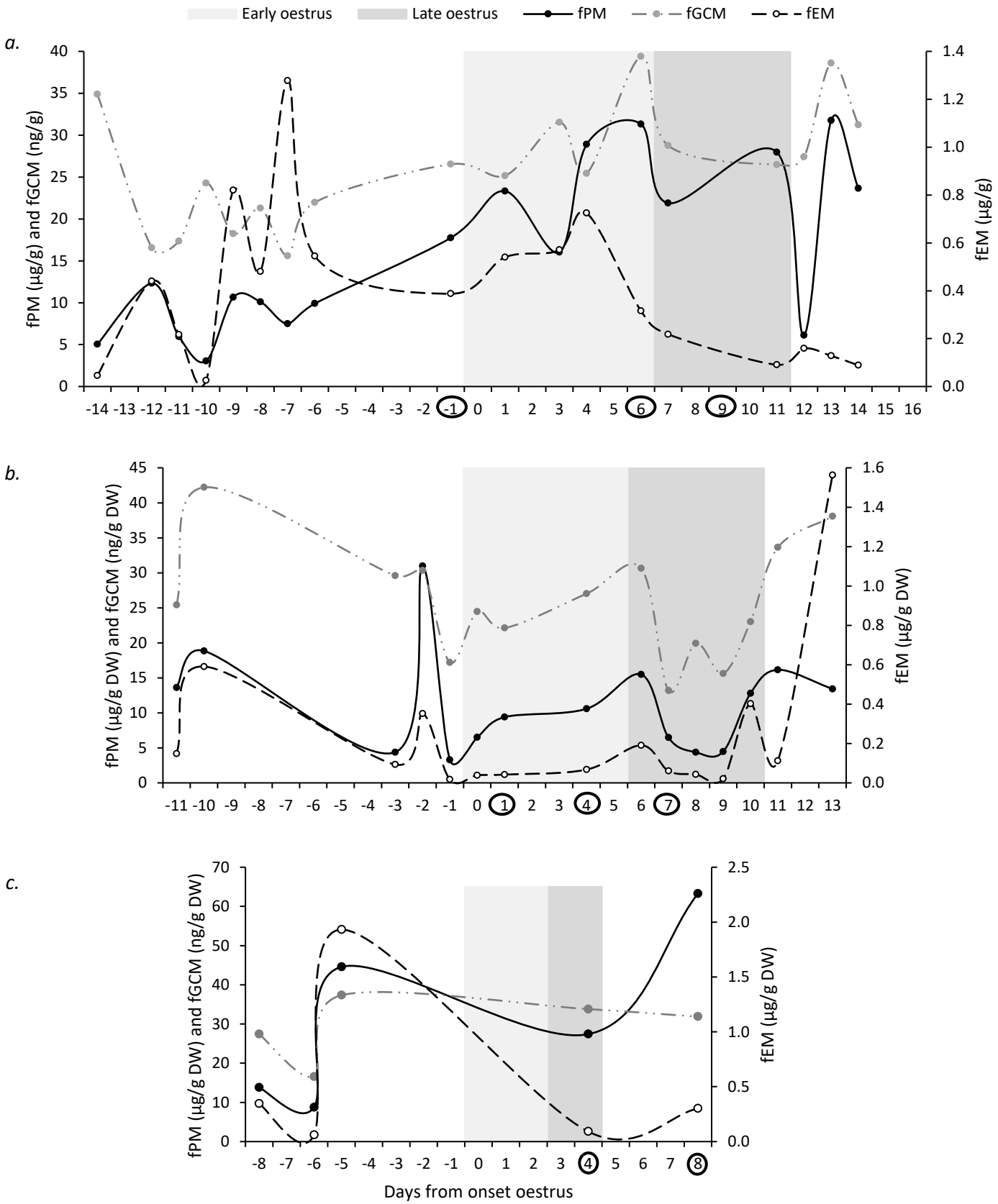


Figure 5.6. *Faecal progesterone, oestrogen and glucocorticoid metabolite concentrations relative to the onset of oestrus for the alpha African wild dog female (a) BRU F1 ♀, (b) PLA F1 ♀, and (c) SAN F1 ♀. Circles indicate days of immobilisation.*

Table 5.4. *Mean ± SEM faecal progesterone, oestrogen and glucocorticoid metabolite concentrations in n=3 African wild dog alpha females and n=7 subdominant females during anoestrus, pro-oestrus, early oestrus, late oestrus and early dioestrus.*

Female	*Faecal hormone*	*Anoestrus*	*Pro-oestrus*	*Early oestrus*	*Late oestrus*	*Dioestrus*	*P-value*
BRU F1 ♀	*fPM (µg/g DW)*	-	9.1 ± 1.5[b]	24.9 ± 3.4[a]	24.9 ± 3.0[a]	20.5 ± 7.6[ab]	0.005
	fEM (µg/g DW)	-	0.5 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.224
	fGCM (ng/g DW)	-	21.9 ± 2.0	30.3 ± 3.3	27.6 ± 1.1	32.41 ± 3.3	0.050
PLA F1 ♀	*fPM (µg/g DW)*	-	14.2 ± 5.1	8.8 ± 1.2	8.7 ± 2.3	14.8 ± 1.4	0.581
	fEM (µg/g DW)	-	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.1	0.8 ± 0.7	0.132
	fGCM (ng/g DW)	-	29.0 ± 4.1	24.5 ± 1.4	20.5 ± 3.1	35.9 ± 2.2	0.097
SAN F1 ♀	*fPM (µg/g DW)*	-	22.4 ± 11.2	-	27.4	63.3	-
	fEM (µg/g DW)	-	0.8 ± 0.6	-	0.1	0.3	-
	fGCM (ng/g DW)	-	27.1 ± 6.0	-	33.8	31.9	-
Anoestrus control (n=7)	*fPM (µg/g DW)*	7.0 ± 0.9	-	-	-	-	-
	fEM (µg/g DW)	0.6 ± 0.3	-	-	-	-	-
	fGCM (ng/g DW)	21.0 ± 2.8*	-	-	-	-	-

Values with different letters for a particular hormone indicate a significant difference between reproductive phases. *fGCM levels were only measured in n=2 subdominant females.

3.3. Other reproductive parameters

No abnormalities of the mammary gland (tumours) were present and the number of pairs of nipples varied between 5 and 7 (Table 5.5). Compared to domestic dogs, the vulva of all these females had a more horizontal position with the vulvar opening pointing ventrally, and in a standing individual, swelling was clearly visible in the region of the vestibule (Fig. 5.7). The vulva had a swollen aspect in all cases except for BRU F2 ♀ (anoestrus control) and SAN F1 ♀ (last day oestrus and early dioestrus; Table 5.5). When standardised to body weight, vulvar size was small in anoestrus (0.4 cm^2/kg), showed a 2.2-fold increase by the end of pro-oestrus (0.9 cm^2/kg), after which it declined again during oestrus (0.6-0.7 cm^2/kg), with a size approaching baseline by the end of oestrus and dioestrus (0.5 cm^2/kg; Table 5.5). Visible vulvar discharge was only present at the first sedation of PLA F1 ♀ (Table 5.5), however the vaginal swab colour showed presence of sanguineous secretions (PLA F1 ♀ and BRU F1 ♀) into early oestrus (Table 5.5).



Figure 5.7. *Swollen vulva and vestibule of PLA F1 ♀ (white arrow).*

Vaginal cytology was similar to that observed in domestic dogs during the phases of anoestrus, late pro-oestrus and early oestrus (Table 5.5). In anoestrus, few epithelial cells were present, all of which were small (parabasal and small intermediary), and old neutrophils and mucus was present (Table 5.5; Fig. 5.8a and b). At the end of pro-oestrus, epithelial cells had increased and transitioned to similar proportions of large, superficial, and keratinised cells. No neutrophils or mucus were present, while bacteria were abundant (Table 5.5; Fig. 5.8b and c). By mid-oestrus, the number of keratinised cells increased in BRU F1 ♀ and reached a maximum (84%) at day +6 (Table 5.5; Fig. 5.8e and f), but unlike the domestic dog, declined again toward late oestrus (Table 5.5; Fig. 5.8g and h). A similar trend was observed for PLA F1 ♀, where the number of keratinised cells decreased from 59% in early oestrus to 33% during late oestrus, and mucus and neutrophils reappeared. On the last day of oestrus (SAN F1 ♀ day +4), the dominant epithelial cell population consisted of medium cells, and the fraction of keratinised cells decreased to 2% (Table 5.5; Fig. 5.8i and j). In early dioestrus, the size of epithelial cells continued to decline, and neutrophils were abundant (Table 5.5; Fig. 5.8k and l).

Vaginoscopy during anoestrus appeared similar to that observed in anoestrus domestic dogs (score 0); a pink, dry mucosa with flat mucosal folds and no oedema. By the end of pro-oestrus (BRU F1 ♀ day -1; Table 5.5), the mucosa was thickened, white and oedematous with presence of haemorrhagic secretions (score 1). By early oestrus (PLA F1 ♀ day +1), mucosal folds shrank slightly, causing secondary folds to appear (score 2). The mucosa still had a white aspect and secretions were now serosanguinous (Table 5.5). Unlike the domestic dog, the mucosal folds

only shrank slightly further over the remainder of the oestrus period, maintaining the appearance of secondary folds (score 3; Table 5.5). Secretions progressively changed to serologic and then disappeared, while the mucosa maintained a pale aspect (Table 5.5). Angulation (score 4), observed in domestic dogs in late oestrus, was not present during oestrus in any AWD females, but appeared later during early dioestrus (SAN F1 ♀ day +8; Table 5.5).

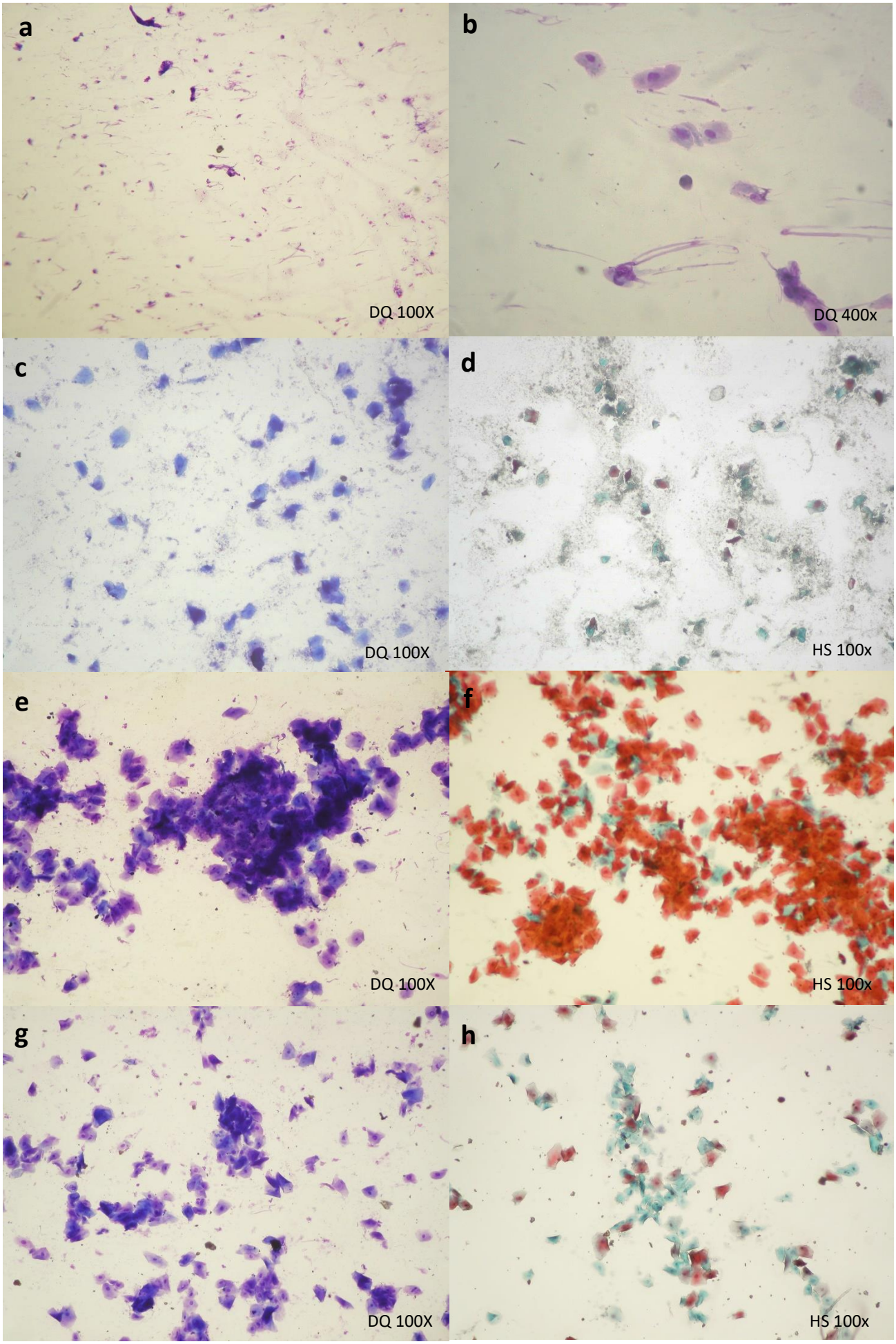
Visualising the ovaries, follicles, corpora lutea and uterine body using ultrasound was largely unsuccessful. As in domestic dogs, the ovaries are surrounded by a bursa (Van den Berghe, personal communication), making visualisation using ultrasound quite challenging. In only a few cases, could the left or right ovary be interpreted with certainty (Table 5.5). In addition, no distinction could be made between follicles and corpora lutea (CL), as both appeared as anechogenic structures (Fig. 5.9), as seen in domestic dogs. Since visualisation of ovarian structures by ultrasound was difficult, results presented in this study should be interpreted with caution.

As in domestic dogs, blood progesterone levels were at baseline levels (<1 ng/ml) during anoestrus and through pro-oestrus to early oestrus, after which they slowly started rising for BRU F1 ♀ (1.9 ng/ml) but not for anovulatory PLA F1 ♀ (0.6 ng/ml) in late oestrus (Table 5.5). Levels were elevated and kept rising through end-oestrus to dioestrus (SAN F1 ♀; Table 5.5). No clear pattern in blood oestrogen levels was evident during the periovulatory period in AWD females (Table 5.5).

Table 5.5. *Other reproductive parameters in female African wild dogs (Lycaon pictus) during different phases of the reproductive cycle.*

	Anoestrus	***End pro-oestrus***	***Early oestrus***			***Late oestrus***			***Early dioestrus***
	PLA F2 ♀	***BRU F1 ♀ day -1***	***PLA F1 ♀ day +1***	***PLA F1 ♀ day +4***	***BRU F1 ♀ day +6***	***PLA F1 ♀ day +7***	***BRU F1 ♀ day +9***	***SAN F1 ♀ day +4***	***SAN F1 ♀ day +8***
Body weight (kg)	25.2	29.5	24.4	-	-	-	-	24.0	-
Pairs of nipples	5	6	6	-	-	-	-	7	-
Vulvar discharge	no	no	red-yellow	no	no	no	no	no	no
Vulva size (H x W; cm²)	9.9	25.7	15.9	16.4	19.1	15.5	20.4	13.0	11.8
Vulva size adjusted for bodyweight (cm²/kg)	0.39	0.87	0.65	0.67	0.65	0.64	0.69	0.54	0.49
Vaginal swab colour	white	red	red	light red	white	light yellow	white	white	white
Vaginal cytology									
Small cells (%)	100	1	0	1	0	2	2	1.5	51
Medium cells (%)	0	3	2	5	0	17	8	48	36
Large cells (%)	0	31	17	31	7	32	16	20.5	9.5
Superficial cells (%)	0	31	22.5	18	9	17	24	3.5	1.5
Keratinised cells (%)	0	34	58.5	45	84	33	50	2	2
Bacteria	+	+	++	++	-	++	-	-	-
Mucus	+	-	-	++	+	++	+	-	++
Neutrophils	few	no	no	few	few	yes	few	yes	yes
Vaginoscopy									
Colour mucosa	pink	white	white	white	white	light pink	white	white	pink
Secretions	no	sanguineous	serosanguinous	serosanguinous	no	serologic	no	serologic	no
Oedema	0	1	2	3	3	3	3	2	4
Ultrasound									
Right ovary - size (mm)	7.5 x 22	not visible	not visible	7 x 18	8 x 16[#]	not visible	9 x 22[#]	6 x 19	not visible
Right ovary – follicles/CL (number - mm)	none	not visible	not visible	2 - 4	not visible	not visible	not visible	1 - 5	not visible
Left ovary - size (mm)	not visible	not visible	10 x 13	9 x 14	8 x 21[#]	8 x 18	10 x 23[#]	10 x 15	8 x 18
Left ovary - follicles/CL (number - mm)	not visible	not visible	1 - 3[#]	1 - 3	not visible	1 - 3	not visible	1 - 10	1 - 10
Uterine body - Ø (mm)	3	not visible	10	not visible	not visible	not visible	3[#]	not visible	not visible
Blood progesterone (ng/ml)	0.4*	0.8*	0.9*	0.8*	1.6*	0.6*	1.9*	10.5	16.6
Blood oestrogen (pg/ml)	12.6*	104.8*	10.1*	56.8*	54*	7.2*	9*	8.22	98.8

*[#]Image interpretation difficult; *Samples temporarily thawed due to freezer failure*


a
DQ 100X
b
DQ 400x
c
DQ 100X
d
HS 100x
e
DQ 100X
f
HS 100x
g
DQ 100X
h
HS 100x

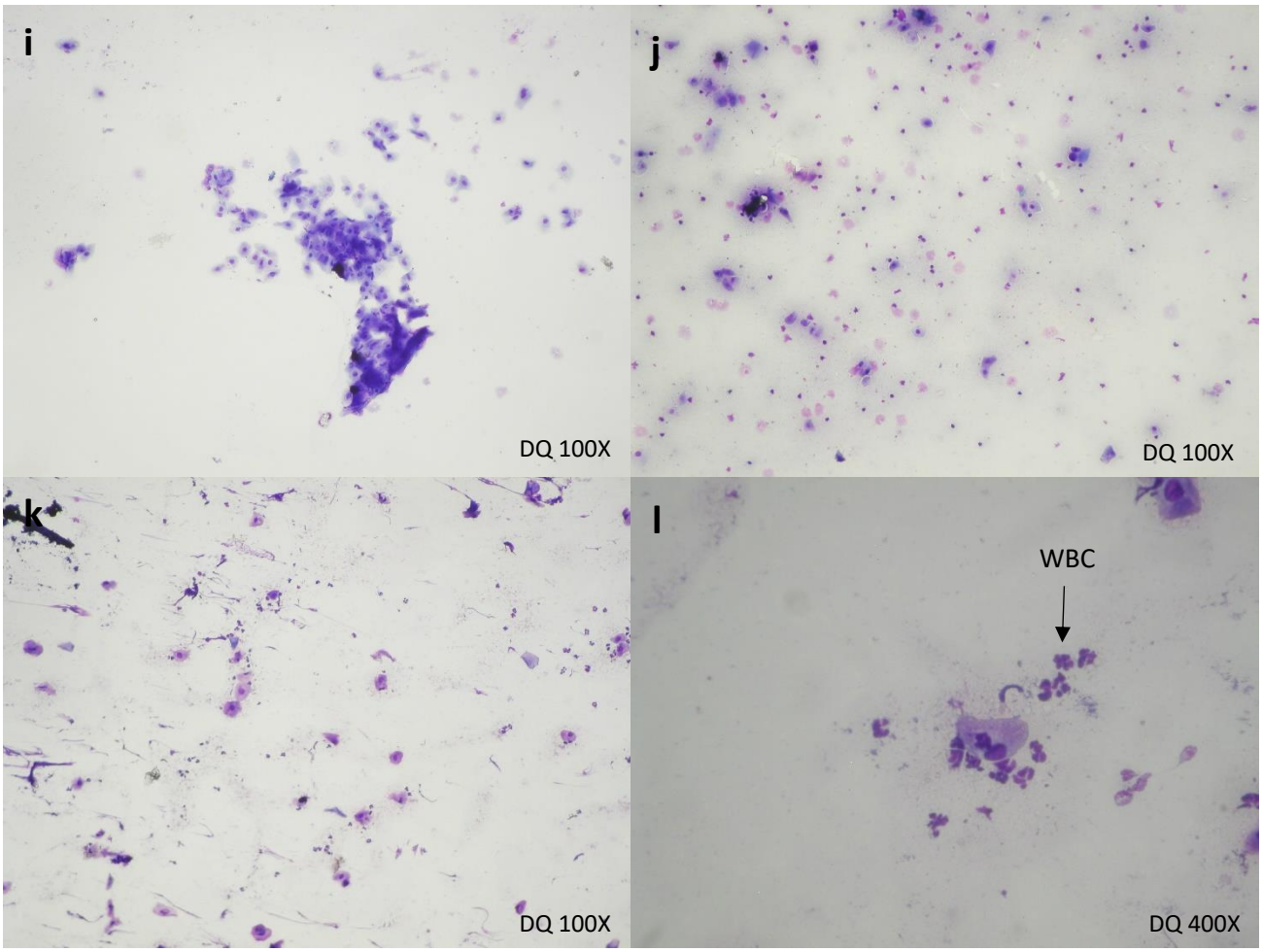


Figure 5.8. *Vaginal cytology in African wild dog females during (a,b) anoestrus (Pla F2 ♀); (c, d) late pro-oestrus (Bru F1 ♀day -1); (e,f) early oestrus (Bru F1 ♀day +6); (g,h) late oestrus (Bru F1 ♀day +9); (i,j) late oestrus (last day oestrus, San F1 ♀day +4); (k,l) early dioestrus (San F1 ♀day +8). DQ, Diff-Quick stain; HS, Harris-Shorr stain; WBC, White blood cell (neutrophil)*

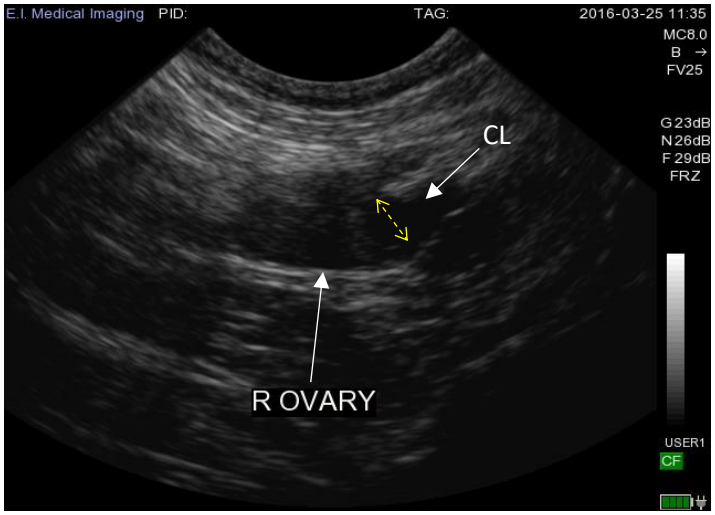


Figure 5.9. *Ultrasound image of the right ovary of the African wild dog alpha female SAN F1 ♀during late oestrus, showing an anechogenic structure 5 mm in diameter (yellow arrow; presumably a CL).*

4. Discussion

The non-invasive measurement of reproductive hormones in faeces has great potential to aid wildlife captive and assisted breeding, conservation and management. This study demonstrated that a 2- to 3-fold increase in the frequency of several key behaviours (male to female affiliative behaviour, male initiating behaviour, sexual follow, ride-up and copulation) coupled with significant rises in faecal progesterone metabolites can discriminate late oestrus in AWD females from other reproductive phases during the periovulatory period. The ability to non-invasively distinguish late oestrus from early oestrus and other reproductive phases is crucial for timing of AI in the AWD, as this phase corresponds to the attainment of oocyte maturity and window of fertilization in canids, which typically ovulate immature oocytes that require 48 - 60 h to mature in the oviduct. Other invasive diagnostic procedures performed under anaesthesia, such as vaginal cytology and vaginal endoscopy, were insufficient to clearly distinguish late oestrus from early oestrus or pro-oestrus, due to infrequent sampling and distinct differences with that observed in domestic dogs.

The variable duration of the oestrus period between females in this study, demonstrates that performing AI at a fixed time interval after the onset of pro-oestrus or oestrus, or after a rise in fPM levels, as has been suggested for the red wolf (Walker *et al.* 2002), is unlikely to be successful. Therefore, other parameters need to be considered to estimate timing of the fertile period. The most consistent parameter to determine the moment of late oestrus in this study was behaviour. Four types of sexual (sexual follow, initiating behaviour, ride-up and copulation) and one non-sexual behaviour (male to female affiliative behaviour) were significantly higher in late oestrus compared to early oestrus. Initiating behaviour and sexual follow where particularly high, as the alpha male was following and interacting with the female continually. Since such behaviours were already occurring in pro-oestrus and early oestrus, some quantification is necessary to detect the 3.4- to 4.8-fold increase during late oestrus. These results are consistent with other studies (Van Heerden & Kuhn 1985, Creel *et al.* 1997, Monfort *et al.* 1997, Van der Weyde *et al.* 2015). However to date, ours is the first study to describe clear differences between early and late oestrus; which is critical to proper timing of AI. Van Heerden & Kuhn (1985) described an incessant follow of the alpha female by the alpha male, intense and frequent urine marking, and mate guarding during oestrus. However, behaviour in this study was descriptive and not quantitative, so differences

between pro-oestrus and oestrus were not clear. As in many other species including domestic dogs (Concannon 2011), the transition of oestrus to dioestrus was obvious in our study, with an abrupt decrease or arrest in all sexual behaviours, as well as marking behaviour and male-female resting patterns.

The main limitation associated with the use of male - female behaviour to determine the fertile period, is that it cannot be used to evaluate groups of single-sex females (where AI would probably have the most practical use in captive breeding programs). Behaviours such as vulvar licking, scent marking, and mounting have also been described previously in single-sex packs (Van der Weyde *et al.* 2015), so the utility of these behaviours should be explored further. However, one of the advantages of AI is that it permits infusion of genetic diversity within a pack without disrupting the social hierarchy. As such, AI could augment the paternity of a litter recognised as 'self' by existing pack members. Thus, in mixed-sex groups, brief separation of the alpha female during the fertile period may be needed for a single AI to increase the chance of foreign frozen-thawed sperm to fertilise oocytes over fresh sperm from multiple copulations by the alpha male. In this study, most of the sexual behaviour was concentrated within 1 to 2 h after feeding each day. This is a significant finding since predictions about the onset of the fertile period can be made after only a relatively short daily observation time.

Faecal hormone profiles were variable between females. The PLA pack alpha female did not show an increase in either faecal or serum progesterone levels during oestrus, indicating an anovulatory cycle (Meyers-Wallen 2007). However, the faecal progesterone and oestrogen profiles of the 2 other females were similar to that seen in domestic dogs (England 2010, Concannon 2011), as well as profiles previous described in female AWDs (Van Heerden & Kuhn 1985, Monfort *et al.* 1997, Van der Weyde *et al.* 2015). Important here, is that in late oestrus, fEM levels tended to return to baseline levels, while fPM levels showed a significant 2.5- to 3-fold increase. These results need to be interpreted with caution as they are only derived from 2 females, one of which one had a limited number of faecal samples. In addition, previous studies show diverse results. Creel *et al.* (1997) did not observe differences in faecal progestin concentrations between the anoestrus and oestrus periods, after which they increased during gestation, while oestrogens increased during oestrus. However, all oestrus samples were grouped together without a clear separation between pro-oestrus and oestrus,

or without taking longitudinal profiles or individual differences into account; which might explain this discrepancy. Van der Weyde *et al.* (2015) on the other hand, saw a trend similar to our study, but with a slightly lower (less than 2-fold) increase in fPM levels from baseline to oestrus. The use of wet instead of dry faecal samples and a different assay (RIA) might explain this difference.

Blood progesterone levels seemed to follow a similar pattern as faecal progesterone. However, absolute values were much lower compared to those in domestic dogs. It is unlikely that this was due to the brief accidental thawing of our stored serum samples. Canine progesterone is known to be stable during prolonged storage in hemolyzed plasma (Reimers *et al.* 1983), and in plasma stored in different collection tubes for up to 14 days, or after 10 freeze-thaw cycles (Reimers *et al.* 1991). In addition, similar low levels have been reported previously during single blood collection in AWD females, with 0.0 ng/ml in anoestrus, 0.0-1.1 ng/ml in pro-oestrus, and 0.0-11.3 ng/ml in dioestrus (Newell-Fugate *et al.* 2012). Blood oestrogen levels did not exhibit a clear pattern in our study, probably caused by infrequent sampling. However, in other canids oestrogen concentrations are less reliable indicators of ovulation and the fertile period (Von Heimendahl & England 2010).

Interestingly, observed patterns of vaginal cytology and vaginoscopy in this study differed to that of domestic dogs. In domestic dogs, increasing oestrogen levels in pro-oestrus cause mitotic divisions of vaginal epithelium. This leads to an increase in cell layers and a keratinisation of the superficial layers. Maximum keratinisation (98-100%) is typically seen throughout the oestrus phase, followed by an extensive rapid desquamation on the first day of dioestrus (Concannon 2011). Using a Harris-Shorr stain, in our study, maximum keratinisation of 84% was observed during mid-oestrus, after which underlying non-keratinised cells reappeared again. As a result, the cytological profile in late oestrus was similar to pro-oestrus, making this technique insufficient to discriminate oestrus in AWDs. Vaginal cytology has previously been reported in AWD females, but serial evaluations were not performed (Newell-Fugate *et al.* 2012). Vaginal endoscopy is a very useful method in domestic dogs to pinpoint the fertile period (Von Heimendahl & England 2010). During anoestrus, the vaginal mucosa is pink with no oedema present. In pro-oestrus, due to rising oestrogen levels, an increase in oedema can be seen with the formation of rounded longitudinal mucosal folds. The mucosal wall shows a paler aspect due to the increase of cell

layers coinciding with keratinization. At the time of the LH surge, there is a decrease in oestrogen levels together with an increase in progesterone. This results in a reduction of tissue oedema resulting in a shrunken and wrinkled appearance (angulation) around the time of ovulation (Von Heimendahl & England 2010). In this study, persistently higher levels of vaginal oedema were seen in AWD females compared to domestic dogs, without obvious changes during the oestrus period. In addition, angulation was not observed until dioestrus. Therefore, the results of these techniques should be interpreted with care, when used to confirm the fertile period during AI trials in AWDs.

In domestic dogs, ultrasound examination of the ovary can be challenging but can predict ovulation in 91.7% of bitches (Fontbonne & Malandain 2006). In pro-oestrus, follicles are visible as small anechogenic structures, and increase to 6-9 mm prior to ovulation (Fontbonne & Malandain 2006). At the time of ovulation, follicles either completely disappear or become smaller and irregular (Fontbonne & Malandain 2006). However, since there is no obvious difference between pre-ovulatory follicles and early postovulatory corpora lutea, daily ultrasound examination is usually necessary (Wallace *et al.* 1992, Fontbonne & Malandain 2006). Furthermore, as the ovary is surrounded by a bursa, it is less visible compared to other species (Fontbonne & Malandain 2006). The same difficulties arose in this study. The ovary was difficult to visualise, and in cases where anechogenic structures where visible (3-10 mm), the limited number of ultrasound examinations per AWD made it impossible to determine if they were follicles or corpora lutea. Therefore, due to the frequent examination required, the use of ultrasound to determine timing of ovulation in AWD females appears to be of limited value.

It is unclear why the PLA female didn't ovulate in this study. It can be argued that this was caused by stress associated with multiple anaesthetic interventions, or the anaesthesia itself as reported in felids (Howard & Wildt 2009). In domestic dogs, glucocorticoids decrease plasma LH levels (Kemppainen *et al.* 1983), which could thus inhibit ovulation. Consequently, stress is suspected to play a role in some anovulatory cycles in domestic dogs (Goodman 2002, Wilborn & Maxwell 2012). Anaesthesia can increase glucocorticoid levels in AWDs (Chapter 4; de Villiers *et al.* 1997). In our study however, fGCM levels did not show any obvious peaks related to anaesthesia, though these may have been missed due to limited sampling. Moreover, fGCM levels did not clearly differ significantly across the different reproductive

phases, making it difficult to conclude that anaesthesia-related stress caused anovulation in the PLA pack alpha female. It is possible that the absence of ovulation influenced vaginal cytology and vaginoscopy images from this female. However, the appearance of both cornified cells and oedema is influenced by increasing oestrogen levels, and crenulation is due to decreasing oestrogen:progesterone ratio (Concannon 2011, Wilborn & Maxwell 2012). In addition, domestic dogs with anovulatory cycles usually have vaginal cytology that resembles oestrus (cornified cells; Meyers-Wallen 2007). In this female, behavioural signs were not altered by anovulation, indicating the importance of measuring faecal progesterone concentration to confirm ovulation, as for domestic dogs (Meyers-Wallen 2007). In addition, the collection of urine or blood to perform LH assays can be useful in future studies during immobilisation of oestrus females. This can determine whether and when the LH surge occurs (Santos et al. 2012). Harnas staff subsequently reported that this alpha female produced a litter of puppies (at the time 4-5 weeks old) in July. This suggests that the alpha female was in heat again around mid-March, approximately 1.5 months after the anovulatory cycle observed in this study; implicating a 'split-heat' as seen on occasion in domestic dogs (Meyers-Wallen 2007, Wilborn & Maxwell 2012).

In conclusion, a 2- to 5-fold increase in particular behaviours such as affiliative behaviour, sexual follow, male initiating behaviour, ride-up, and copulation; a 2.5- to 3-fold increase above baseline in faecal progesterone concentration; and faecal oestrogen levels that decline to baseline, appear to be suitable criteria to determine the fertile period in African wild dog females, and help guide the timing of artificial insemination attempts. However, without frequent highly-invasive sampling, blood steroid hormone concentrations, vaginal cytology, vaginoscopy and ultrasound images need to be interpreted with great care. Further research with a larger cohort of animals is needed to confirm the findings of this study, and to further develop a robust protocol for non-invasive detection of the fertile period for artificial insemination in this species.

Chapter 6

General discussion

The development of assisted breeding techniques such as sperm freezing, and artificial insemination (AI) can greatly assist captive breeding, conservation and management in any wildlife species. However, the application of these techniques is not always immediately possible due to deficient knowledge in male and female reproductive physiology (including sperm physiology), and difficulties associated with captive management in some species. This has led to diverse and sometimes negative opinions in the zoological community about the use of assisted breeding techniques in wildlife generally (Holt 2008), but also in the African wild dog (AWD). Firstly, the complex social structure exhibited by AWDs, makes some captive institutions hesitant to perform immobilisations/temporary pack separations (needed for health assessment, sperm collection and potentially AI), especially as reports of injuries during reintroduction are not uncommon (Vlamings 2011, Foster 2014, Quick 2014). Secondly, previous attempts to freeze sperm from this species yielded poor post-thaw sperm quality (Hermes *et al.* 2001, Johnston *et al.* 2007), which gave the impression sperm freezing was just not possible for the AWD. Perhaps most importantly, knowledge about both male and female reproductive physiology is still largely missing; with basic questions about mechanisms of reproductive suppression in males, and physiology of peri-ovulatory events in females, still unanswered. This thesis therefore, aimed to (i) improve the knowledge of male reproductive physiology, specifically the effect of social rank on subordinate male fertility; (ii) optimise a sperm freezing protocol to provide improved post-thaw sperm quality; (iii) provide behavioural tools to assist captive management, specifically temporary pack separation, immobilisation and reintroduction; and (iv) develop non-invasive parameters to understand and time the fertile period in females. This information combined can lead the way to the application of AI in this species.

The effect of social rank on subordinate male fertility was investigated in Chapter 2. Results demonstrated that subordinate males are at higher risk of urine contamination during sperm collection by electroejaculation in the pre-breeding season, leading to lower progressive motility and normal morphology of spermatozoa. However, in the breeding season, sperm quality and other reproductive parameters of subordinate and dominant males did not differ. This has important implications for sperm banking initiatives, as all AWD males of reproductively mature age can be considered as suitable candidates for sperm freezing. This means valuable alleles of different genes can be preserved in the germplasm of subordinate

sibling pack members that would otherwise be lost if such males were sub/infertile. Chapter 3 demonstrated that a sperm freezing protocol consisting of a two-step dilution TRIS - 20% EY extender with 5% glycerol and 0.5% Equex STM, coupled with a TRIS-citrate-fructose thawing solution, yields significantly improved post-thaw quality and longevity of AWD spermatozoa compared to previous studies (Hermes *et al.* 2001, Johnston *et al.* 2007). Sperm motility persisted for up to 8 h and viability remained stable for more than 4 h after thawing and neither DNA integrity nor morphology of spermatozoa were damaged by the freeze-thaw process. This suggests that this freezing protocol provides post-thaw spermatozoa of sufficient quality for use in AI (Linde-Forsberg *et al.* 1999, Peña & Linde-Forsberg 2000), making it suitable for worldwide frozen transport and sperm banking in the AWD. The results of both these studies provide some essential information needed to start sperm banking in the species. However, the major limitation to sperm freezing in our study seems to be the failure to consistently collect good quality samples. Chapter 2 and other studies have shown that sperm quality in the pre-breeding season is generally of very low quality (Newell-Fugate *et al.* 2012), and therefore insufficient for sperm freezing. Moreover, even in the breeding season, general semen quality is relatively low. This was confirmed in Chapter 3, in which less than half of the collected samples were of sufficient quality (≥60% total sperm motility) to be frozen. This was not only due to limited motility, but also other major limitations that included urine contamination, absence of an ejaculate, absence of spermatozoa and low semen volume. Thus, further research is needed to improve sperm collection success in the AWD. As suggested in Chapter 3, avoiding the use of alpha-2 adrenergic receptor agonists for immobilization when performing electroejaculation, coupled with draining and flushing the bladder prior to semen collection, can help to avoid urine contamination of samples (Johnston *et al.* 2007, Talukder & Hikasa 2009). In addition, other factors that might influence sperm collection success, such as testing and comparing different electroejaculation protocols or even different semen collection methods, need to be explored. Chemical ejaculation using alpha-2 agonists such as medetomidine and dexmedetomidine has been described in felids, including domestic cats (Filliers *et al.* 2010, Zambelli *et al.* 2010, Swanson *et al.* 2017), jungle cats (*Felis chaus*; Kheirkhah *et al.* 2017), and lions (*Panthera leo*; Lueders *et al.* 2012, Luther *et al.* 2017). The administration of these alpha-2 agonists induces the release of semen into the urethra, which is collected by urethral catheterization, usually providing a highly concentrated good quality sperm sample. However, no reports are available looking into the

use of this or similar alternative chemical ejaculation techniques in canids. In addition, other factors that affect sperm quality need to be explored further. In other carnivores, body weight, age and breeding history (Rijsselaere *et al.* 2007), as well as the level of inbreeding (Pukazhenthi *et al.* 2006, Lockyear *et al.* 2016) and diet (Santymire *et al.* 2015) are all related to sperm quality. In addition, low ejaculation frequency and ejaculation after a long period of sexual abstinence can result in low sperm quality at first sperm collection (Imrat *et al.* 2014). Three of the packs we evaluated did not contain a female during the breeding season (Chapter 2), but this did not seem to negatively affect the increase in testis or prostate size from pre-breeding to breeding season, nor suppress sperm production. Given the breeding season in captive AWDs in the Northern hemisphere differs by 6 months (Van den Berghe *et al.* 2012), it suggests breeding condition may be regulated by photoperiod. As such, it is possible that latitude and available daylight might influence the quality of sperm collected from males housed at different locations.

The quality after freeze-thawing of our AWD spermatozoa was at the lower end of the acceptable range for AI compared to domestic dog standards (Thomassen *et al.* 2006). Thus, further optimisation of our freezing protocol would be beneficial. Future research analysing the lipid composition of the sperm membrane, could provide greater understanding of membrane physiology during cryopreservation and as such lead to improvements. In humans, sperm of oligo- and asthenozoospermic men contain lower levels of docosahexaenoic acid (DHA), total polyunsaturated fatty acids (PUFA) and n-3 fatty acids, a lower n-3:n-6 ratio, and higher levels of saturated fatty acids (SFA; Conquer *et al.* 1999, Gulaya *et al.* 2001, Aksoy *et al.* 2006, Tavilani *et al.* 2007). Moreover, sperm cell fatty acid composition has been proposed as a predictor of post-thaw sperm quality (Martinez-Soto *et al.* 2013). In boars, sperm quality (motility, viability, normal morphology, and plasma membrane integrity) is positively correlated with total lipids, cholesterol, PUFA, DHA, and n-3 PUFA composition (Am-in *et al.* 2011). As in humans, male-to-male differences in post-thaw sperm survival may be linked to the amount of long-chain PUFA (Waterhouse *et al.* 2006). Silver fox (*Vulpes vulpes*) sperm is more resistant to freeze-thawing than blue fox (*Alopex lagopus*) sperm, and shows higher post-thaw levels of docosapentaenoic acid (DPA), desmosterol, cholesterol and a higher PUFA/SFA ratio (Miller *et al.* 2005). Differences in cryogenic success can also be seen between the sperm of African (*Loxodonta africana*; high cryogenic success) and Asian elephants

(*Elephas maximus*; low cryogenic success), with African elephant sperm containing significantly higher levels of DHA and DPA (Swain & Miller 2000). Flying-fox (*Pteropus spp.*) sperm is sensitive to cold shock and acrosome damage after freezing/thawing and shows a low PUFA/SFA ratio (Melville *et al.* 2012). Marsupial (eastern grey kangaroo, koala and common wombat) spermatozoa from the cauda epididymis on the other hand, show a very high ratio of PUFA/SFA and are relatively insensitive to cold shock, with higher levels of DHA in koala and wombat sperm compared to the kangaroo, which relates to cryogenic success (Miller *et al.* 2004). These examples clearly show a correlation between low levels of sperm PUFA and DHA with low cryogenic success and/or low quality of fresh semen. To overcome this problem and increase sperm DHA levels (and as such semen quality and cryogenic success) dietary supplementation with PUFA has been trialled in men (Conquer *et al.* 2000), boars (Rooke *et al.* 2001, Castellano *et al.* 2010), bulls (Gholami *et al.* 2010), roosters (Kelso *et al.* 1997, Surai *et al.* 2000), rams (Esmaeili *et al.* 2012) and stallions (Brinsko *et al.* 2005); but has yielded conflicting results. Direct supplementation of PUFAs to the semen extender has also been trialled, especially in production animals. With the exception of one report (Abavisani *et al.* 2013), most studies report a significant improvement in post-thaw semen quality (Kaeoket *et al.* 2010, Nasiri *et al.* 2012, Takahashi *et al.* 2012, Towhidi & Parks 2012, Ejaz *et al.* 2014). Apart from the one study mentioned above looking at the post-thaw lipid composition of sperm membranes in two fox species (Miller *et al.* 2005), no such research has been performed in other canids. Knowledge of the lipid composition of AWD sperm membranes could help us understand differences in sperm quality and cryogenic success between different males and could help us develop more finely-tuned AWD-specific extenders and/or additives for semen freezing.

Chapter 4 demonstrated that Dog Appeasing Pheromone (DAP) can be a useful tool in captive management of AWDs, as it prevented the rise in faecal androgens seen in placebo treated animals after separation, immobilisation and reintroduction procedures. Moreover, on the day of pack reintroduction, DAP treated packs tended to show lower rates of contact-dominance and active-submission behaviour, but higher rates of non-contact dominance behaviour. These results suggest that DAP could reduce hormones & behaviours leading to aggression in captive AWDs. However, the underlying mechanism governing DAP's effect on androgen output is still unclear. We have postulated that elevated prolactin may play a role

by partial suppression of the H-P-G axis (Siracusa *et al.* 2010). Therefore, further research is needed to confirm this association and measure other hormones influential in suppression/activation of the H-P-G axis. Also, the isolation, characterisation, synthesis and application of AWD-specific appeasing pheromones, might yield a more pronounced effect especially on behaviour (Rekwot *et al.* 2001, Pageat & Gaultier 2003, Brennan & Zufall 2006). Such research warrants further investigation. Pheromones might also have other important applications in AWD reproduction, conservation and management (Rekwot *et al.* 2001, Brennan & Zufall 2006). For example, the application of foreign scent marks has been used to limit ranging behaviours of free-ranging AWDs (Jackson *et al.* 2012).

Determining the timing of the fertile period is challenging in all wild canids that cannot be handled without immobilisation. Some canids such as the grey (*Canis lupus*), red (*C. rufus*), and maned wolves (*Chrysocyon brachyuris*) can be trained more easily than AWDs, and simple procedures including blood collections are sometimes possible with a manual restraint (Seager *et al.* 1975, Walker *et al.* 2002, Teodoro *et al.* 2012). This permits the serial collection and analysis of blood progesterone, oestrogen and LH concentrations; thereby enabling peri-ovulatory events to be monitored and timing of ovulation to be predicted (Walker *et al.* 2002, Thomassen & Farstad 2009, Von Heimendahl & England 2010). Due to the skittish nature of AWDs however, such training is impossible. Some zoological institutions have managed to train their captive AWDs, but this is typically limited to brief intramuscular injections through a cage (Isaacs 2014, Kutilek 2014). Therefore, Chapter 5 aimed to validate non-invasive parameters to estimate the fertile period in AWD females. We found that increased frequency of specific behaviours coupled with significant rise in faecal progesterone and a decline in oestrogen metabolite concentrations are suitable parameters to determine the timing of the fertile period in AWD females. However, sample size in our study was low, and data from additional females should be collected to strengthen these results. In red wolves, it is advised to perform AI 5-6 days after the initial rise in faecal progesterone levels (Walker *et al.* 2002). By contrast, we found that due to the highly variable length of oestrus between individual AWDs, it is necessary to evaluate behavioural combined with complete profiles of faecal progesterone and oestrogen to discriminate the late oestrus. Our research is the first step toward non-invasive detection of the fertile period in AWD females, enabling us to estimate the timing of AI.

A logical next step would be AI trials, initially using fresh sperm samples of good quality, followed by frozen-thawed sperm once successful pregnancies have been achieved. To maximise chances of pregnancy when using frozen-thawed sperm, intra-uterine AI is preferred over intra-vaginal AI in canids (Linde-Forsberg *et al.* 1999, Thomassen *et al.* 2006). This can be performed either by surgery, or by transcervical rigid fibre endoscope (EIU) or rigid Norwegian AI catheter. For EIU, the endoscope should be introduced into the vagina through the dorsal commissure of the vulva at an angle of 45°, thereby avoiding the clitoris and urethral ostium (Macedo *et al.* 2012). When the vagina is reached, the endoscope must be positioned horizontally and advanced passing the mucosal dorsal folds until the cervix is visualised. Introduction of a flexible catheter through the endoscope into the uterus through the cervix should permit the intrauterine insemination of spermatozoa and increase the chance of pregnancy success (Linde-Forsberg *et al.* 1999). Our preliminary investigations (not included in Chapter 5 of this thesis) indicate that transcervical passage of a catheter using endoscopy (without injection of spermatozoa), was relatively easy to perform, indicating this technique can be used for AI in AWD females.

In summary, this thesis provides essential knowledge and important techniques to facilitate sperm banking and artificial insemination initiatives in the African wild dog, which can greatly assist conservation and management of the species. Further research may help improve sperm collection success, provide additional information on sperm membrane physiology during freezing, and illuminate the underlying mechanism of DAP in mediating aggression, as well as confirm our findings regarding peri-ovulatory events in the female.

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