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PRACTICAL MANAGEMENT OF THE ENDANGERED AFRICAN WILD DOG (*Lycaon Pictus*): IMPLEMENTATION OF RESEARCH METHODOLOGIES, MANAGEMENT TOOLS AND VALIDATION OF NON-INVASIVE ENDOCRINOLOGY APPLICATIONS

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Practical management of the endangered African wild dog (*Lycaon pictus*): implementation of research methodologies, management tools and validation of non-invasive endocrinology applications

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To my father

“When the last tree has been cut down, the last fish caught, the last river poisoned, only then will we realize that one cannot eat money.”

Native American saying

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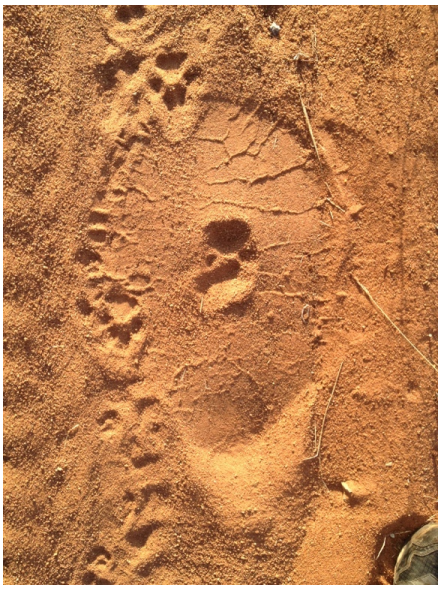
ABSTRACT

The porpoise of this study was to implement research methodologies and assess effectiveness and impact of management tools to promote best practices for the long term conservation of the endangered African wild dog (*Lycaon pictus*). Different methods were included in the project framework to investigate and expand the applicability of these methodologies to free-ranging African wild dogs in the southern African region: ethology, behavioural endocrinology and ecology field methodologies were tested and implemented. Additionally, research was performed to test the effectiveness and implication of a contraceptive implant (Suprenolin) as a management tool for the species of a sub-population hosted in fenced areas. Attention was especially given to social structure and survival of treated packs. This research provides useful tools and advances the applicability of these methods for field studies, standardizing and improving research instruments in the field of conservation biology and behavioural endocrinology. Results reported here provide effective methodologies to expand the applicability of non-invasive endocrine assessment to previously prohibited fields, and validation of sampling methods for faecal hormone analysis. The final aim was to fill a knowledge gap on behaviours of the species and provide a common ground for future researchers to apply non-invasive methods to this species research and to test the effectiveness of the contraception on a managed metapopulation.

Chapter I

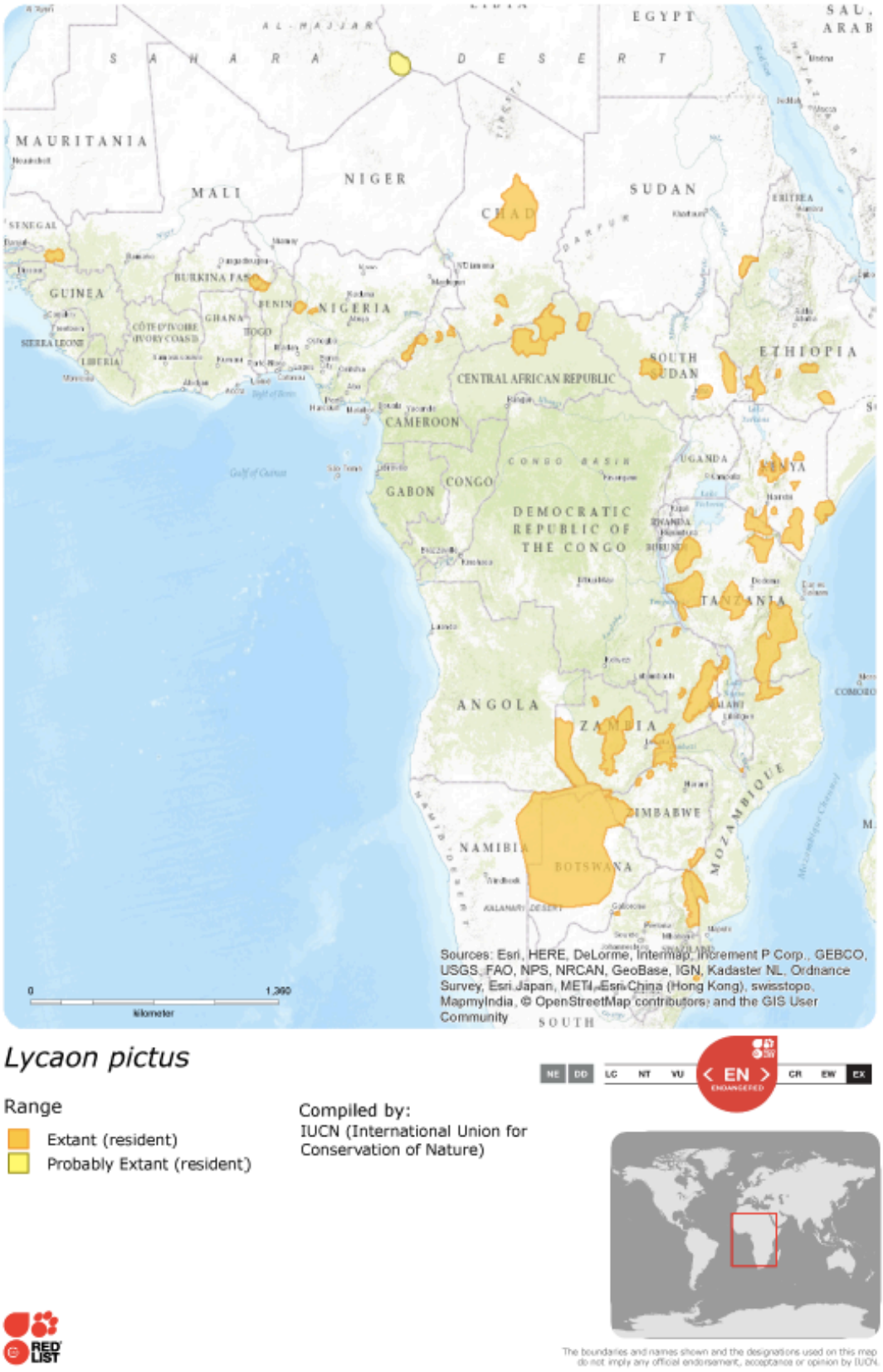
General introduction



1. *Species background*

The African wild dog (*Lycaon pictus*) is the only species of the genus Lycaon, belonging to the family Canidae, is among the most threatened carnivore species in Africa (Creel and Creel, 2002; Newell-Fugate et al., 2012; Woodroffe, 2011). The species was listed as Endangered under the Red List of Endangered Species (IUCN – International Union for Conservation of Nature) in 1990 (Woodroffe, 2011). Historical data indicate that African wild dogs were formerly distributed throughout sub-Saharan Africa, from desert to mountain summits across 39 African countries. During the last 20 years, reports indicate its presence in only 14 countries over fragmented areas (figure 1) (Creel and Creel, 2002; McCreery, 2000). Potentially viable populations are considered to occur in only six countries, of which most extensive territories are found in South Africa, Botswana and Tanzania, but populations are highly fragmented and largely confined to protected areas (Mills and Gorman, 1997; Newell-Fugate et al., 2012; Woodroffe, 2011). Consequently, the size of the breeding population is directly related to the number of packs that make up the population (McCreery, 2000). Assessing the number of mature individuals of the species is challenging, due to the elusive nature and the large home ranges of these animals. The current general population estimate is established at 3000-6000 individuals (Woodroffe, 2011).

Figure 1: African wild dog distribution map (Woodroffe, 2011).



Lycaon pictus

Range

- Extant (resident)
- Probably Extant (resident)

Compiled by:
IUCN (International Union for Conservation of Nature)

NE DD LC NT VU EN ENDANGERED CR EW EX

The boundaries and names shown and the designations used on this map do not imply any official endorsement, acceptance or opinion by IUCN.

The wild dog can be recognised by a three-colours coat, typically including several shades of tan, black and white patches (Fig. 2). The unique pattern of each animal allows recognition and identification of individuals by sight (Maddock and Mills, 1994). (Creel and Creel, 1995; Creel, 1996). Wild dogs are considered not sexually dimorphic, with both sexes having relatively long legs, head and body length averaging 76 - 112 cm and, overall, a slim build weighing 18 – 28 kg. Wild dogs' distinctive characteristic are relatively large rounded ears, unlike many other canids (Creel and Creel, 2002). There is some debate to define their nearest related species (Girman et al., 1993; Wayne et al., 1997). However, the black-backed jackal (*Canis mesomelas*) may be the wild dogs nearest relative (Zrzav, 2004).

Figure 2: African wild dog, Alpha male *Shrek*, Morena pack. Photo: Gabriella Postiglione

Wild dogs live at relatively low densities of 2-35 dogs/1000 km^2 across a range of different ecosystem (Creel and Creel, 2002). They are highly gregarious carnivores that live, hunt, and rest almost exclusively in a closed social unit (Frame et al., 1979; Creel and Creel, 1995) defined as a *pack*, namely a potential reproductive unit (Frame et al., 1979; Reich, 1981). Commonly, siblings of the same sex can emigrate at 2-3 years of age and join opposite-sex group to form a new pack (McCreery, 2000). Wild dogs are considered obligate cooperative breeders, as social dominants nearly completely monopolise copulations, and reproduction has been considered rare (Creel and Creel, 1995; Woodroffe, 2011). Individuals other than parents help care for offspring, in a common effort to raise one single litter during each reproductive season (Marneweck et al., 2019).

2. *Current threats to the species survival*

African wild dogs (AWD) have historically suffered from a high level of human persecution, with state sponsored eradication campaigns decimating the species and eradicating it from most of its formal range (Woodroffe, 2011). Except for wild dog populations in large protected areas in Tanzania and southern Africa, most populations are heavily fragmented and consist of less than 100 individuals. Nowadays, the main threat for the species is human-induced habitat fragmentation, together with both direct (shooting, poisoning, snaring and road kills) and indirect (habitat degradation) anthropogenic causes of mortality continue to be responsible for their decline (Courchamp and Macdonald, 2001; Gusset et al., 2010). Other threats for the species are represented by persecution by other carnivores

(Cozzi et al., 2012; McCreery, 2000), and diseases (Newell-Fugate et al., 2012).

3. *South African metapopulation*

To develop a conservation action plan to improve the status of AWD's in southern Africa, a wild dog meta-population was created starting 1998 to establish a second viable population of AWD outside the Kruger National Park. The initial goal of establishing a series of wild dog subpopulations with at least nine packs in South Africa outside of Kruger was achieved in under 10 years (Gusset et al., 2010; Potgieter and Davies-Mostert, 2012). Reintroduction efforts have met with mixed success, but generally provide hope that the wild dogs decline can not only be stopped, but reversed (Creel and Creel, 2002). The goal of the wild dog meta-population is not only to ensure the long-term survival and conservation of the wild dog in Southern Africa, but also to encourage biodiversity (Potgieter et al., 2015). As part of the meta-population management, wild dogs are moved between the meta-population reserves in single sex groups to mimic natural dispersal patterns and to subsequently maintain genetic integrity. With these movements, animals are frequently forced to bond artificially to form new packs and the meta-population has been expanded beyond the borders of South Africa (Marneweck et al., 2019).

One of the main problems associated with translocations, group enlargement, group merging, and group formation of African wild dogs is intra-pack aggression (Marneweck et al., 2019).

The favourable outcome of the artificial creation of a new pack before release is critical in the reintroduction and translocation process (Gusset et al., 2006). To achieve a good level of bonding and to reduce aggression, groups of wild dogs are held separately in adjacent holding enclosures (bomas). In this way, groups are allowed to interact only through the fence, being in olfactory, auditory and visual contact, while physical contact is prevented at this stage (Potgieter and Davies-Mostert, 2012). Spatial relationships and social interactions (Gusset et al., 2006) and spatial and acoustic behaviours (Potgieter et al., 2015) have been associated with the probability of integrating foreign conspecifics to form a new pack.

4. Aims and Objectives

The development of practical welfare assessment protocols has been reached most extensively in farmed animals; nevertheless, the principles underlying such protocols can be adjusted to suit different species and management (Main et al., 2012; Majewski et al., 2012). The severity of a welfare challenge has been argued to be strictly connected to the similarity of the environment experienced to that in which the animals evolved, and for which their goals and mechanisms for achieving these goals are adapted (Turner and Dwyer, 2007). In this context, all methods of management have the potential to affect animal welfare including the use of fences, artificial management of prey-predator species. The suffering wildlife endures because of humans is a collective responsibility that presents a moral imperative for animal welfarists and conservationists (Bradshaw and Bateson, 2000).

The rationale of the current research considers priority the integration of behavioural, endocrine, health and ambient measures to provide robust and detailed indicators of animal wellbeing. Following extensive research on the metapopulation management and the African wild dog conservation, the need for standardisation and methodology assessment for gathering vital information for conservation of the species was clear. The present research aims at developing and validating multidisciplinary procedures adaptable to both wild and captive conditions, allowing for overlapping of future results and thus ameliorating the general efficacy of researches. With this study, both new and traditional approaches and methodologies have been developed and tested under wild conditions, to address the need of testing the efficiency and usefulness of these methods, helping future researchers in choosing proper methods to enhance the species survival.

5. *References*

Courchamp, F., Macdonald, D.W., 2001. Crucial importance of pack size in the African wild dog *Lycaon pictus*. Animal Conservation 4, 169–174. https://doi.org/10.1017/S1367943001001196

Cozzi, G., Broekhuis, F., Mcnutt, J.W., Turnbull, L.A., Macdonald, D.W., Schmid, B., 2012. Fear of the dark or dinner by moonlight? Reduced temporal partitioning among Africa's large carnivores. Ecology 93, 2590–2599. https://doi.org/10.1890/12-0017.1

Creel, S., 1996. Conserving wild dogs. Trends in Ecology & Evolution 11, 337. https://doi.org/10.1016/0169-5347(96)81137-2

Creel, S., Creel, N.M., 2002. The African wild dog: behaviour, ecology and conservation. Princeton University Press. https://doi.org/10.1016/S0169-5347(02)00039-3

Creel, S., Creel, N.M., 1995. Communal hunting and pack size in African wild dogs, *Lycaon pictus*. Animal Behaviour 50, 1325.

Frame, L.H., Malcolm, J.R., Frame, G.W., van Lawick, H., 1979. Social organization of African wild dogs (*Lycaon pictus*) on the Serengeti plains, Tanzania 1967–1978. Zeitschrift für Tierpsychologie 50, 225–249.

Gusset, M., Slotow, R., Somers, M.J., 2006. Divided we fail: the importance of social integration for the re-introduction of endangered African wild dogs (*Lycaon pictus*). Journal of Zoology 270, 502–511.

Gusset, M., Stewart, G., Bowler, D., Pullin, A., 2010. Wild dog reintroductions in South Africa: a systematic review and cross-validation of an endangered species recovery programme. https://doi.org/10.1016/j.jnc.2009.11.001

Main, D.C.J., Mullan, S., Atkinson, C., Bond, A., Cooper, M., Fraser, A., Browne, W.J., 2012. Welfare outcomes assessment in laying hen farm assurance schemes. Animal Welfare 21, 389–396.

Majewski, E., Hamulczuk, M., Malak, A. 2012. Cost-effectiveness assessment of improving animal welfare standards in the European Agriculture. International Association of Agricultural Economists (IAAE) Triennial Conference, Foz do Iguaçu, Brazil, 18-24 August, 2012.

Marneweck, C., Becker, P.A., Beverley, G., Davies-Mostert, H.T., du Plessis, C., Forssman, K., Graf, J., Gusset, M., Hofmeyr, M., Kelly, C., Kilian, P.J., Marchal, A.F.J., Marneweck, D.G., Marnewick, K., Owen, C., Postiglione, G., Slotow, R., Somers, M.J., Szykman Gunther, M., van Dyk, G., Whittington-Jones, B., Parker, D.M., 2019. Factors affecting the success of artificial pack formation in an endangered, social carnivore: the African wild dog. Animal Conservation. https://doi.org/10.1111/acv.12490

McCreery, E.K., 2000. Spatial relationships as an indicator of successful pack formation in free-ranging African wild dogs. Behaviour 137, 579–590. https://doi.org/10.1163/156853900502222

Mills, M.G.L., Gorman, M.L., 1997. Factors affecting the density and distribution of wild dogs in the Kruger national park. Conservation Biology 11, 1397–1406.

Newell-Fugate, A.E., O. Nöthling, J., J. Bertschinger, H., 2012. Seasonal changes in steroid hormone profiles, body weight, semen quality, and the reproductive tract in captive African wild dogs (*Lycaon pictus*) in South Africa. General and Comparative Endocrinology 178, 272–281. https://doi.org/10.1016/j.ygcen.2012.05.008

Paquet, P., Darimont, C., 2010. Wildlife conservation and animal welfare: two sides of the same coin? Animal Welfare 19, 177-190.

Potgieter, K.R., Davies-Mostert, H.T., 2012. A simple visual estimation of food consumption in carnivores. PLoS ONE 7. https://doi.org/10.1371/journal.pone.0034543

Potgieter, K.R., O'Riain, M.J., Davies-Mostert, H.T., 2015. Behavioural cues can be used to predict the outcome of artificial pack formation in African wild dogs (*Lycaon pictus*). African Journal of Wildlife Research 45, 215–222. https://doi.org/10.3957/056.045.0215

Reich, A., 1981. The behavior and ecology of the African wild dog (*Lycaon pictus*) in the Kruger national park (Ph.D.). Yale University, Connecticut, United States.

Somers, M.J., Graf, J.A., Szykman, M., Slotow, R., Gusset, M., 2008. Dynamics of a small re-introduced population of wild dogs over 25 years: Allele effects and the implications of sociality for endangered species' recovery. Oecologia 158, 239–47. https://doi.org/10.1007/s00442-008-1134-7

Turner, S., Dwyer, C., 2007. Welfare assessment in extensive animal production systems: challenges and opportunities 4. Animal Welfare 16, 189-192.

Vlamings, B.H.A.C., 2011. Dog appeasing pheromone®: A useful tool to minimize stress and aggression of African wild dogs (*Lycaon pictus*)? Prestigious Master: Neuroscience and Cognition. Graduate School of Life Sciences, University of Utrecht, the Netherlands.

Woodroffe, R., 2011. Ranging behaviour of African wild dog packs in a human-dominated landscape. Journal of Zoology 283, 88–97. https://doi.org/10.1111/j.1469-7998.2010.00747.x

Zrzavý, J., Řičánková V.2004. Phylogeny of recent *Canidae* (Mammalia, Carnivora): relative reliability and utility of morphological and molecular datasets. Zoologica Scripta 33, 311-333.

Chapter II

Definition of a standardized ethogram for the African wild dog (*Lycaon pictus*)

1. *Abstract*

Standardization is crucial in every research field and ethology does not differ. Despite this need, there is a tendency in studies that include animal behaviour to do not follow proper ethology methods to define the behaviour of focus. The lack of definitions and standardisation in the field of ethology and especially for the African wild dog (*Lycaon pictus*), limits the possibility of comparing ethology data gathered with different methods. The present research included a first phase of field work for behavioural data collection, a second phase of references review, and a third phase of ethological data condensation for the achievement of the final goal: the creation of an extensive ethogram for the species African wild dog. The final results constitute a standardised ethogram composed of N=153 behaviours, divided in N=15 non-mutually exclusive classes. The ethogram was designed to uniform the application of scrupulous ethological methods for future ethology study on this endangered carnivore, to enhance the species conservation in the wild and the animal's management in captivity. To the author knowledge, this is the sole extensive ethogram for the species, and the first extensive review of the literature regarding African wild dog's behaviour.

2. *Introduction*

Behavioural studies examine the action of an individual over time. Depending on the objectives of a study, a variable number of subjects' actions are extrapolated from the continuous stream of activity and reduced into a series of defined actions that can be effectively quantified (Bernstein, 2010). The process of selecting these elements of behaviour is the first crucial part of any ethology study: the creation of an ethogram (Bernstein, 2010; Gerencsér et al., 2013; MacNulty et al., 2007). This "dictionary of behaviour" consists of a list of behaviours exhibited by the chosen species (Bernstein, 2010). The more precise the observations are, the more sophisticated the interpretations can be about the biology of a certain individual or species (Gerencsér et al., 2013). Without detailed ethograms, dissimilar behaviours might be grouped, driving into misleading assumptions (Overall, 2014). One problem with ethograms is that they may be as different as the number of investigators that deal with a species, resulting in data that cannot be compared, even if collected with observation conducted on the same animals under the same conditions (Bernstein, 2010). Most researchers include in their studies partial ethograms or skip this step and include few behavioural definitions, leading to the creation of studies that remain disconnected, generating a limited incremental gain in knowledge.

Standardization of behavioural terminology codes and measures can create a significant advance in the field of ethology (Stanton et al., 2015) and even in behavioural genetics, neuro-behavioural development, and others, building on a scientific method that is valid and repeatable (Overall, 2014).

Furthermore, the study of behaviour can make a substantial contribution to conservation, from small population decline and dispersal, to predict the consequences of environmental change, by understanding the behavioural change of a species (Sutherland, 1998).

Attempts have been made to develop species- and taxon- specific ethograms (e.g. felids: Stanton et al. 2015; equids: McDonnell, 2003; common marmoset [*Calithrixjacchus jacchus*] Stevenson and Poole, 1976) but to the authors knowledge, no attempt has been made towards the creation of a standardized extensive ethogram for the species Lycaon. Considering that African wild dog (*Lycaon pictus*) (AWD) has been listed as *Endangered* under the IUCN Red List of Endangered Species since 1990, with approximately 6,600 adults, of which only 1,400 are mature individuals, the species warrant conservation concern (Woodroffe and Sillero-Zubiri, 2012). The advancement of our knowledge on the species behaviour and the standardization of the methodologies applied in the field of ethology is crucial for their survival in the wild as well as their management in captivity (Stanton et al., 2015). As Bernstein mentioned in his chapter on the Enciclopedia of behaviour (2010) “If all investigators used the same ethogram, we would have no problem” thus, the aim of this study was to create an extensive and standardized ethogram for the species *Lycaon*.

3. *Materials and methods*

The research was divided in three phases to achieve the elaboration of a comprehensive ethogram of the species: the first phase included the behavioural observations of four packs of African wild dogs; the second phase consisted of an extensive literature review to collect published behavioural data on the species as described by Stanton et al. (2015); the third phase included the construction of the final standardized ethogram. Details of the process are described below.

o Behavioural observations

o Study area

The study was conducted in South East Botswana and North of South Africa. There are three recognized seasons: hot, dry (August-October); hot, wet (November-March); and cool, dry (April-July). Two settings where included: Limpopo Lipadi Private Game and Wilderness Reserve, Tuli Block, Botswana (LL), and Khamab Nature Reserve (KNR), South Africa. LL consists of 22,050 ha of fully fenced mopani savannah. It is open to private owners for self-drive or guided safari, using up to 10 vehicles. The reserve contains 12 permanent waterholes and 10 natural water ponds where water was present from October 2nd, 2017, to March 2018. AWD subjects were fee-rooming and had access to the entire area. The animals were never

feed. The KNR consists of 100,000 ha of fully fenced Kalahari desert landscape. According to the data disclosure policy, details regarding the reserve (e.g. water distribution and landscape details) will be used exclusively for the necessary data analysis and will not be listed in the present chapter. Both reserves have electric fences with two electric grids at the end of the fence, to discourage the AWDs from leaving the protected area. Prey availability in both reserves was related to prey distribution and included impala (*Aepyceros melampus*) steenbok (*Raphicerus campestris*) blue wildebeest (*Connochaetes taurinus*), waterbuck (*Kobus ellipsiprymnus*) greater kudu (*Tragelaphus strepsiceros*) and potentially other animals.

- Subjects

A total of N=71 African wild dogs were included in the present study. Of these, N=36 were adults (> 1 year old), N=17 were juveniles (animals born in 2017, thus being 1 year old at the time of the study) and N=18 pups (< 1 year old). All subjects were identified by their unique coat patterns, ear marks, and tail coloration. All subjects were born in the wild. Subjects details can be found in table 1. Packs composition is summarized in table 2. LL subjects formed two packs for a total of 12 adult AWDs, seven males and five females. From June 2017 to December 2017, the *Morena* pack comprised eight animals, the breeding pair (M=Shrek and F=Two spots) three males (Torre, Whity, Ring) and two females (Milly and Wimpy) from the 2015 litter, and one female (Wacha) from the 2013 litter. During this period, one VHF (Very High Frequency) collar was active on the male Ring, allowing the localisation of the pack with a telemetry system (R-1000

Telemetry Receiver, Communications Specialist, Inc. USA). In November 2017, the two youngest females of the Morena pack (Milly and Whimpy) dispersed. They were occasionally monitored, but the lack of a VHF collar did not allow the use of telemetry, thus limiting the number of sightings. On February 14th, 2018, four outsider AWDs arrived on the Morena pack area. These animals where immediately identified with photo ID as three males and one female and monitored. The four AWDs where named *Valentines* pack that, between February 14th and 25th embraced the previously dispersed sisters form the *Morena* pack, Milly and Wimpy.

In KNR, the subjects of study were 55 free ranging AWD, namely 18 pups born in 2018, 17 juveniles born in 2017, and 36 adults (> 1 year of age). In this region animals formed two packs, named *South* and *Botswana* pack (details table 1 and table 2). All juveniles and pups where born in KNR, while the origin of the adults is unknown. Pups gender was not detected, and they are excluded from table 1. All the packs composition and structure were natural (e.g. no artificial pack formation was performed for at least 6 years).

- Behavioural data collection

Packs were located by combining data from the satellite collar with data from telemetry, always from the ground. One week prior to the start of the study, the researcher allowed the animals to habituate to the presence of the vehicle for increasing periods of time, although all AWDs have previously had contact with similar vehicles. During these sessions, photographs were taken to identify single individuals and create photo ID kits for each pack.

Observations were made from a vehicle positioned 20-80 meters from the animals. On occasion, when a resting dog was disturbed, it would rise, look at the vehicle, possibly emit a low-level alarm growl, as documented by Robbins and McCreery (2003), and then lie down further away. Each pack was observed for a period of two months, using *ad libitum* method (Altmann, 1974), from June 1st, 2017, until September 1st, 2018. When packs were located, and the area considered suitable for viewing, behavioural observations were made throughout the day (05:00 – 21:00), during active and resting phases. Data were collected until observations were no longer possible e.g. the pack moved into thick bush and / or visual contact was lost. Entries were recorded observing all pack members, including pups.

- Literature review

The second phase of the study included an extensive literature review, performed following the method described by Stanton et al. (2015) with minor modifications. Literature searches were conducted using the following academic data bases: Science direct, Scopus, Wiley Online Library, ProQuest, and Google Scholar. Searches were organised using combinations of the species scientific name (*Lycaon pictus*), common names (African wild dog, pained dog, hunting dog, hunting wolf), with the following keywords: ethology, behaviour, ethogram. The difference between American and British spelling of the term behaviour (e.g. "behavior" and "behaviour") was tested. Terms were entered in the most general search field, which differed between databases. Each manuscript

was evaluated under the inclusion criteria described in table e. Manuscript were classified by obtaining the following information: citation, study type, behavioural categories (if applicable), behaviour name, definitions, degree of definition completeness, use of an ethogram. Manuscript that fulfilled the inclusion criteria were systematically screened for behavioural descriptions and definitions. Should a manuscript include any reference to another source for a specific behaviour, that reference was included in the review following the same criteria. Retrieved data were analysed for similarities and differences. Rigor during this process was applied in order to select appropriate terminology and definitions.

- Creation of a standardized ethogram

For the creation of the AWD's ethogram, all behaviours were condensed and organised into a catalogue. Behaviours recorded during the observational phase were matched with behaviours gathered with the literature review following the criteria described below. Physical characteristics essential to each behaviour were identified and components of these physical characteristics were evaluated on regard of their meaning, clarity and ability to accurately communicate a consistent description. Equivalent behaviours with different designations were identified and pooled under a single title and description. The final title was chosen considering its frequency and clarity. As an example, since the the behaviour "Hoo-call" appeared three times, twice as "hoo-call" and once as "Hoo", the title "hoo-call" was selected for the final ethogram. Considering the paucity of studies classified under the inclusion criteria, decision was

made to include each behaviour that was identified both on phase one (field observation) and phase two (literature review). Components of the description were evaluated singularly for each selected definition. Should a behaviour be described in more than one selected reference, the final definition was created by overlapping the different descriptions, giving priority to physical characteristics as well as to the clarity of the description itself.

Behavioural categories were defined based on the literature review, behavioural descriptions retrieved, and based on functionality principles. The latter were applied according to Mainardi (1992), that described two methods to catalogue behaviours using functionality or casualty. Behavioural categorization based on functionality implies the differentiation of behaviours based on their primary function (e.g. aggression, locomotion). Selection of behavioural categories was accomplished applying the same criteria used for the behavioural definitions. When no other behavioural categories were found, the categories described by Stanton et al. (2015) where included.

4. Results

o Behavioural observations

Field observations allowed the creation of an ethogram composed of N=116 behaviours. For the purpose of the present study behaviours were recorded *ad libitum,* although quantification of frequency and duration was not performed. The author recorded each behaviour and subsequently took note

of a short description. All actions visualized during the data collection were included in the ethogram.

- Literature review

Literature web search lead to a total of 22,973 hits. Details of keywords combinations, web searcher used and resulting hits can be found in table 4. The use of the keyword “behaviour” was tested for determining any difference in the selection of the American *vs* British spelling. Databases results did not differ in terms of numeric results and the British spelling was chosen for the porpoise of the search. The combination of keywords that resulted in highest numbers of hits were: “Lycaon pictus” AND “Behaviour” (N=7130), “African wild dogs” AND “Behaviour” (N=4578), followed by “Lycaon pictus” AND “Ethology” (N=2639). The web searcher GoogleScholar reported the highest number of results. Web results were merged, and duplicates were eliminated. Criteria detailed in table 3 where then applied to select results for inclusion. Following this process, 21 results qualified for inclusion.

Selected references details are reported in table 5. References included: 15 journal articles, five PhD dissertation and one book. Seven were based on studies carried out in captive environments and 13 on free-roaming animals. Six studies included an ethogram, of which three PhD dissertations (one on captive and two on free-ranging animals) and three research articles conducted in captivity. In one case, a research article included references to a previous study for the ethogram creation.

In terms of definitions provided, the selected studies reported a total of N=194 behaviours with associated descriptions. Behaviours that were listed but not described were excluded. Examination also revealed many definitions to be variations of other behaviours, differing by method of description, these behaviours were merged in single definitions and their titles pulled. A total of N=142 single behaviours remained following this process. Of these, N = 119 were found once, and not repeated within the rest of the selected studies. N=17 behaviours were found in two sources, and N=6 in three sources.

- Creation of a standardized ethogram

The following phase included the fusion of the literature data with the field work observation results: the literature review results were matched with the catalogue created during the behavioural data collection. N=11 behaviours described during the field observations did not match any reference description. These behaviours were: *Approach; Drink; Leave; Nursing; Run; Sniffing Air; Spy-Hop; Trot; Walk; Chewing*. Additionally, the non-behavioural entry *other* was included. N=37 behaviours included in the literature review catalogue were not observed in the field phase. The final ethogram obtained by the unification of the literature review and the behavioural observations is reported in table 6.

Behavioural categories (N=15) where found in three of the selected references, however, following the selection and merging criteria, N=14 categories were elected. Considering the paucity of references that included behavioural categories for African wild dog studies, it was decided to

include in the categories definition data described by Stanton et al. (2015). The final behavioural categories selected are: *Affiliative; Aggressive; Exploratory; Locomotory; Maintenance; Play; Postural; Reproductive; Social Assertion; Submissive; Vocalisation; Abnormal.* Other definitions of behavioural categories are reported in table 7. Behaviours are listed in the respective categories in table 8.

5. *Discussion*

The current study represents the first extensive ethogram for the species African wild dog *Lycaon pictus*. To the author knowledge, no extensive review of the literature regarding African wild dog's behaviour has been published to date. In this essay direct field behavioural data were merged with the results of an extensive literature review. A comprehensive ethogram was created, including N=153 behaviours of which N=142 already described in three references, 17= described in two references and N=6 described in one reference; N=12 behaviours were non-previously described. All behaviors have been defined with a short title and described with a clear and concise definition. The behaviours were categorised in 12 non-exclusive classes to improve the ethogram accessibility and flexibility for its application on captive and wild settings.

The web search revealed a high number of hits non-relevant to the study, highlighting the ambiguity of the common names used for *Lycaon*. Additionally, the review results suggest that few studies that refer to the African wild dog's behaviour follow proper ethology methods. Compared to the huge number of hits obtained with the web search, a small number of

studies matched the inclusion criteria. Regardless the double matches and the articles that were not focused on *Lycaon pictus*, a vast amount of publications included behavioural measurements, or referred to behaviour (i.e. *special behaviour*, *social behaviour*, *ranging behaviour*, *sociality*) but did not include a description of the behaviours of interest, leaving the definition as self-explanatory or implied**.** In other cases, researchers included a short description of the behaviour in the text, avoiding the creation of a proper ethogram, and only one paper included an ethogram with references to a previous study. In the present study, decision was made to include non-mutually exclusive behavioural classes, in order to increase the flexibility of the ethogram for future applications. No description was found for classes of dominance behaviours in African wild dogs, while one definition was found for *social assertion* (McCreery, 1999), thus behaviours classified during the behavioural observation as "dominance behaviours" were categorized under the class *social assertion*.

Description of the hunting sequence was not included in the present research. Decision was made to include the most recent definition of hunt (Creel and Creel, 1995, 2002; Creel et al., 2016), without fragmenting the behaviour in distinct phases. Details on the different hunting techniques and description of the hunting phases can be found in a PhD thesis by Reich (1981). Behaviours studied by field observations that could mismatched with refences were: *Approach; Drink; Leave; Nursing; Run; Sniffing Air; Spy-Hop; Trot; Walk; Chewing*, and the non-behavioural entry *other.* The latter represents an alternative that could be scored on a working ethogram and considers the possibility that the animals might express behaviours not previously described and included in the present study.

6. *Conclusion*

The present study offers a unified up to date ethogram to be used on African wild dogs' studies with a behavioural implication. This ethogram is designed to be user-friendly and highly flexible, leaving the possibility of defining the behavioural classes as needed, but guiding through the process of behavioural classification and definition. This ethogram was designed to uniform the application of scrupulous ethological methods. African wild dog is a highly social canid (Jackson et al., 2017; Knobel and du Toit, 2003; Walker et al., 2017) whose behaviour received scarce and fragmented description, and even when AWDs' behaviours were reported, they were not organically classified in a usable ethogram. By providing a complete ethogram for AWD and sharing it to the scientific community, we hope to have provided a useful tool, which could guarantee comparability among different behavioural studies on AWD, making easier to standardise both description and results. A robust, comparable and clear approach could, as a consequence, lead to a better comprehension of African wild dogs' needs and ecology, and enhance their welfare and conservation.

Table 1 – AWD subjects' details

Reserve	Pack	Name	Sex	Year of birth	Collar	Collared on
Limpopo lipadi	Morena	Shrek	M	~2007	VHF	--
Limpopo lipadi	Morena	Two Spots	F	2008	Satellite	10/01/2018
Limpopo lipadi	Morena	Whacha	F	2013	VHF	10/01/2018
Limpopo lipadi	Morena	Wimpy	F	2015	no	--
Limpopo lipadi	Morena	Milly	F	2015	no	--
Limpopo lipadi	Morena	Whity	M	2015	no	--
Limpopo lipadi	Morena	Torre	M	2015	Satellite	27/08/2018
Limpopo lipadi	Morena	Ring	M	2015	VHF	2015
Limpopo lipadi	Valentines	Blacky	M	unknown	no	--
Limpopo lipadi	Valentines	Musky	M	unknown	no	--
Limpopo lipadi	Valentines	Pato	M	unknown	no	--
Limpopo lipadi	Valentines	Gigi	F	unknown	no	--
Khamab	Botswana	Bawdm1	M	unknown	no	--
Khamab	Botswana	Bawdm2	M	unknown	Satellite	07/07/2018
Khamab	Botswana	Bawdm3	M	2017	no	--
Khamab	Botswana	Bawdm4	M	2017	no	--
Khamab	Botswana	Bawdm5	M	unknown	no	--
Khamab	Botswana	Bawdm6	M	unknown	no	--
Khamab	Botswana	Bawdm7	M	2017	no	--
Khamab	Botswana	Bawdm8	M	unknown	no	--
Khamab	Botswana	Bawdm9 Rubio	M	unknown	no	--
Khamab	Botswana	Bawdm10	M	unknown	no	--
Khamab	Botswana	Bawdm11	M	unknown	no	--
Khamab	Botswana	Bawdf1	F	unknown	no	--
Khamab	Botswana	Bawdf2	F	unknown	no	--
Khamab	Botswana	Bawdf3	F	2017	no	--
Khamab	Botswana	Bawdf4	F	2017	no	--
Khamab	Botswana	Bawdf5	F	2017	no	--
Khamab	Botswana	Bawdf6	F	2017	no	--
Khamab	South	Splittie	M	unknown	no	--
Khamab	South	Awdm2	M	2017	no	--
Khamab	South	Awdm3	M	2017	no	--
Khamab	South	Awdm4	M	2017	no	--
Khamab	South	Awdm5	M	2017	no	--
Khamab	South	Awdm6	M	2017	no	--
Khamab	South	Awdm7	M	2017	no	--
Khamab	South	Awdm8	M	2017	no	--
Khamab	South	Awdm9	M	2017	VHF	08/07/2018
Khamab	South	Eva 1	F	unknown	no	--
Khamab	South	Awdf2	F	2017	no	--
Khamab	South	Awdf3	F	2017	no	--
Khamab	South	Awdf4	F	unknown	no	--
Khamab	South	Awdf5	F	unknown	no	--
Khamab	South	Awdf6	F	unknown	no	--
Khamab	South	Awdf7	F	unknown	no	--
Khamab	South	Awdf8	F	unknown	no	--

Khamab	South	Awdf9	F	unknown	no	--
Khamab	South	Awd18	F	unknown	no	--
Khamab	South	Awd19	M	unknown	no	--

Table 2 – packs composition

	Limpopo Lipadi		Khamab	
	Morena	**Valentines**	**Botswana**	**South**
Adults	12	4	10	10
females	5	1	2	8
males	7	3	8	2
Juveniles	--	--	7	10
females	--	--	4	2
males	--	--	3	8
Pups	--	--	8	10
Pack members	12	4	25	30
Total	16		55	

Table 3 – References inclusion criteria

Criteria		Description
Inclusion		
	Species	Only manuscripts describing African wild dog's behaviours were considered for inclusion when providing behavioural description in tabular form or included in the text.
	Ethogram	The article contain an ethogram, or a list of behaviours recorded and described, included in either a tabular form or within the text
	Publication	Articles have been published in peer-reviewed journals. Postgraduate thesis (PhD and MSc) and books were included, when the quality of the behavioural description was relevant
	English language	Manuscripts have been either published in English or have been translated reliably
Exclusion		
	Failure to list behaviours	Articles that reference previous ethograms but failed to list or provide a brief description of any of the behaviours recorded in their study were excluded
	Veterinary manipulation	Behaviours expressed in direct response to medical treatments were not considered, unless identified as existing within the natural repertoire of the species
	Author duplications	If multiple articles written by the same Autor listed the same behaviours, only one article was included in the study
	Unavailability	Manuscripts included were found through university, colleague, and general internet access, as well as direct communication with the authord. If a manuscript could not be retrieved in this manner, it was excluded.

Table 4 – Web searches results

Key words combinations			Science Direct	Scopus	Wiley Online Library	ProQuest	GoogleScholar	Genus totals
"Lycaon pictus"	and	"Behaviour"	507	103	807	1063	4650	7130
"Lycaon pictus"	and	"Ethogram"	15	0	12	75	210	312
"Lycaon pictus"	and	"Ethology"	67	679	32	401	1460	2639
"African wild dog"	and	"Behaviour"	261	99	295	903	3020	4578
"African wild dog"	and	"Ethogram"	5	0	4	59	131	199
"African wild dog"	and	"Ethology"	41	0	25	293	980	1339
"painted dog"	and	"Behaviour"	14	2	31	124	158	329
"painted dog"	and	"Ethogram"	0	0	1	1	4	6
"painted dog"	and	"Ethology"	0	0	2	1	24	27
"painted wolf"	and	"Behaviour"	4	0	1	15	0	20
"painted wolf"	and	"Ethogram"	0	0	0	3	0	3
"painted wolf"	and	"Ethology"	0	0	0	1	1	2
"hunting dog"	and	"Behaviour"	220	38	273	2479	2730	5740
"hunting dog"	and	"Ethogram"	5	0	3	43	0	51
"hunting dog"	and	"Ethology"	19	0	13	125	441	598

Table 5 – References selected after applying the inclusion criteria

References	Type	Journal	Environment	Method	Ethogram (Y/N)	Selected (N)
Creel and Creel (1995)	journal article	*Animal Behaviour*	wild	all occurrence	no	1
Creel and Creel (2002)	book	--	wild	all occurrence	no	1
Creel et al. (2016)	journal article	*Ecology*	wild	all occurrence	no	1
de Villiers et al. (2003)	journal article	*Journal of Zoology London*	captive	focal and ad libitum	no	3
Frame et al. (1979)	journal article	*Zeitschrift für Tierpsychologie*	wild	focal animal	no	2
Jordan et al. (2013)	journal article	*Ethology*	wild	all accorrence	no	3
Jordan et al. (2014)	journal article	*Animal Behaviour*	wild	critical incident sampling	no	1
Knobel and du Toit (2003)	journal article	*Applied Animal Behavioural Science*	captive	not described	no	3
Leonard (2008)	PhD Thesis	--	captive	not described	yes	8
McCreery (1999)	PhD Thesis	--	wild	all occurrence	yes	71
McCreery (2000)	journal article	*Behaviour*	wild	proximity scan sample	no	1
O'Malley (2013)	journal article	*Behaviour*	captive	instantaneous scan sampling and all occurrence	yes	2
Parker (2010)	PhD Thesis	--	wild	not described	no	1
Price (2010)	journal article	*Bioscience Horizons*	captive	scan sampling	yes	7
Rafacz and Santymire (2014)	journal article	*Zoo biology*	captive	instantaneous scan sampling	yes	22
Reich (1981)	PhD Thesis	--	wild	not described	no	3
Robbins (2000)	journal article	*Behaviour*	wild	not described	no	26
Robbins and McCreery (2003)	journal article	*Biological Conservation*	wild	not described	no	1
Van Heerden (1981)	journal article	*The Onderstepoort Journal of Veterinary Research*	captive	not described	no	6
Walker et al. (2017)	journal article	*Proceedings of the Royal Society B*	wild	critical incident sampling	no	3

Table 6 – comprehensive ethogram

Title	Definition	References		Personal Observations
Active submission	Approach conspecific crouching, grinning, tail wagging, and sometimes head bobbing	(McCreery, 1999)		X.
Aggression-Pace	Walking parallel to other dogs with stiff foreleg pars, head down, piloerection	(Rafacz and Santymire, 2014)		X.
Allo-Groom	Lick or nibble conspecific anywhere except the mouth, not reciprocal	(Rafacz and Santymire, 2014)	(McCreery, 1999)	X.
Anal Dragging	Subject sits with its hind legs extended forward and drag itself forward using its forelegs	(Van Heerden, 1981)		X.
Approach	Subject moves toward a conspecific with precise direction, head is on the same axis of the shoulders, ears upwards			X.
Approach Object	Subject moves close (less than 1 body length) to an object without touching it. The behaviour ends when the dog moves away more than one body lenght	(Leonard, 2008)		X.
Attack	A sudden attack from some distance, nipping/biting by the aggressor will follow	(Knobel and du Toit, 2003)		
Avoid	Subject moves quickly away or take several steps away, or move body back as if withdrawing from partner while remaining stationary	(McCreery, 1999)		X.
Bark - Alarm	The most growl-like bark in spectral appearance and auditory quality. They express greater motivational intensity than alarm growls, but serve a similar function	(Robbins, 2000)		X.
Bark - Alarm - Tremolo	Tremolo alarm barks indicate a higher level of arousal than common alarm barks. They are also more harmonic and longer in duration, with a more uniform distribution of energy over time. These high intensity barks can be given when lions are detected or when humans approach on foot. Can be repetitive.	(Robbins, 2000)		
Bark - Attack	The shortest bark. Can be entrained in a series of increasing syllable length and amplitude. To the unaided ear, they have a nasal quality. Attack barks can be issued by pack members chasing hyenas	(Robbins, 2000)		
Bark - Clear	Commonly associated with domestic dogs sounds, can be given by adults in alarm, and when adults interact affiliatively with pups. Given when highly aroused	(Robbins, 2000)		
Bark - Howl	the longest barks with the least frequency spread. Uttered with a tremolo. Given by individual dogs while running excitedly during an interpacket encounter	(Robbins, 2000)		
Bark - Threat	Shorter than alarm barks and disyllabic. The first part of the first syllable is often stressed. May express motivational ambivalence. Can be given toward pups soliciting food and appeared to signal muted aggression	(Robbins, 2000)		X.
Bark - Yelp	Bark with the highest fundamentals and the greatest frequency ranges. Can be given when packs mobbed hyenas. Can be an affiliative yelp bark toward pups at the den.	(Robbins, 2000)		
Begging cries	Form of food solicitation, also used in context of greeting ceremonies; can be viewed as a form of social solicitation. Can also indicate distress	(Robbins, 2000)		X.
Begging gurgles	Produced during picks of arousal. Consists of begging cries encapsulating noisy components	(Robbins, 2000)		X.
Bite	Snapping jaws shut on legs, belly, or anus to halt other animal	(Rafacz and Santymire, 2014)		X.
Bite attempt	Subject snap at approaching animal. Can be expressed by during courtship	(Reich, 1981)	(McCreery, 1999)	X.
Bite defensive	From a submissive posture, attempt to bite a conspecific	(McCreery, 1999)		X.
Bite muzzle	Bite to muzzle of conspecifics	(McCreery, 1999)		X.
Bite neck	Bite to neck of conspecifics	(McCreery, 1999)		X.
Bite object	Subject places its mouth around the object	(Leonard, 2008)		
Bite throat	Bite to throat of conspecifics	(McCreery, 1999)		X.
Biting car	Biting visitor car tyres, tow bar covers or other parts of the vehicle	(Price, 2010)		

Body bob	Momentarily lowering body toward ground while approaching or being approached by conspecific	(McCreery, 1999)			X.
Body lift	Lift conspecific off ground by placing the head or body under the trunk or between fore or hindlegs	(McCreery, 1999)	(Van Heerden, 1981)		X.
Body rolling	mainly by males next to a sleeping or resting female and often followed copulation or repeated mounting. Often occurs in places where materials such as urine, faeces or food remnants were detectable.	(Van Heerden, 1981)			X.
Body rub	With the head, rub any body part of conspecific (excluding act of chinning)	(McCreery, 1999)			
Bow	Flex the forelegs and extend the hindlegs	(McCreery, 1999)			X.
Bump with nose	Forcefully poke or bump a conspecific with muzzle	(McCreery, 1999)			X.
Charge	Rapid, abrupt directional move toward a conspecific from some distance away (in contrast to lunge)	(McCreery, 1999)			X.
Chase	Chasing another subject, usually with ears back and piloerection	(Rafacz and Santymire, 2014)	(Knobel and du Toit, 2003)	(McCreery, 1999)	X.
Chewing	Repeated biting or gnawing of an object (bone, stone, branch or any other item)				X.
Chin to neck	Place\rest chin on the dorsal surface of the neck	(McCreery, 1999)			X.
Chin/Cheek rub	The subject makes physical contact between the neck or torso and the conspecific	(Leonard, 2008)			
Chinning	Rub face and muzzle of a conspecific with underside jaw	(McCreery, 1999)			
Coalition	Event when individuals combine their forces; individual coming to the aid of the other(s)	(de Villiers et al., 2003)			X.
Crouch	Lower hindquarters to ground with the back arched when approached by conspecific, may defecate and\or urinate	(McCreery, 1999)			X.
Defecation	elimination of faces, ssubject is standing with front legs and hind legs flexed	(Rafacz and Santymire, 2014)			X.
Defecation - Object	The dog deposits faecal material on the ground on or within one body length of the object	(Leonard, 2008)			X.
Defensive gape	From submissive posture, with the mouth open and teeth unexposed, attempt to ward off aggressive conspecific	(McCreery, 1999)			X.
Dig	Scratching ground with one or both front paws to make a hole	(Rafacz and Santymire, 2014)			X.
Direct stare	Ears pushed hard forward, head erect or lowered, intense directional gaze	(McCreery, 1999)			X.
Drink	Ingesting water				X.
Ears back	Ears pushed back, folded (inside edge touches outside edge hiding meatus) or not folded	(McCreery, 1999)			X.
Ears horizontal	Ears parallel to ground, meatus facing forward or downward, may be pushed forward	(McCreery, 1999)			X.
Ears pushed hard forward	Ears erected and pushed hard, facing forward	(McCreery, 1999)			X.
Ears up	More or less relaxed position with ears facing forward	(McCreery, 1999)			X.
Ears up-horizontal	Ears held in any position between straight up with meatus facing to the side and horizontal	(McCreery, 1999)			X.
Fight	Aggression with physical contact between two subjects snapping at each other, often placing their chins on the ground, attaches include strife directed especially toward the muzzle, a side of the face, the genital area	(Reich, 1981)			
Forage	Ingesting food	(O'Malley, 2013)			X.
Greeting ceremony	the onset of the greeting ceremony is frequently initiated by a single animal running up to another with head shoulder-height, mouth in a gape, and ears folded back, while initiating muzzle-to-muzzle contact. High intensity displays involve ritualized parallel walking accompanied by low amplitude rumbles and buzz gurgle moans that, with increasing motivational intensity, are replaced by twitters, begging cries, yelp/squeals, whines, and whimpers. 'Symbolic solicitation for food'	(Robbins, 2000)	(McCreery, 1999)	(Walker et al., 2017)	X.

Growls - Alarm	Shorter than social growls with more energy contained in the first half of the vocalisation. Serve to warn the pack when confronted with a possible threat. Sometimes given repetitively with intersillable intervals from 0.5-6.3 s and bouts lasted from 13.5 to 106. s	(Robbins, 2000)	(McCreery, 2000)		X.
Growls - Social	Heard during antagonistic interactions with other pack members	(Robbins, 2000)			
Head bob	Move head side to side or up and down while approaching or being approached by conspecific, in its most extreme form, head movement highly erratic	(McCreery, 1999)			X.
Head lowered	Neck extended with head held at the shoulder height or below	(McCreery, 1999)			X.
Head rotation	Lateral movement of head while slightly tilting it side to side.	(McCreery, 1999)			X.
Hoo-Call	Long distance contact call. occurs with the head held lower than the shoulders and a gaping mouth that oscillates slightly with each emission. Pulsed, usually monotonic, concatenations of single syllables. typically issued in bouts.	(Robbins and McCreery, 2003)	(Frame et al., 1979)	(Robbins, 2000)	X.
Hunt	Pursuit at a fast run for more than 50 m bringing prey at bay or making a kill	(Creel and Creel, 1995)	(Creel and Creel, 2002)	(Creel et al., 2016)	X.
Inspect Faeces	Animals inspects an existing deposit of faeces; includes approaching and sniffing	Rafacz and Santymire and Santymire 2014			X.
Intervention	One animal separates two or more subjects of which one or more had directed aggressive behaviours towards the other(s)	(de Villiers et al., 2003)			
Investigation of a scent mark	An individual sniffed (muzzle directed at, and lingering within 30 cm of deposit) or licked it (made direct contact using the tongue)	(Jordan et al., 2013)			X.
Joust	Both participants rear up onto hindlegs to place forelegs upon one's another shoulders	(McCreery, 1999)	(Rafacz and Santymire, 2014)		X.
Lay	Resting on the ground in sternal or lateral position with or without contact with other animals, no visible movement of the torso, head not touching the ground	(Rafacz and Santymire, 2014)	(Price, 2010)		X.
Leap	Jump and propel the body to facilitate contact with conspecific; hindlegs may not leave the ground	(McCreery, 1999)			X.
Leave	Subject moves away from one or more conspecifics without running				X.
Lick object	Subject brings its tongue in contact with the object	(Leonard, 2008)			X.
Lip licking	Quick movement of the tongue over one's lips	(McCreery, 1999)			X.
Look sway	Turn head away from conspecific as if avoiding eye contact	(McCreery, 1999)			X.
Look toward	turn or lift head up as if directing attention to conspecific	(McCreery, 1999)			X.
Lower forequarters to ground	Lower forequarters and possibly the chin onto the ground; may be extreme form of "bow"	(McCreery, 1999)			X.
Lower hindquarters to ground	Lower haunches toward ground (may sit) in response to a conspecific	(McCreery, 1999)			X.
Lunge	Rapid, abrupt directional move toward a nearby conspecific, piloerect, ears back	(McCreery, 1999)	(Rafacz and Santymire, 2014)		
Moans - Buzz	Biggest syllable of any vocalization; context specific utterances aired during the initial excitation phase of high intensity greeting ceremonies and begging cry displays. Can encapsulate noisy gurgles	(Robbins, 2000)			
Moans - Full	Shortest syllable and narrowest frequency range of the moans. Appear to indicate frustration. Given by subjects trying to access kills and while digging at the opening of dens in which pups were resting	(Robbins, 2000)			X.
Mob	Initiator attacks (e.g. lunges or notes) conspecific and others join in	(McCreery, 1999)			
Mount	Clasp conspecific's hindquarters with forelegs	(McCreery, 1999)			
Mouth closed	Lips not parted	(McCreery, 1999)			X.
Mouth gape	Mouth open, only lower teeth visible	(McCreery, 1999)			X.

Mouth gape-grin	Mouth open, lips retracted, teeth half exposed	(McCreery, 1999)		X.
Mouth grimace	Mouth more or less closed, lips retracted exposing incisors	(McCreery, 1999)		X.
Mouth open	Mouth open, classification uncertain	(McCreery, 1999)		X.
Mouth snarl	Mouth closed, upper lip curled up exposing premolars	(McCreery, 1999)		
Mouth snarl-grin	grin with upper lip curled up exposing premolars	(McCreery, 1999)		
Mouth toothless	Mouth open teeth not exposed	(McCreery, 1999)		X.
Mouth yawn	Mouth fully open, lips briefly retracted exposing teeth	(McCreery, 1999)		
Mutual greet - muzzle licking	Reciprocal licking and biting of mouth area, can be solicitation for food	(McCreery, 1999)	(Robbins, 2000)	X.
Mutual greet - muzzle to muzzle	Brief pointing or touching of muzzles when no more than one adult head length away, mouth may be opened or closed, ears relaxed	(McCreery, 1999)		X.
Mutual greet - parallel walking	Walking with bodies oriented in same direction with muzzles side by side	(McCreery, 1999)		X.
Neck present	Turn head on axis to expose the throat when approached by or approaching conspecific	(McCreery, 1999)		X.
Nose to body	point muzzle to any part of the body excluding groin, muzzle and neck regions	(McCreery, 1999)		X.
Nose to groin	thrust or place muzzle to groin of conspecific	(McCreery, 1999)		X.
Nursing	lactating female feeds the offspring, thereby the pups lactate by placing their mouths around the nipples and suckling			X.
Offensive threat	Aggressor snarls at the recipient with raised lips	(Knobel and du Toit, 2003)		
Other	Any behaviour not otherwise categorized			
Out of sight	Animal not visible	(O'Malley, 2013)		X.
Overmarking	The placement of a deposit on an existing deposit (urine or faeces) so that the two were at least partially overlapping	(Jordan et al., 2013)		X.
Pacing	Walking back and fort over the same small area	(Rafacz and Santymire, 2014)		
Pack call	can include hoos, whines, whimpers, yelp/squeals, growls, barks. These calls were chorused by both packs simultaneously or issued in volleys. Can be given once outside the context of an interpack encounter	(Robbins, 2000)		
Partnership	Two or more aggressors act in concert against another party	(de Villiers et al., 2003)		X.
Passive submission	Roll shoulders first on back, with mouth slightly open, lips retracted, and ears flattened in response to an approaching conspecific	(McCreery, 1999)		X.
Paw touch	Animal makes physical contact between either right or left front paw and an object or conspecific	(Leonard, 2008)		
Pawing	lift forepaw as to make contact with conspecific but without contact	(McCreery, 1999)		
Pinning	press muzzle to head of recipient forcing it to the ground	(McCreery, 1999)		
Play Bow	Animal is crouched down, touching or nearly touching forelimbs to the ground with rear end high in the air. Orientation is directly towards a play partner	(Rafacz and Santymire, 2014)		X.
Play chase	Chasing another subject, usually with ears forward and not piloerect	(Rafacz and Santymire, 2014)		X.
Play disruption	Aubject intervenes in bouts of play among conspecifics	(Reich, 1981)		
Play invitation	Stamping on forelegs with ears up, facing others	(Rafacz and Santymire, 2014)		X.
Pulse - Whine	Whining softly, rapidly in presence of others	(Rafacz and Santymire, 2014)		X.

Rally initiation	Initiation posture: head lowered, mouth open, and ears folded back	(Walker et al., 2017)			X.
Rear back	Rear up on hindlegs to place forelegs on shoulders of conspecific not reciprocated	(McCreery, 1999)			X.
Redirect behaviour	Refocus attention on object or another dog when interacting with conspecific	(McCreery, 1999)			X.
Rest	Lying down, head down. Body can be in contact with others	(Price, 2010)	(McCreery, 1999)		X.
Rub on car	Rubbing body against the back of visitor's vehicle bumpers/exhausts	(Price, 2010)			
Run\Gallop	Forward locomotion, head aligned with the body axis, different positions of the ears (backwards or elevated); phases with both front and back legs extended where no paw is in contact with the ground				X.
Run (trot) away	quick locomotion when moving away from pursuit of conspecific	(McCreery, 1999)			X.
Scanning	quadrupedal stance and actively scanning the environment	(Price, 2010)			X.
Self groom	Licking, scratching own body, rolling and squirming on back	(Rafacz and Santymire, 2014)			X.
Sitting	Back part of the body in contact with the ground, usually occurs when animals is scratching itself	(Rafacz and Santymire, 2014)			X.
Sneeze	Audible, abrupt exhalation of air through the nose	(Walker et al., 2017)			X.
Sniff car exhaust	Sniffing/licking car exhausts	(Price, 2010)			
Sniff conspecific	Movement of the muzzle over\around any part of body of aconspecific	(McCreery, 1999)			X.
Sniff object	Animals brings the nose to or less than one head length of the object	(Leonard, 2008)	(Price, 2010)		X.
Sniffing air	dog inhales air tough the nose with the head raised and nose not in proximity of any object or animal. Ears can be pointed backwards or straight up				X.
Spy-Hop	While standing up, the subject lift front paws and back, moving. The entire body upright an standing on forelegs				X.
Squeals - Begging	Similar to whistle squeals, given during greeting ceremonies and begging cry displays as expression of solicitation as well as appeasements. Also accompany spar twitters	(Robbins, 2000)			
Squeals - Whistle	Similar to yelps but with syllable length over three times that of yelps. Given in similar context as yelps	(Robbins, 2000)			
Stalk	Approach with measured walk, with lowered head, while directing attention toward a conspecific or a subgroup	(McCreery, 1999)	(Rafacz and Santymire, 2014)		X.
Standing	Standing still on all four paws, usually addressing the look to the surroundings or the other animals	(McCreery, 1999)			X.
Submission-Beg	Whining, cringing, tail wagging, falling over to expose underside, for food	(Rafacz and Santymire, 2014)			
Submission-whine	Long, high-pitched whining, dog crouching sideways to other dog	(Rafacz and Santymire, 2014)			X.
Tail durled	Curled up and over the body	(McCreery, 1999)			X.
Tail down	Hanging straight down	(McCreery, 1999)			X.
Tail parallel	Raised parallel to the body axis	(McCreery, 1999)			X.
Tail straight up	Raised perpendicular to body axis	(McCreery, 1999)			X.
Tail tuck	Arched between the legs with the tip under the hindquarters or belly	(McCreery, 1999)			X.
Tail wag	Side to side swoosh-like or twitching motion	(McCreery, 1999)			X.
Tandem marking	Both members of a pair scent-mark the same location in quick succession.	(Jordan et al., 2014)	(Van Heerden, 1981)	(Parker, 2010)	X.
Trot	Forward locomotion, the head can be aligned with the body axis or elevated above the shoulder, different positions of the ears (backwards or elevated); two paws in diagonal pairs touch alternately the ground				X.
True mating	Mount associated with penis intrusion and a tie which lasts from 50 seconds to 2 minutes. The female stands firmly on her four legs with head slightly raised, the male is above and behind her, often with the head pressed into her scapular region, clamping her with his forelegs. During the coitus the female can assume a semi-sitting position.	(Van Heerden, 1981)			X.

Twitters – Attack	Lower components are more pronounced with lower fundamentals. Mostly noisy low frequency twitters that sound nasal. Given when chasing hyenas, during inter-pack encounter, and by resting pups when approached by adults	(Robbins, 2000)			
Twitters – Mob	Often disyllabic. Given by adults while mobbing other pack members, pursuing prey, chasing hyenas and vultures from a kill, and by resting pups when approached by adults as a warning to lie elsewhere	(Robbins, 2000)			X.
Twitters – Social	High-pitched staccato-like emissions. Low and high frequency ranges, used as vocal accompaniments in the greeting ceremony. Can be given during resting periods when pack members reposition themselves or approach one another; can indicate a high level of arousal.	(Robbins, 2000)			
Twitters – Spar	Twitters with low frequency components more frequent than high. Given by subadults during vigorous play, and by adults in appeasement or when engaged in social investigation during pack formation.	(Robbins, 2000)			X.
Urinate object	Subject urinates with one leg raised or by squatting on or within one body length of the object	(Leonard, 2008)			
Urination	Elimination of urine	(Rafacz and Santymire, 2014)			X.
Urine scent mark	Elimination with leg postures including cocked leg (single hind leg raised/cocked once); raised leg (both hind legs raised independently at least once); and handstand (both hind legs raised simultaneously in a hop).	(Jordan et al., 2013)	(Van Heerden, 1981)	(Frame et al., 1979)	X.
Varied ear postures	Ear postures change rapidly or ears move independently	(McCreery, 1999)			X.
Walk	Forward locomotion, head can be aligned with the body axis or elevated above the shoulder, different positions of the ears (backwards or elevated); at least one paw is always touching the ground				X.
Whimpers	Low to high frequency sound, with related syllables. Usually repetitive, given when dogs approached one another during resting periods. Also given when adults call pups from the den, and during greeting ceremonies. It represents one of the most common affiliative vocalizations	(Robbins, 2000)			X.
Whines	Low to high frequency, unlike whimpers not repeated. Can sound like meows and mews issued by adults in greeting ceremonies. Appear to be a form of solicitation or frustration	(Robbins, 2000)			X.
Yelps	Short, sharp sounds given in submission by subordinate dog or pups either in anticipation of, or in response to, pain probably to inhibit potential injury. Also given in appeasement and solicitation during greeting ceremonies and begging cry displays. Some times given when chasing hyenas	(Robbins, 2000)			X.

Table 7 – definition of behavioral categories

Category	Definition
Affiliative	"Friendly" behaviours that may communicate the animal's intention to associate with other individuals in a peaceful manner
Aggressive	Can potentially inflict harm on the recipient
Exploratory	Investigative behaviours associated with the subject general interest in the environment or specific stimulus.
Locomotory	Behaviours that enable the movement from one location to another. Comprises walk, trot,run\gallop
Maintenance	Behaviours associated with the physiological requirements of the animal. Comprises drinking, defecating, eating
Play	Activity that can be solitary or social, comprising from a mild to vigorus physical activity, serving minimal immediate functions
Postural	Behaviours in which the subject is stationary or performing minimal movements
Reproductive	Sexual behaviours associated with courtship, mating and reproduction
Social Assertion	Behaviour occurring during an intersexual context, associated with the confrontation of two or more individuals
Submissive	Include passive and active submission
Vocalisation	Sounds and/or calls produced by the subject using its vocal apparatus.
Abnormal	Repetitive, dysfunctional behaviours with no apparent goal

Table 8 – classification of behaviours under respective non-mutually exclusive categories

Affiliative	Aggressive	Exploratory	Locomotory	Maintenance	Play	Postural
allogroom	aggression-pace	approach object	approach	defecation	joust	chewing
bark - clear	attack	bite object	hunt	drink	pawing	other
begging cries	bark - alarm - tremolo	chewing	dig	nursing	play bow	out of sight
begging gurgles	bark - attack	chin/cheek rub	leave	urination	bite muzzle	redirect behaviour
chin/cheek rub	bark - howl	defecation - object	pacing	Forage	bite neck	rest
coalition	bark - threat	lick object	Run/gallop		nose to groin	scanning
defecation - object	bark - yelp	paw touch	stalk		partnership	self-groom
ears back	bite	sniff object	trot		pawing	sitting
greeting ceremony	bite attempt	urinate - object	walk		pinning	sneeze
growls - alarm	charge		anal dragging		play bow	sniff conspecific
head bob	chase		body rolling		play chase	sniffing
hoo-call	ears pushed hard forward		approach object		play invitation	sniffing air
inspect faeces	fight				rear back	spy-hop
investigation of a scent mark	growls - social				bite throat	standing
leap	intervention					tail curled
look away	lunge					tail down
look toward	mob					tail parallel
lower hindquarters to ground	mouth grimace					tail straight up
moans - buzz	mouth snarl					tail tuck
mouth gape	mouth snarl-grin					tail wag
mouth gape-grin	bite neck					varied ear postures
mutual greet - muzzle licking	offensive threat					
mutual greet - muzzle to muzzle	partnership					
mutual greet - parallel walking	play disruption					
overmarking	bite throat					
pack call	twitters - attack					
play chase	twitters - mob					
play invitation						
pulse - whine						
rally initiation						
rear back						
sniff conspecific						
tandem marking						
twitters - social						

twitters - spar

urinate - object
urine scent mark
whimpers
whines

Reproductive	Social Assertion	Submissive	Vocalisation	Abnormal
anal dragging	bite muzzle	active submission	bark - alarm	biting car
body rolling	body lift	avoid	bark - alarm - tremolo	rub on car
mount	body rub	bite defensive	bark - attack	sniff car exhaust
overmarking	bump with nose	body bob	bark - clear	
tandem marking	chin to neck	crouch	bark - howl	
true mating	chinning	defensive gape	bark - threat	
	nose to body	lower forequarters to ground	bark - yelp	
	nose to groin	neck present	begging cries	
	pinning	passive submission	begging gurgles	
	stalk	run (trot) away	growls - alarm	
		squeals - whistle	growls - social	
		submission-beg	hoo-call	
		submission-whine	moans - buzz	
		yelps	moans - full	
			pack call	
			squeals - begging	
			squeals - whistle	
			twitters - attack	
			twitters - mob	
			twitters - social	
			twitters - spar	
			whimpers	
			whines	
			yelps	

8. *References*

Altmann, J., 1974. Observational study of behaviour: sampling methods. Behaviour 49, 227–267.

Bernstein, I.S., 2010. Ethograms, activity profiles and energy budgets. Enciclopedia of Behaviour.

Creel, S., Creel, N.M., 2002. The African wild dog: behaviour, ecology and conservation. Princeton University Press. https://doi.org/10.1016/S0169-5347(02)00039-3

Creel, S., Creel, N.M., 1995. Communal hunting and pack size in African wild dogs, *Lycaon pictus*. Animal Behaviour 50, 1325–1339. https://doi.org/10.1016/0003-3472(95)80048-4

Creel, S., Creel, N.M., Creel, A.M., Creel, B.M., 2016. Hunting on a hot day: effects of temperature on interactions between African wild dogs and their prey. Ecology 97, 2910–2916. https://doi.org/10.1002/ecy.1568

de Villiers, M.S., Richardson, P.R.K., van Jaarsveld, A.S., 2003. Patterns of coalition formation and spatial association in a social carnivore, the African wild dog (*Lycaon pictus*). Journal of Zoology 206, 377–389.

Frame, L.H., Malcolm, J.R., Frame, G.W., van Lawick, H., 1979. Social Organization of African Wild Dogs (*Lycaon pictus*) on the Serengeti Plains, Tanzania 1967–1978. Zeitschrift für Tierpsychologie 50, 225–249.

Gerencsér, L., Vásárhelyi, G., Nagy, M., Vicsek, T., Miklósi, A., 2013. Identification of behaviour in freely moving dogs (*Canis familiaris*) using inertial sensors. PLoS One 8, e77814. https://doi.org/10.1371/journal.pone.0077814

Jackson, C.R., Groom, R.J., Jordan, N.R., McNutt, J.W., 2017. The effect of relatedness and pack size on territory overlap in African wild dogs. Movement Ecology 5, 10. https://doi.org/10.1186/s40462-017-0099-8

Jordan, N.R., Apps, P.J., Golabek, K.A., McNutt, J.W., 2014. Top marks from top dogs: tandem marking and pair bond advertisement in African wild dogs. Animal Behaviour 88, 211–217. https://doi.org/10.1016/j.anbehav.2013.12.001

Jordan, N.R., Golabek, K.A., Apps, P.J., Gilfillan, G.D., McNutt, J.W., 2013. Scent-mark identification and scent-marking behaviour in African wild dogs (*Lycaon pictus*). Ethology 119, 644–652. https://doi.org/10.1111/eth.12105

Knobel, D.L., du Toit, J.T., 2003. The influence of pack social structure on oral rabies vaccination coverage in captive African wild dogs (*Lycaon pictus*). Applied Animal Behaviour Science 80, 61–70. https://doi.org/10.1016/S0168-1591(02)00136-3

Leonard, D.A., 2008. An evaluation of environmental enrichment for two highly social and endangered canid species, the African wild dog (*Lycaon pictus* and Mexican wolf (*Canis lupus baileyi*) (Ph.D.). Saint Louis University, Ann Arbor.

MacNulty, D.R., Mech, L.D., Smith, D.W., 2007. A proposed ethogram of large-carnivore predatory behavior, exemplified by the wolf. Journal of Mammalogy 88, 595–605. https://doi.org/10.1644/06-MAMM-A-119R1.1

McCreery, E.K., 2000. Spatial relationships as an indicator of successful pack formation in free-ranging African wild dogs. Behaviour 137, 579–590. https://doi.org/10.1163/156853900502222

McCreery, E.K., 1999. The formation of new reproductive units in free-ranging African wild dogs (*Lycaon pictus*). (Ph.D.) University of California, United States, California..

McDonnell, S., 2003. The equid ethogram: a practical field guide to horse behavior. The Blood-Horse Inc. ed. Hong Kong.

O'Malley, C.I., 2013. Individual behavior types and social dynamics in a pack of African painted dogs (*Lycaon pictus*) at Brookfield Zoo (M.S.). Ann Arbor, United States.

Overall, K.L., 2014. The ethogram project. Journal of Veterinary Behavior: Clinical Applications and Research 9, 1–5. http://dx.doi.org/10.1016/j.jveb.2013.12.001

Parker, M.N., 2010. Territoriality and scent marking behavior of African wild dogs in northern Botswana (Ph.D.). University of Montana, United States, Montana.

Price, L.J., 2010. A preliminary study of the effects of environmental enrichment on the behaviour of captive African wild dogs (*Lycaon pictus*). Bioscience Horizons 3, 132–140. https://doi.org/10.1093/biohorizons/hzq017

Rafacz, M.L., Santymire, R.M., 2014. Using odor cues to elicit a behavioral and hormonal response in zoo-housed African wild dogs. Zoo Biology 33, 144–149. https://doi.org/10.1002/zoo.21107

Reich, A., 1981. The behavior and ecology of the African wild dog (*Lycaon Pictus*) in the Kruger national park (Ph.D.). Yale University, United States. Connecticut.

Robbins, R.L., 2000. Vocal communication in free-ranging African wild dogs (*Lycaon pictus*). Behaviour 137, 1271–1298.

Robbins, R.L., McCreery, E.K., 2003. Acoustic stimulation as a tool in African wild dog conservation. Biological Conservation 111, 263–267. https://doi.org/10.1016/S0006-3207(02)00294-X

Stanton, L.A., Sullivan, M.S., Fazio, J.M., 2015. A standardized ethogram for the *felidae*: A tool for behavioral researchers. Applied Animal Behaviour Science 173, 3–16. http://dx.doi.org/10.1016/j.applanim.2015.04.001

Stevenson, M.F., Poole, T.B., 1976. An ethogram of the common marmoset (*Calithrix jacchus jacchus*): general behavioural repertoire. Animal Behaviour 24, 428–433.

Sutherland, W.J., 1998. The importance of behavioural studies in conservation biology. Animal Behaviour 56, 801–809.

Van Heerden, J., 1981. The role of integumental glands in the social and mating behaviour of the hunting dog *Lycaon pictus* (Temminck, 1820). The Onderstepoort Journal of Veterinary Research 48, 19–21.

Walker, R.H., King, A.J., McNutt, J.W., Jordan, N.R., 2017. Sneeze to leave: African wild dogs (*Lycaon pictus*) use variable quorum thresholds facilitated by sneezes in collective decisions. Proceedings of the Royal Society B: Biological Sciences 284. https://doi.org/10.1098/rspb.2017.0347

Woodroffe, R., Sillero-Zubiri, C., 2012. *Lycaon pictus*. The IUCN red list of threatened species, e. T12436A16711116

Chapter III

Implementation of measurement techniques of Africal wild dog (*Lycaon pictus*) faecal glucocorticoid metabolites concentration

1. Abstract

Non-invasive hormone monitoring is an established methodology to wildlife studies, currently used to assess the response to perceived stressors (Palme et al., 2005; Sheriff et al., 2011; Ganswindt et al., 2012), and to understand factors influencing population dynamics (Santymire and Armstrong, 2010). This technique must be carefully validated to ensure that results reflect the physiological responsiveness of the species (Ganswindt et al., 2012; Touma and Palme, 2005) and to avoid biases related to environmental factors (Mesa-Cruz et al., 2014; Webber et al., 2018). The current study was designed to: test faecal glucocorticoid metabolites on free-ranging African wild dogs, a non-invasive method proven effective for the species (de Villiers et al., 1997; Monfort et al., 1997; Santymire and Armstrong, 2010; Vlamings, 2011); to perform an environmental validation; to develop an effective and costless field method to avoid the use of highly technical dehydration methods. Results showed that social rank and age did not play a significant role in faecal glucocorticoids metabolites (fGCM), although the infrequent collection could have played a role in the present results. Under 20 minutes post defecation, no significant effect of time was detected of fGCM concentrations, while a significant increase was evident with 57% fGCM concentration increase at 45 minutes post defecation, followed by a decrease to stable concentrations between 6 and 96 hours. Consequently, future studies should consider these results when choosing sampling methods and select a suitable post-defecation time interval. Results proved the field-friendly methods for faecal dehydration effective, avoiding the need for storing and transporting faecal material to a laboratory equipped

with a lyophiliser, and consequently reducing the associated costs. These results could potentially expand the use of fGCM EIA to remote field studies were power limitation and laboratory equipment so far used to be a logistical barrier.

2. *Introduction*

The African Wild Dogs (*Lycaon pictus*) (AWD) has been listed as Endangered from 1990, with the species being virtually eradicated from northern and western Africa, and greatly reduced in central and north-east Africa (Woodroffe and Sillero-Zubiri, 2012). The current status of the species is susceptible to slipping quickly into the Critically Endangered category (Davies-Mostert et al., 2016) as a result of threats including extreme sensitivity to habitat fragmentation (Creel and Creel, 2002; Woodroffe and Sillero-Zubiri, 2012) and heavy human competition in forms of direct persecution, road mortalities and accidental snaring (Davies-Mostert et al., 2016). Human-animal conflict has been described to be responsible for up to 93% mortality of *Lycaon* in a given population (Rasmussen and Macdonald, 2012).

Some degree of behavioural plasticity has been described for a sub-population of African wild dogs in response to anthropogenic activity, with packs responding to extreme risk with temporal shifts and scattered rather than clumped organization when at rest (Rasmussen and Macdonald, 2012), edging disruptive behavioural expressions described for packs that tend to annul (McCreery, 2000).

Considering the high level human pressure on the species, and the uncertain stressor impact of anthropogenic activity, it becomes vital to understand African wild dogs stress physiology, including potential adaptation and associated thresholds to ensure and promote the species survival (Van der Weyde et al., 2016). Prolonged exposition to stressors can influence the hypothalamic-pituitary-gonadal axis and induce long term modifications in

glucocorticoids concentrations and subsequently provoke disruptive physiological effects, including reproductive suppression, muscle atrophy and immune suppression (Ganswindt et al., 2012; Wingfield and Sapolsky, 2003). Faecal hormone analysis provides information about factors influencing wildlife population dynamics, and determination of the health status of free-ranging populations to assist with wildlife management. (Santymire and Armstrong, 2010), but in situ studies that rely on non-invasive faecal steroids assessments can be subject to biases caused by the potential changes in faecal hormones concentration cuased by fiel conditions (Mesa-Cruz et al., 2014). Glucocorticoids are metabolized in the liver and gut prior to excretion (Palme et al., 2005) and undergo a series of progressive digestions enacted by faecal bacteria enzyme even post defecation, consequently compromising the reliability of the steroid monitoring (Washburn and Millspaugh, 2002; Webber et al., 2018). Dehydration and freezing of samples proved effective to block the bacterial digestion and to avoid this bias in faecal steroid concentration (Palme et al., 2005). In numerous species, timing and degree of alteration of the fGCM concentrations have been investigated: faecal samples of males and females African elephants (*Loxodonta Africana*) showed a distinct decrease in mean fGCM concentrations after 168h, following an initial increase up to 23% for males (Webber et al., 2018). A decrease in fGCM concentrations post-defecation has been noted in brown hyaena *Parahyaena brunnea* (Hulsman et al., 2011) and Nile crocodile (Ganswindt et al., 2014). In contrast, fGCM concentrations of jaguar were proved to remain relatively stable for up to 5 days in both the wet and dry season (Mesa-Cruz et al., 2014). On baboons (*Papio ursinus*), where faecal GCM degradation was less than 10% over 30

days of storage at ambient temperature (Beehner and Whitten, 2004). Although the exact process of bacterial activity causing this alteration is still not fully understood, the need for standardizing sampling methodologies is fundamental to obtain valuable data (Touma and Palme, 2005). The current research objectives were: a) to assess adrenocortical function in African wild dogs of different sex, age classes, ranks and living in different locations; b) to determine post-defecation changes in fGCM, in unpreserved AWD faeces under natural conditions to improve best sampling practice c) to test alternative approaches to the gold standard freeze-drying methodology for AWD faecal samples for fGCM: drying under sunlight, using a solar oven, or a mechanical dehydrator.

3. Materials and methods

3.1 Study area

Sampling for the present study was conducted in two natural reserves, one in South East Botswana and one in Northern South Africa: Limpopo Lipadi Private Game and Wilderness Reserve, Tuli Block, Botswana (LL), and Khamab Nature Reserve (KNR), South Africa. LL reserve consists of a 22050 he of fully fenced Mopani savannah. The KNR consists of 100,000 hectares, fully fenced Kalahari desert landscape. According to the data disclosure policy agreed with the KNR, water distribution and landscape details will be used exclusively for the data analysis and will not be listed in the present chapter. Both reserves have electric fences with two electrical grids at the end of the fence, discouraging the AWD from leaving the

protected area. Subjects were fee-rooming and had access to the entire area; animals were never fed. Prey availability was related to prey composition and distribution, including impala (*Aepyceros melampus*) in LL, steenbok (*Raphicerus campestris*) blue wildebeest (*Connochaetes taurinus*), waterbuck (*Kobus ellipsiprymnus*) greater kudu (*Tragelaphus strepsiceros*) and potentially other animals in both reserves.

3.2 Subjects

All subjects were identified by their unique coat patterns and earmarks. LL subjects were: 2 animals classified for the porpoise of this study as Old (>5 years) (alpha female=8 years; alpha male >7 years old), ten adults (> 1 year of age), of which five males and four females. All LL African wild dogs were born in the wild and divided into two packs. The Morena pack comprised eight animals, the alpha pair and six adults born from the alpha pair. From June 2017 to December 2017 the pack was composed of eight animals: male and female alpha, five siblings from their litter of 2015 (two females and three males), and one female from a litter of 2013. In November 2017, the two youngest females dispersed. On February 14th, 2018, four unknown AWD entered the LL area and were identified with photo ID as four males and one female of unknown origin. From that moment the newly arrived dogs were included in the data sampling and monitored opportunistically. The four AWD where named Valentines pack. The two dispersal sisters form the Morena pack joined the Valentines pack between February 14th and 25th.

In KNR, subjects of the study were 61 free-ranging AWD, 18 pups born in 2018, 17 juveniles born in 2017, and 26 adults of estimated 2-5 years of age. All juveniles and pups where born in KNR, while the origin of the adults is unknown. Gender ratios, social composition and group size of African wild dogs varied per site. Pups gender was not classified and were not included in the subjects (for the pack sex-ratio and pack composition details see table 1 Chapter 2). Summary of the subjects is presented in Table 1.

3.3 Samples collection

Faecal samples were opportunistically collected from October 10, 2017 to August 23, 2018, at LL, Botswana, and from July 7, 2018, to September 9, 2018, at KNR, South Africa. Faecal material was opportunistically collected within 20 minutes post defecation when animals spontaneously moved away. When animals were in close proximity to the dropping (less than 100m) and did not leave the site for longer than 20 minutes post defecation, samples were not collected.

N=26 faecal samples were collected, N=20 from 15 known individuals (table 1) and N=6 from unknown individuals. N=7 samples were collected in KNR, and N=19 samples were collected in LL. In KNR, N=4 samples were collected from the Botswana pack, N=2 samples from the Khamab pack, and N=1 from the South pack. In LL, N=18 samples were collected from the Morena pack and N=1 from the Valentines pack. N=11 samples were collected from males, N=9 samples from females, and N=6 samples were collected from non-identified subjects (table 2).

The entire dropping was collected, placed into sealable plastic bags and immediately put on ice. Samples were frozen within two hours at -20°C until further processing. To assess the possible influence of the time interval between defecation and collection, post defecation – collection interval was recorded for 23 samples and classified into four time-intervals (T=0-5: collection occurring from 0 to 5 minutes post defecation; T=5-10 from 5 to 10 minutes; T=10-15: from 10 to 15 minutes; T=15-20 from 15 to 20 minutes).

3.4 Samples processing

Frozen samples were crushed and mixed on a cool surface without allowing the material to thaw. 5 g subsamples were taken from each faecal mass and frozen at -20°C to be lyophilised via freeze-dryer for 48h or until all moisture was removed from the samples. These samples constituted the matrix for the fGCM concentration assessment and the pre-test baseline for the dehydration experiment described below. The concentration of fGCM related to differences in age classes, rank, and study sites were tested.

3.5 Technical experiments

Two separate experiments were performed to test for a) the stability of faecal steroid concentrations post-defecation, and b) alternative approaches to drying faecal material to avoid the use of freeze drying.

3.5.1 Stability of faecal steroid concentration post-defecation experiment

To test for the potential effect of direct sunlight and exposure to highly variable temperatures on fGCM concentrations, one sample was divided into 30 equal sub-samples, randomly organised in 10 sets of triplicates. One set was immediately refrozen to constitute the baseline (T=0), and the rest were stored in plastic containers exposed to open air in direct sunlight for 0.5, 1, 2, 6, 12, 24, 48, 96, 192 hours. African wild dogs tend to defecate once a day (Creel et al., 1997) often during the morning rally (personal observations), consequently, to mimic the drying regime under natural conditions, sub-samples were placed in direct sunlight in the early morning and were left in open air both at night and during the day. Successively, for each respective time interval, triplicates were frozen at -20°C and lyophilised by freeze-drying. The sample used for the degradation experiment was collected and put on ice 15 minutes following defecation.

3.5.2 Faecal samples dehydration methods experiment

For the experiment 10 samples were thawed, mixed and divided into four aliquots. Next, sub-samples were randomly assigned to four sets to be treated with different drying methods: 1) dried under full sun (*solar*); 2) dried using a home-made *solar oven* (figure 1); 3) dried with a mechanical meat drier (*biltong dryer*) (figure 2); 4)lyophilized. Samples dried with the three alternative methods were weighed every four hours from 0700 to 1900 and considered dry when subsequent weights differed by ≤ 0.01 g. Within each treatment, samples were rotated every 4 h between 0700 and 1900 to

ensure similar exposure to heat for each sample-aliquot. Temperature was measured every 4 hours, maximum and minimum temperature for each treatment was recorded. When dried, samples were sealed and stored at room temperature (20-25 °C) until assayed.

3.5.3 fGCM extraction

Samples from the different tests were pulverised and sieved through a mesh in order to separate any undigested material (Fiess et al., 1999) Freeze-drying and grinding is considered the golden standard method for the preparation of faecal samples for fGCM quantification as it preserves the glucocorticoids (Washburn and Millspaugh, 2002). Between 0.050 and 0.055g of faecal powder (extract weight noted) was extracted by adding 3 ml of 80% ethanol in distilled water. After the suspensions were vortexed for 15 minutes, the mixture was centrifuged at 1500 x g for 10 minutes. Supernatants were saved into sealable micro-centrifuge tubes for storage at -20°C until assayed (Ganswindt et al., 2010).

3.6 Hormone analysis

Faecal steroid extracts were measured for fGCM concentrations using a competitive enzyme immunoassay (EIA), cortisol-3-CMO:BSA antibody and cortisol-3-CMO-DADOO-biotin label, previously validated for African wild dogs (Vlamings, 2011). All samples were assayed in duplicate, with nonspecific binding and blank controls in quadruplicate, a full standard curve and low and high controls in duplicate. Sensitivity of the assay was 0,6 ng/g dry weight (DW). Intra- and inter-assay coefficients of variation,

determined by repeated measurements of high- and low-quality controls ranged between 5,67 – 6,90% and 9,39-13,49%, respectively. Assay procedures followed published protocols (Webber et al., 2018) and were conducted in the Endocrine Research Laboratory, University of Pretoria, South Africa.

3.7 Statistical analysis

For the alteration of faecal steroid concentration post-defecation test, repeated measures analysis of variance (ANOVA) was used to test for differences in fGCM concentrations post-defaecation. Each time interval at which freezing occurred was considered a separate treatment. Pair-wise *t*-tests were conducted *post hoc* to identify between which of these treatments' significant differences in fGCM concentrations could be found. Simple linear regression was utilized in order to assess differences in fGCM concentrations determined for the various sampling sites. This was followed by Tukey's *post-hoc* tests conducted at 95% family-wise confidence levels. In cases of all pair-wise multiple comparison procedures, the α-level was adjusted by applying the procedure described by Holm (1979). Data for the three groups of the dehydration test (solar, solar oven, biltong drier) were analyzed independently. Each standard and treatment faecal glucocorticoid measurement for each of the four treatment was compared. Time of collection between 0 and 20 minutes post defecation was tested with repeated measures analysis of variance (ANOVA). Statistical significance for all tests was set at alpha (α) = 0.05 and significance inferred at $P<0.05$.

All statistical analyses were run using algorithms in *R* with the use of the *R Studio* interface (Fox et al., 2018).

3.8 Ethical considerations

All applicable international, national and institutional guidelines for the care and use of animals were applied during the present study. This study was conducted with the approval of the Animal Welfare Committee COBA – Università di Bologna (Prot. N. 0003606), the Ministry of Environment, Natural Resources Conservation and Tourism, Republic of Botswana (Research Permit reference: ENT 8/36/4 XXXX II (4)), as well as the permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (ACT NO 35 of 1984), issued by the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (Ref. 12/11/1/1/20 – 847).

4. Results

Faecal steroid extracts were measured for fGCM concentrations using an established EIAs for African wild dogs (Vlamings, 2011) All fGCM concentrations are expressed in ng/g of dry weight (ng/g DW).
Animals age was classified under three categories: *Yearlings*= 1 year; *Adults*= 2-5 years, *Old*= >5 years. Age classes results are reported in figure 3. fGCM concentrations did not differ significantly among age classes (Yearlings= 35,55 ± 7,45; adults= 44,65 ± 11,70; Old= 36,27 ± 16,10. Median ± SEM). fGCM did not show significant differences in relation to social rank. Overall fGCM concentrations were 28% higher for LL animals

(46,06 ± 10,68 median ± SEM) than for KNR animals (33,11 ± 5,47 median ± SEM).

To assess the influence of the exact timing of collection, time intermeddled between defecation and collection was recorded. N= 5 samples were collected at T= 0-5 (37,98 ± 4,10 median ± SEM); N=5 samples at T= 5-10 (57,55 ± 9,69 median ± SEM); N=13 at T=15-20. (37,50 ± 11,83 median ± SEM). No sample was collected at T=10-15. ANOVA revealed that collection time did not contribute to the variance of the fGCM readings (*P*=0.573) (figure 4).

4.1 Stability of faecal steroid concentration post-defecation experiment

During the experiment, the ambient temperature measured in proximity to the sub-samples ranged between 14,3 - 43,3 °C. Repeated measures ANOVA gives time as a significant predictor of change in fGCM concentrations (*P*=0.0433). The treatment induced an increase in fGCM concentration of ~57% at T=0.5 of direct sunlight exposition, from an initial median concentration of 24,83 ng/g DW at t=0, to 38,16, followed by a steady decrease from T0.5 to T=2 (22,93 ng/g DW). Concentration subsequently stabilises between 6 and 96 hours (T=6: 21,86 ng/g DW; T=96: 21,88 ng/g DW), to undergo a non-significant increase at 96 and 192 h (T=96: 21,52 ng/g DW; T=192: 28,28 ng/g DW (Figure 5).

4.1.1 Faecal samples dehydration experiment

Each sub-set of samples underwent different treatment for dehydration. 1) *Solar*: sub-samples treated with direct sunlight were thoroughly dry after 135h. The temperature ranged between 14,3 - 43,3 °C. Between 84 and 122h of treatment, these samples weight increased on an average of 0,002 ± 0,001 g during the night and subsequently decreased during the day. 2) *Solar oven*: samples were fully dry in 125h. The temperature inside the *solar oven* ranged between 14,3 - 36,8 °C. Weight increase of 0,002 ± 0,001 g was detected between subsequent weighing at night and in the morning between 60 and 108h of treatment. 3)*Biltong dryer*: samples mechanically dehydrated were fully dry in 71h. During the treatment, the temperature ranged between 29,3°C and 31,5°C.

Results for the sub-samples exposed to the drying treatments were independently compared with results obtained with the pre-test baseline. Treatments did not affect the accuracy of faecal glucocorticoid measurements in African wild dog species (Figure 6) (Freeze-dry *vs* Solar: Z=-0,866; *P*= 0,432; Freeze dry *vs* Solar oven: Z= -1,172; *P*= 0,262; Freeze-dry *vs* Biltong dryer: Z= - 1,682; *P*= 0,105).

5. Discussion

fGCM concentrations were successfully obtained using an EIA test previously validated for the African wild dog (Vlamings, 2011). Rank-related fGCM concentrations did not show significant differences, unlike results described in previous studies, were subordinates of both sexes had

lower basal fGCM concentrations than dominants (Creel et al., 1997; Creel, 2001; Creel, 2005). Alphas are considered to maintain a functional hypothalamic-pituitary-gonadal axis despite chronically elevated glucocorticoid (Creel et al., 1997; Creel, 2001; Creel and Creel, 2002). Elevated glucocorticoids in dominants were first inferred to be related with higher aggressiveness of these animals compared to subordinates (Creel, 1996), but other studies gathered better insight on the relation between aggressiveness and ranking, demonstrating that aggressive behaviours were related to ranking only during the reproductive season (Creel et al., 1997) while fGCM were higher in dominant dogs year-round (Creel, 1996; Creel and Creel, 2002). The long-term responsiveness of the adrenal axis of African wild dog is still not fully understood (Van der Weyde et al., 2016). Social group stability in primates has been linked to conflicting results regarding glucocorticoid concentrations, with phycological correlates of dominance differing in stable and unstable hierarchies (Sapolsky, 1992, 1990). A similar explanation could potentially justify for the glucocorticoids result presented here, where differences in pack stability may have interfered with the adrenocortical response, as suggested by Creel, (2005). Another possible factor could have potentially been age and related social skillfulness (de Villiers et al. 1997) that participating along with the stable pack ranking, might have resulted in lower than expected fGCM concentrations for alphas dogs of the LL Morena pack. To test for the effect of age, data from animals belonging to the Morena pack older than 5 years were tested against adults and yearlings. No significant differences were observed, but it is worth considering that faecal samples containing aggression-induced peaks in glucocorticoids metabolites may have been

missed due to infrequent sample collection. Interestingly, fGCM concentrations of yearlings, revealed a narrow variation regardless of the paucity of the data. No details regarding specifically fGCM of in-situ AWD yearlings were found in the literature, while a study including this age class conducted in captive wild dogs refers to age classes as non-significant. Further studies should focus on assessing fluctuations in fGCM concentrations of animals over the different seasons in relation to pack stability and social grouping, as well as age classes.

When comparing fGCM from the two different study sites, median concentrations were 28% higher for animals of LL compared to KNR, although statistical differences were not evident. This discrepancy could be independent of stress and be instead related to a difference in the dietary intake, a consequence of the difference in prey availability in the two study sites. Dietary intake can impact the degree of reabsorption of metabolites, time of pooling and exogenous augmentation of glucocorticoid levels (Vynne et al., 2011). Another variable to consider is the time of the year and associated temperature differences: KNR samples were collected over winter (Southern hemisphere) while LL samples were collected over the entire year. Potential seasonal variability in the stability of fGCM concentrations in the African wild dog should be further assessed.

Exposure to natural condition including direct sun or shade, can potentially influence the sample dehydration (Washburn and Millspaugh, 2002) and impact bacterial activity, responsible for the post-defecation alteration of faecal samples (Ganswindt et al., 2014; Hulsman et al., 2011; Palme et al., 2005). A short-term variation on fGCM concentration was not detected within the samples collected at 0-5, 5-10, or 15-20 minutes post defecation,

proving that scat can be collected quickly after defecation. To further investigate the stability of faecal steroid concentration post-defecation under natural conditions, a degradation experiment was planned. Our results indicate a significant change in fGCM concentrations (P=0.0433) over time. Faecal sample collected 15 minutes post defecation and exposed to direct sunlight underwent an increase of ~57% of fGCM concentrations after 30 minutes of treatment. After 6 hours of treatment, fGCM concentrations reached comparable levels determined at T=0. Respective fGCM concentrations did not change significantly between 6 and 192 h, with narrow variation between 6 and 96 hours. These results show a similar trend, but different timing compared to a test conducted by Crossey et al. (2018). These authors quantified fGCM alteration post defecation in African wild dogs under artificial conditions exposing the samples to potential degradation under controlled environment at room temperature (12-20 °C). Results showed that fGCM remained stable until 24h post defecation, with a significant increase of approximately 155% 96h post-defecation. Natural environmental conditions (e.i. exposition to UV-rays and wind) may have been responsible for the different timing in the sample degradation as a consequence of the effect of these conditions on the bacterial activity. Additionally, AWD scat used by these authors were collected under human care, where animals were presumably not fed with venison, resulting in a higher fat-concentration diet that might have affected the fGCM stability. FGMs stability post-defaecation has been studied in several mammal species, with conflicting results: for the African buffalo (*Syncerus caffer*) fGCM concentration increment has been identified between 2 and 4 hours post defecation (Ganswindt et al., 2012), and during the initial 24h period

post defecation for white-tailed deer (*Odocoileus virginianus*) (Washburn and Millspaugh, 2002). On the contrary, fGCM concentrations were found to decrease post defecation in brown hyaena (*Parahyaena brunnea*) (Hulsman et al., 2011) and Nile crocodile (Ganswindt et al., 2014), and to remain relatively stable up to 5 days in jaguar faeces (Mesa-Cruz et al., 2014), while lower variation over time was fund in sun-dried faeces of leopards (*Panthera pardus*) compared to samples left to dry in the shade (Webster et al. 2018). Further studies are needed to accurately define the potential effect of natural elements (e.i. rainfall, humidity) on fGCM alteration on African wild dog over time. Results from the degradation test should be considered for future collection protocol, where it is advisable to plan collection rather immediately (e.g. under 20 minutes) post-defecation, or between 6 and 96h post defecation.

Results of the dehydration methods experiment proved the viability of these methods over the freeze-dryer process. fGCM concentration of samples dehydrated with direct sunlight (*solar*), using a home-made *solar oven* and a mechanical meat dryer (*biltong dryer*) did not significantly differ from results obtained from lyophilised samples. The effectiveness of the three field-friendly dehydration methods can potentially expand the ability of researches to include fGCM analysis in conservation and physiology study conducted in remote areas. Depending on the logistics, future research could include fGCM assessment using direct sunlight or solar oven dehydration if electric power limitation is a concern. Alternatively, when unpredictable weather conditions (i.e. wind, rainfall) or wildlife presence could potentially compromise the solar dehydration, having a small source of power available, samples dehydration with the mechanical meat dryer (biltong

dryer) should be considered. The latter could be preferable when time limitation is present, as samples resulted dry in 71h, against the 125h and 135h of the solar oven and solar treatments.

Additionally, the use of these alternative drying methods excludes the necessity for transporting frozen material, reducing the logistical and instrumentational needs, expenses, and associated legal requirements. Dehydrated samples can be stored at room temperature, avoiding the need of unpractical and often expensive transportation of frozen material. Furthermore, a freeze-dryer is not necessarily part of the equipment of a standard laboratory.

6. Conclusions

1 – fGCM concentrations of alpha and yearling differed from previous studies, underlying the need for further investigations regarding rank, social stability and social skillfulness as potential variables for glucocorticoids concentrations in African wild dogs.

2- Based on the results of the fGCM degradation experiment, it is recommendable to standardise African wild dog faecal samples collect under 20 minutes post defecation, or between 6 and 24 hours to avoid samples degradation and consequent effects on fGCM concentrations.

3- Dehydration of faecal samples with direct sunlight, a solar oven or a mechanical meat dryer proved effective for fGCM EIA. These economic and straightforward alternatives to highly technical intense methods (lyophilisation with freeze-drying) could distinctively increase the applicability of the non-invasive hormone monitoring approach in studies on wildlife, in this case, African wild dog.

7. Tables

Table 1 – Details of identified subjects (Tot. N=15)

Reserve	Pack	Subject	Sex	Age
LL	Morena	Shrek	M	Old
LL	Morena	Two Spots	F	Old
LL	Morena	Wimpy	F	Adult
LL	Morena	Milly	F	Adult
LL	Morena	Whity	M	Adult
LL	Morena	Wacha	F	Adult
LL	Morena	Torre	M	Adult
LL	Morena	Ring	M	Adult
KNR	Khamab	Collar	M	Yearling
KNR	Khamab	Brokie	M	Yearling
KNR	Bots	Red Bawdm4	M	Adult
KNR	Bots	Alpha	F	Adult
KNR	South	Alpha	F	Adult
KNR	Bots	Bawdf6	F	Yearling
KNR	Bots	Bawdf10	M	Yearling

Table 2 - Samples collected from identified subjects (Tot. N=20) with details regarding origin, sex and age of subjects.

	KNR	LL
Females	**3**	**6**
Adult	2	3
Old		3
Yearling	1	
Males	**4**	**7**
Adult	1	6
Old		1
Yearling	3	

Figure 1 – solar oven. Solar oven was built on a base of card-board boxes covered with tin foil to achieve maximum sunlight intensification. Samples were placed in the inner compartment and left in the solar oven until dry.

Figure 2 – biltong dryer – samples were placed in a mechanical meat drier on the central compartment. The meat drier is equipped with a heating element, a fan, and air vent at the bottom.

Figure 3 – box plot for fGCM concentrations related to age classes.

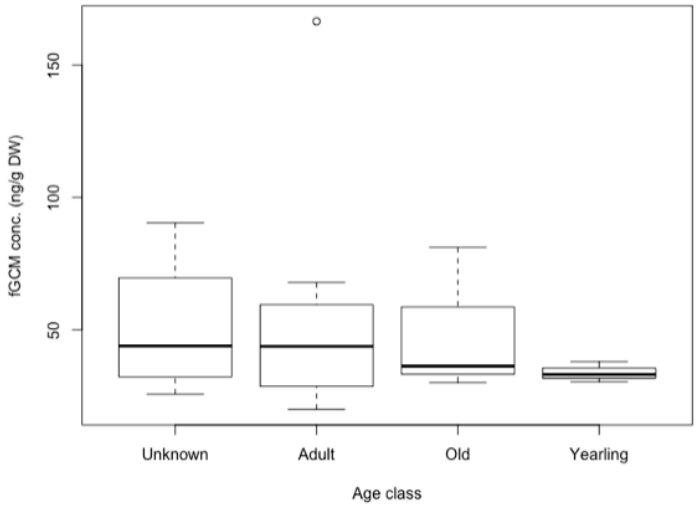


Figure 4 - Box plot for fGCM concentrations at time intervals between defecation and collection (0 to 5, 5 to 10, 15 to 20 minutes)

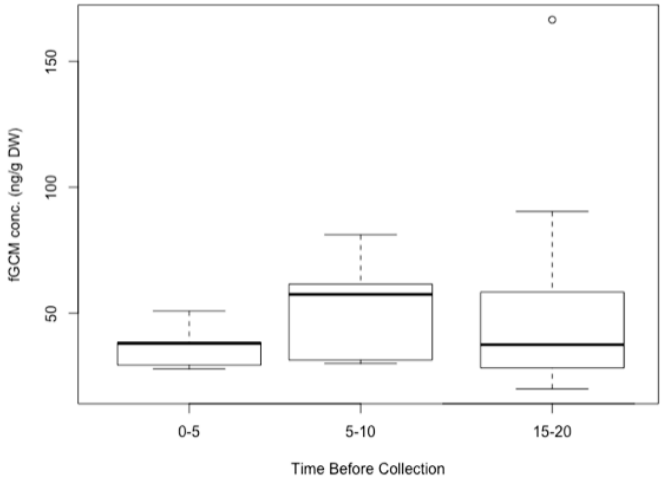


Figure 5 – average absolute change (%) in mean faecal glucocorticoid metabolite (ng/g) concentrations of faecal sample stored in open air under direct sunlight at time intervals from 0 to 192 hours.

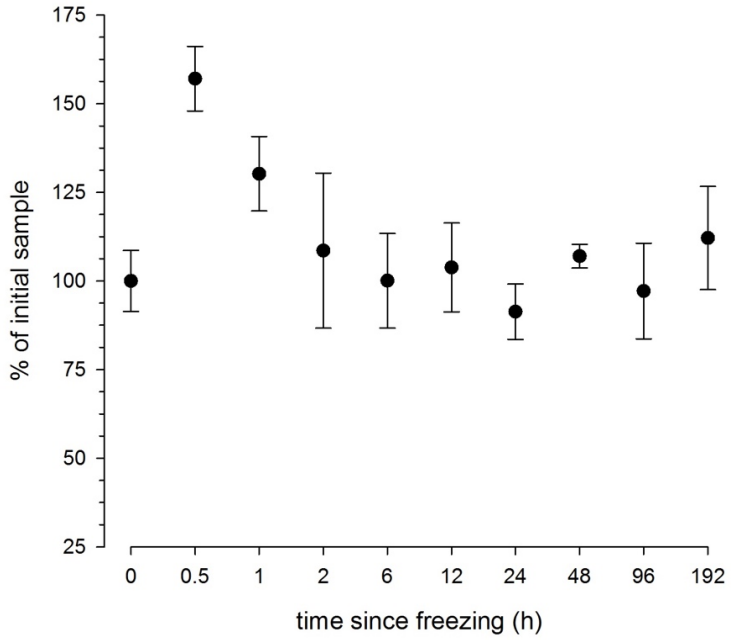
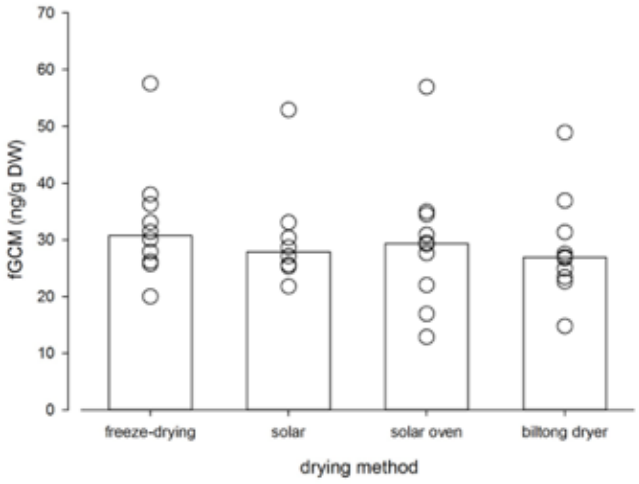


Figure 6 - faecal glucocorticoid metabolite (ng/g DW) concentrations of four sub-groups of faecal samples treated with different dehydration methods: freeze-drying, solar, solar oven, biltong-mechanical dryer. The treatments did not affect the accuracy of faecal glucocorticoid measures in African wild dog scat samples.



8. *References*

Beehner, J., Whitten, P., 2004. Modifications of a field method for faecal steroid analysis in baboons. Physiology & Behavior 82, 269–277. https://doi.org/10.1016/j.physbeh.2004.03.012

Creel, S., 2005. Dominance, aggression, and glucocorticoid levels in social carnivores. Journal of Mammalogy 86, 255–264.

Creel, S., 2001. Social dominance and stress hormones. Trends in Ecology & Evolution 16, 491–497. https://doi.org/10.1016/S0169-5347(01)02227-3

Creel, S., 1996. Conserving wild dogs. Trends in Ecology & Evolution 11, 337. https://doi.org/10.1016/0169-5347(96)81137-2

Creel, S., Creel, N.M., 2002. The African wild dog: behaviour, ecology and conservation. Princeton University Press. https://doi.org/10.1016/S0169-5347(02)00039-3

Creel, S., Creel, N.M., Mills, M.G.L., Monfort, S.L., 1997. Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. Behavioural Ecology 8, 298–306.

Crossey, B., Ganswindt, A., Chimimba, C.T., 2018. Faecal glucocorticoid metabolite concentrations and their alteration post-defaecation in African wild dogs (*Lycaon pictus*) from South Africa. Wildlife Biology 2018.1

Davies-Mostert, H.T., Page-Nicholson, S., Marneweck, K., Marnewick, D.G., Cilliers, D., Whittington-Jones, B., Killian, M.G.L., Parker, D., Power, J., Rehse, T., Child, M.F., 2016. A conservation assessment of *Lycaon pictus*. In: The Red List of Mammals of South Africa, Swaziland and Lesotho.

South African National Biodiversity Institute and Endangered Wildlife Trust, South Africa.

de Villiers, M.S., van Jaarsveld, A.S., Meltzer, D.G.A., Richardson, P.R.K., 1997. Social Dynamics and the Cortisol Response to Immobilization Stress of the African Wild Dog, *Lycaon pictus*. Hormones and behavior 31, 3–14.

Fiess, M., Heistermann, M., Hodges, J.K., 1999. Patterns of urinary and faecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). General and Comparative Endocrinology 115, 76–89. https://doi.org/10.1006/gcen.1999.7287

Fox, J., Weisberg, S., Friendly, M., Hong, J., Andersen, R., Firth, D., Taylor, S., R Core Team, 2018. Effects package: Effect displays for linear, generalized linear, and other models.

Ganswindt, A., Münscher, S., Henley, M., Palme, R., Thompson, P., Bertschinger, H., 2010. Concentrations of faecal glucocorticoid metabolites in physically injured free-ranging African elephants *Loxodonta africana*. Wildlife Biology 16, 323–332. https://doi.org/10.2981/09-081

Ganswindt, A., Tordiffe, A.S.W., Stam, E., Howitt, M.J., Jori, F., 2012. Determining adrenocortical activity as a measure of stress in African buffalo (*Syncerus caffer*) based on faecal analysis. African Zoology 47, 261–296.

Ganswindt, S.B., Myburgh, J.G., Cameron, E.Z., Ganswindt, A., 2014. Non-invasive assessment of adrenocortical function in captive Nile crocodiles (*Crocodylus niloticus*). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology 177, 11–17. https://doi.org/10.1016/j.cbpa.2014.07.013

Holm, S., 1979. A simple sequentially rejective multiple test procedure. Scandinavian Journal of Statistics 6, 65–70.

Hulsman, A., Dalerum, F., Ganswindt, A., Muenscher, S., Bertschinger, H.J., Paris, M., 2011. Non-invasive monitoring of glucocorticoid metabolites in brown hyaena (*Hyaena brunnea*) feces. Zoo Biology 30, 451–458. https://doi.org/10.1002/zoo.20325

McCreery, E.K., 2000. Spatial relationships as an indicator of successful pack formation in free-ranging African wild dogs. Behaviour 137, 579–590. https://doi.org/10.1163/156853900502222

Mesa-Cruz, J.B., Brown, J.L., Kelly, M.J., 2014. Effects of natural environmental conditions on faecal glucocorticoid metabolite concentrations in jaguars (*Panthera onca*) in Belize. Conservation Physiology 2, cou039–cou039. https://doi.org/10.1093/conphys/cou039

Monfort, S.L., Wasser, S.K., Mashburn, K.L., Burke, M., Brewer, B.A., Creel, S.R., 1997. Steroid metabolism and validation of noninvasive endocrine monitoring in the African wild dog (*Lycaon pictus*). Zoo Biology 16, 533–548. https://doi.org/10.1002/(SICI)1098-2361(1997)16:6<533::AID-ZOO6>3.0.CO;2-B

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S.M., Möstl, E., 2005. Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in faecal samples. Annals of the New York Academy of Sciences 1040, 162–171. https://doi.org/10.1196/annals.1327.021

Rasmussen, G.S.A., Macdonald, D.W., 2012. Masking of the zeitgeber: African wild dogs mitigate persecution by balancing time. Journal of Zoology 286, 232–242. https://doi.org/10.1111/j.1469-7998.2011.00874.x

Santymire, R.M., Armstrong, D.M., 2010. Development of a field-friendly technique for faecal steroid extraction and storage using the African wild dog (*Lycaon pictus*). Zoo Biology 29, 289–302. https://doi.org/10.1002/zoo.20248

Sapolsky, R.M., 1992. Stress, the aging brain and the mechanism of neuron death. MA: MIT Press., Cambridge.

Sapolsky, R.M., 1990. Adrenocortical function, social rank and personality among wild baboons. Biological Psychiatry 28, 862–878.

Sheriff, M.J., Dantzer, B., Delehanty, B., 2011. Measuring stress in wildlife: techniques for quantifying glucocorticoids. Oecologia 166, 869–887. https://doi.org/0.1007/s00442-011-1943-y

Touma, C., Palme, R., 2005. Measuring faecal glucocorticoid metabolites in mammals and birds: the importance of validation. Annals of the New York Academy of Sciences 1046, 54–74. https://doi.org/10.1196/annals.1343.006

Van der Weyde, L.K., Martin, G.B., Paris, M.C.J., 2016. Monitoring stress in captive and free-ranging African wild dogs (*Lycaon pictus*) using faecal glucocorticoid metabolites. General and Comparative Endocrinology 226, 50–55. https://doi.org/10.1016/j.ygcen.2015.12.022

Vlamings, B.H.A.C., 2011. Dog appeasing pheromone®: A useful tool to minimize stress and aggression of African wild dogs (*Lycaon pictus*)? (M.S.) Graduate School of Life Sciences, University of Utrecht, the Netherlands.

Vynne, C., Baker, M.R., Breuer, Z.K., Wasser, S.K., 2011. Factors influencing degradation of DNA and hormones in maned wolf scat. Animal Conservation 1–11. https://doi.org/10.1111/j.1469-1795.2011.00503.x

Washburn, B.E., Millspaugh, J.J., 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. General and Comparative Endocrinology 127, 217–222. https://doi.org/10.1016/S0016-6480(02)00056-4

Webber, J.T., Henley, M.D., Pretorius, Y., Somers, M.J., Ganswindt, A., 2018. Changes in African Elephant (*Loxodonta africana*) faecal steroid concentrations post-defaecation. Bothalia - African Biodiversity & Conservsation. https://doi.org/10.4102/abc.v48i2.2312

Wingfield, J.C., Sapolsky, R.M., 2003. Reproduction and resistance to stress: when and how. Journal of Neuroendocrinology 15, 711–724. https://doi.org/10.1046/j.1365-2826.2003.01033.x

Woodroffe, R., Sillero-Zubiri, C., 2012. *Lycaon pictus* (No. e. T12436A16711116). The IUCN red list of threatened species.

Chapter IV

Implementation of measurement techniques of African wild dog (*Lycaon pictus*) faecal reproductive steroids concentration

1. *Introduction*

The evaluation of the reproductive status of wild species using non-invasive methodologies has a number of applications to conservation and management, both in captivity and in the wild. In captivity, monitoring ovarian cycles facilitates scheduling access to males, evaluating the reproductive potential, and implementing assisted reproductive technologies such as artificial insemination (Fiess et al., 1999). In the wild, the possibility of understanding the reproductive endocrine status of a species provides a physiological valuation. This information is invaluable to support the long term conservation efforts, as they allow for assessing impacts of social disruption caused by anthropogenic factors (Fiess et al., 1999), as well as recognising the relationships between behavioural and endocrinological variables influencing reproductive fitness and success (Monfort et al., 1997). The same information may also be of considerable importance in evaluating the effectiveness of fertility control measures (Fiess et al., 1999) such as hormonal implants, used in response to the urging need to mitigate the number of African wild dog dispersal groups outside protected areas (see chapter 6).

African wild dogs are considered mono-oestrous seasonal breeders (Monfort et al., 1997; Newell-Fugate et al., 2012; Van der Weyde et al., 2015), with breeding mostly restricted to the dominant pair and suppressed in subordinates (Monfort et al., 1997; Creel, 2001; Creel and Creel, 2002). The mechanism by which reproduction is inhibited in subordinates has been debated at length: it is still not fully clear whether dominant animals limit reproduction of subordinates by inhibiting reproductive behaviour or by physiologically suppressing the ovulatory cycles, but behavioural

suppression is considered likely (Van den Berghe et al., 2012; Van der Weyde et al., 2015). Physiological validation for faecal androgen metabolites (fAM) and faecal progestagens metabolites (fPM) quantification have been previously proven effective in African wild dog (Monfort et al., 1997), providing a means for characterizing longitudinal reproductive rhythms in males and females. To compare progestogen concentrations among studies can be challenging due to differences in the assay used and methods of collection (Newell-Fugate et al., 2012).

fPM have been assessed for both pseudo-pregnant and pregnant females, fPM concentrations were described to rise steadily from anoestrus to dioestrus in both pseudo-pregnant and acyclic females, with pseudo-pregnant females having significantly higher progestogen concentrations than acyclic females in every phase of the cycle (Van der Weyde et al., 2015). One pregnancy was monitored and a single pregnant female showing a pre-ovulatory surge in progestagens after a peak in oestradiol, coinciding with declining oestrogen concentrations, similar to what Monfort et al. (1997) described.

The use of fPM quantification for pregnancy diagnosis in African wild dogs has been tested only on small sample sizes, where significant differences between one single pregnant and pseudo-pregnant females were not detected, suggesting that assessment of fPM may not be useful for pregnancy diagnosis (Monfort et al., 1997; Van der Weyde et al., 2015). fPM ranges need further investigation: in two studies deploying RIA, non-pregnant luteal FP concentrations were 400 fold higher (Newell-Fugate et al., 2012) than non-pregnant luteal FP concentrations reported for the one other female African wild dog regularly followed (Monfort et al., 1997).

Before non-invasive methods of measuring steroids may be utilized to their full potential, basic field sampling protocols need to be developed to avoid sampling biases or unreliable results (Washburn and Millspaugh, 2002). Faecal bacteria enzymes can alter metabolite concentrations post-defaecation, thus compromising the reliability of steroid monitoring if using non-fresh samples (Millspaugh and Washburn, 2004; Washburn and Millspaugh, 2002; Webber et al., 2018). Validation of collection, storage, processing techniques as well as assays is needed to ensure reliable results in analyzing faecal steroid concentration (Webber et al., 2018). The present study aims at 1) assess the effect of natural conditions over time for fPM and fAM concentrations to develop sampling protocol 2) to test alternative drying methods to freeze drying.

2. *Materials and methods*

o *Study animals and samples collection*

We collected 29 faecal samples from 25 African wild dogs (table 1). All adults and yearlings were wild and housed in natural reserves, in Botswana and South Africa. (see chapter 2, Study area, for details on the location of study). We collected faeces opportunistically upon defecation from identified and unidentified animals. Samples were collected from October 10, 2017 to August 23, 2018, at LL, in Botswana, and from July 7, 2018, to September 9, 2018, at KNR, in South Africa. Faecal material was opportunistically collected within 20 minutes post-defecation when animals spontaneously moved away. When animals were in close proximity to the dropping (less than 100 m) and did not leave the site for longer than 20 minutes post-defecation, samples were not collected. Post defecation to collection time intervals were noted. The entire dropping was collected and placed into sealable plastic bags. All samples were immediately put on ice and subsequently frozen within two hours at -20°C until further processing. At the time of analysis, frozen samples were crushed and mixed on a cool surface without allowing the material to thaw. Subsamples of 5g were taken from each faecal mass and frozen at -20°C to be lyophilised for 48h or until all moisture was removed from the samples. These samples constituted the matrix for the fAM and fPM concentration assessment and the pre-test baseline. Next, we evenly allocated the remaining faecal mass into 5 - 10g sub-samples.

- Stability of faecal steroid concentration post-defecation

  To assess the possible influence of the timing of collection on fAM and fPM concentrations, time intervals between defecation and collection were recorded. Samples were collected between 0 and 20 minutes post defecation.

- Stability of faecal steroid concentration post-defecation experiment

  To test for the potential effect of direct sunlight and exposure to highly variable temperatures on steroids concentrations, one sample was divided into 30 equal sub-samples and randomly organised into 10 sets of triplicates. One set was immediately refrozen to constitute the baseline (t=0), and the rest were stored in plastic containers exposed to open air in direct sunlight for time intervals of 0.5, 1, 2, 6, 12, 24, 48, 96 and 192 hours respectively. African wild dogs tend to defecate once a day (Creel et al., 1997), often during the morning rally (personal observations). Consequently, to mimic the drying regime under natural conditions, sub-samples were placed in direct sunlight in the early morning and were left in open air both at night and during the day. During the experiment, the ambient temperatures measured in proximity to the sub-samples ranged between 14,3 - 43,3 °C. Triplicates were frozen at -20°C and lyophilised by freeze-drying for each respective time interval. The sample used for the degradation experiment was collected and put on ice 15 minutes following defecation.

- Faecal samples dehydration methods experiment

  For the experiment, 10 samples were thawed, mixed and divided into four aliquots. Next, sub-samples were randomly assigned to three sets to be

treated with different drying methods; 1) dried under direct sunlight (solar); 2) dried using a home-made solar oven (figure 1) ;3) dried with a mechanical meat drier (biltong dryer) (figure 2). Samples dried with the three alternative methods were weighed every four hours from 07h00 to 19h00 and were considered dry when subsequent weights differed by ≤ 0.01 g. Within each treatment, samples were rotated every 4 h between 07h00 and 19h00 to ensure similar exposure to heat for each sample-aliquot. The temperature was measured every 4 hours, the maximum and minimum temperature for each treatment were recorded. Temperatures ranged between 14,3 - 43,3 °C for solar treatment, in the *solar oven* between 14,3 - 36,8 °C, and between 29,3°C and 31,5°C in the *biltong dryer*. When dried, samples were sealed and stored at room temperature (20-25 °C) until further analysis.

- fAM and fPM extraction

Samples from the different tests were pulverised and sieved through mesh in order to separate any undigested material (Fiess et al., 1999). Freeze-drying and grinding is considered the golden standard method for the preparation of faecal samples for fAM and fPM quantification as it preserves the glucocorticoids (Webber et al., 2018). Between 0.050 and 0.055g of faecal powder (extract weight noted) was extracted by adding 3 ml of 80% ethanol in distilled water. After the suspensions were vortexed for 15 minutes, the mixture was centrifuged at 1500 x g for 10 minutes. Supernatants were saved into sealable micro-centrifuge tubes for storage at -20°C until assayed (Ganswindt et al., 2010).

- Hormone analysis

Faecal steroid extracts were measured for fAM and fPM concentrations using a competitive enzyme immunoassay (EIA). All samples were assayed in duplicate, with nonspecific binding and blank controls in quadruplicate, a full standard curve and low and high controls in duplicate. Sensitivity of the assay was 4.8 ng/g dry faecal weight for fAM EIA, and 19.2 ng/g dry faecal weight for the fPM EIA. Intra-assay coefficients of variance ranged from 5.67% – 6.90% for the fAM EIA, and 5.96% - 6.53% for the fPM EIA.

The coefficients of variance for inter-assay variance ranged from 9.39% – 13.49% for the fGCM EIA, 14.03% - 14.90% for the fAM EIA, and 14.49% - 15.81% for the fPM EIA. Assay procedures followed published protocols (Crossey et al., 2018; Webber et al., 2018) and were conducted at the Endocrine Research Laboratory, University of Pretoria, South Africa.

- Data analysis

The stability of faecal steroid concentration post-defecation was calculated using the mean steroid concentration determined at t=0 as 100%. Differences in steroid concentration between samples stored at t=0h and 0.5–192 h post-defaecation were examined descriptively as well as by one-way repeated measures ANOVA, followed by post hoc analysis using a Bonferroni t-test, with the application of the Bonferroni correction. Differences in steroid concentrations between drying treatments were examined by Kruskal– Wallis one-way ANOVA on ranks followed by post hoc pairwise comparisons using Dunn's method. The statistical analyses

were performed using the software programme Sigma Plot 12.5. Statistical significance was assumed when $P < 0.05$. Data are presented as means ± SD, medians and ranges were applicable.

- Ethical considerations

All applicable international, national and institutional guidelines for the care and use of animals were applied during the present study. This study was conducted with the approval of the Animal Welfare Committee COBA – Università di Bologna (Prot. N. 0003606), the Ministry of Environment, Natural Resources Conservation and Tourism, Republic of Botswana (Research Permit reference: ENT 8/36/4 XXXX II (4)),as well as the permission to do research in terms of Section 20 of the Animal Diseases Act, 1984 (ACT NO 35 of 1984), issued by the Department of Agriculture, Forestry and Fisheries, Republic of South Africa (Ref. 12/11/1/1/20 – 847).

3. Results

- Stability of faecal steroid concentration post-defecation

To assess the possible influence of the timing of collection on fAM and fPM concentrations, time intervals between defecation and collection were recorded. Collection time did not contribute significantly to variance in fAM concentrations ($R^2 = 0,1628$) (figure 2) nor in fPM ($R^2 = 0,1324$) (figure 3).

- Stability of faecal steroid concentration post-defecation experiment

A repeated measures ANOVA determined time as a significant predictor of change for fAM (P =0.0096) but not for fPM concentrations (P =0.3836). Figure 3 and figure 4 show fAM and fPM median concentrations expressed in µg/g of dry weight (µg/g DW) ±SE. fAM median concentration at t=0 was 0,32 µg/g DW, exposure to direct sunlight induced an increase in fAM concentration of ~35% at t=0.5 (0,43 µg/g DW) and ~52% at t=1 (0,48 µg/g DW). This was followed by a decrease in fAM concentrations from t=1 to t=6 (0,29 µg/g DW). Measured fAM concentrations underwent a non-significant increase between t=6 and t=24 (t=24: 0,37 µg/g DW); and resulted stabile between t=24 and t=96 (0,35 µg/g DW).

fPM median concentration at t=0 was 5,66 µg/g DW, exposure to direct sunlight induced a steady increase in fAM concentration of ~43% at t=1 (8,08 µg/g DW). This was followed by a decrease in fAM concentrations from t=1 to t=6 (5,46 µg/g DW). Measured fAM concentrations underwent a non-significant increase between t=6 and t=24 (t=24: 5,35 µg/g DW).

- Faecal samples dehydration experiment

Drying times under different regimes

The time to reach complete dryness varied for samples within subsets. 1) Solar: sub-samples placed in direct sunlight were determined to be thoroughly dry after 135h, at temperatures ranging between 14,3 - 43,3 °C. Between 84 and 122h of treatment, the weight of these samples increased on average 0,002± 0,001g during the night and subsequently

decreased during the day. 2) Solar oven: samples were determined to be fully dry in 125h. Temperatures inside the solar oven ranged between 14,3 - 36,8 °C throughout the course of the treatment. A weight increase of 0,002 ± 0,001 g was detected between subsequent weighing at night and in the morning between 60 and 108h of treatment. 3) Biltong dryer: samples mechanically dehydrated were fully dry in 71h. During the treatment, temperatures to which the samples were exposed ranged between 29,3°C and 31,5°C.

Results for the sub-samples exposed to the drying treatments were independently compared with results obtained with the pre-test baseline. Treatments affected the accuracy of fAM (figure 5) but did not affect fPM (figure 6) measurements in African wild dog faecal samples.

4. Discussion

fAM and fPM concentrations were successfully obtained using an EIA test previously validated for African wild dog (Vlamings, 2011). The time interval between deposition and collection (from 0 to 20 minutes pos defecation) was not related to variance in fAM and fPM concentration, indicating the possibility of collecting samples under 20 minutes post defecation. Stability of faecal steroid concentration post-defecation was tested to assess the effect of natural condition over time on faecal samples. For faecal matter to dry under natural conditions, numerous elements play a role in determining the duration until it is fully dry. Size, texture and water content of the sample, the local weather conditions, the number of hours the material is exposed to sunlight and the presence of rain need to be

considered. Consequently, a longer drying time provides bacteria more time to alter the steroid composition in the faecal matrix (Webber et al., 2018).

Our results showed that fAM concentrations in freeze-dried African wild dog were comparable with concentrations obtained from samples dehydrated in direct sunlight (solar), in solar oven, and using a mechanical meat dryer (biltong).

5. *Conclusions*

The present study identified correct time intervals for faecal collection in relation to time interval post defecation. Future studies should consider these results when choosing appropriate sampling methods to avoid biases due to samples degradation potentially related to dehydration and bacterial activity.

Results showed the potential of using alternative methods to avoid the need of keeping samples frozen until transportation to a laboratory equipped with a freeze-dryer. Furthermore, the use of this alternative methods allows for expansion of research in remote areas with limited access to electricity and consequently, reduced capacity of storying samples at -20°C, as required by the golden standard freeze-drying method. Transportation of frozen samples tends to be an additional limitation for field research, especially when studying animals in remote areas. The possibility of dehydrating samples on site with these field friendly method should be considered and will potentially boost the effort toward better understanding of this species under natural habitat, promoting conservation and ensuring the important knowledge on the species.

6. *Tables*

Table 1: Origin of faecal samples

	Adult	Pup	Yearling	Tot
Female	8	--	1	9
Male	8	3	3	14
Unknown	6	--	--	6
Total	22	3	4	29

Figure 1: fAM concentration related to time of collection

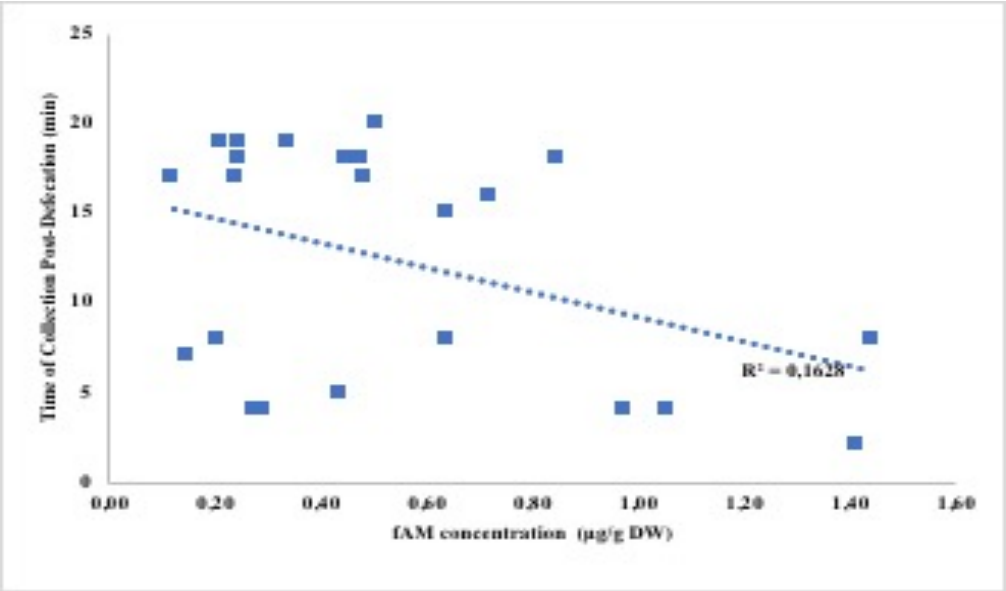


Figure 2: fPM concentration related to time of collection

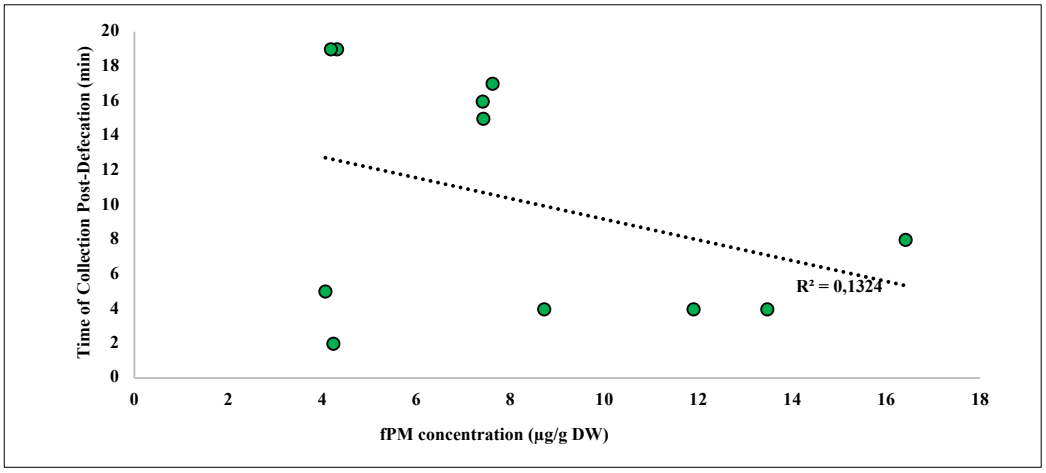


Figure 3: fAM concentration degradation over time

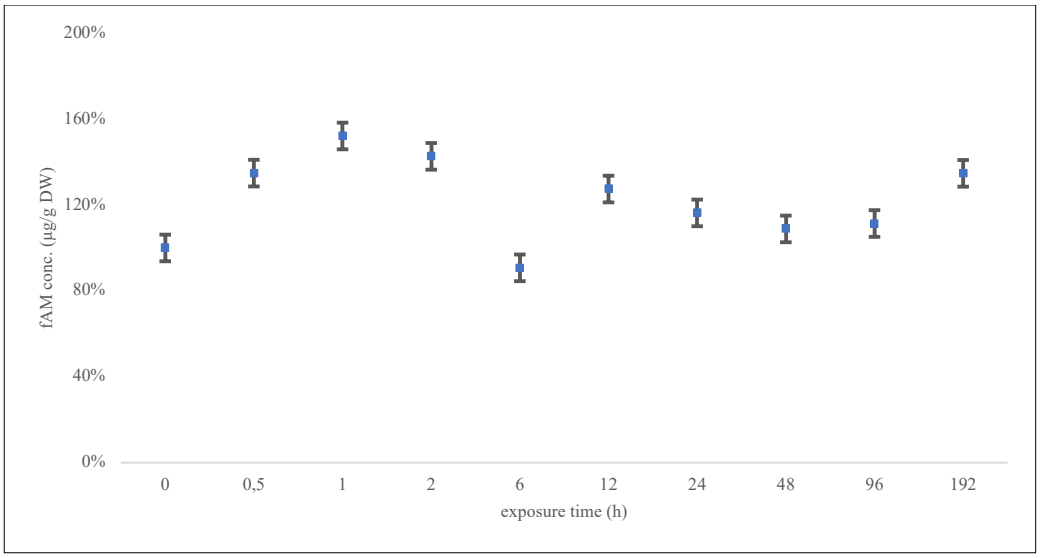


Figure 4: fPM concentration degradation over time

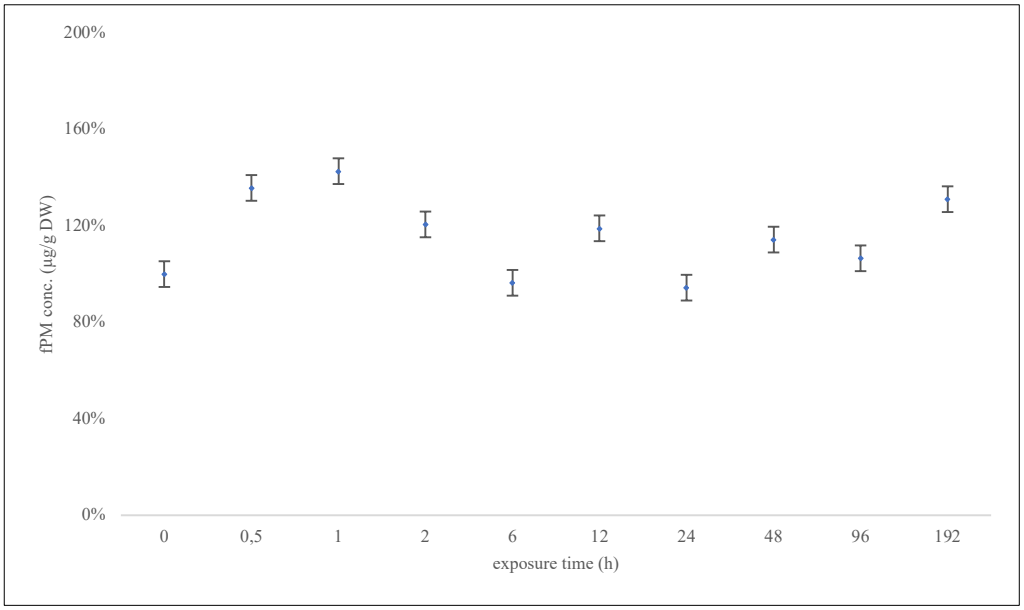


Figure 5:fAM concentrations dehydration experiment

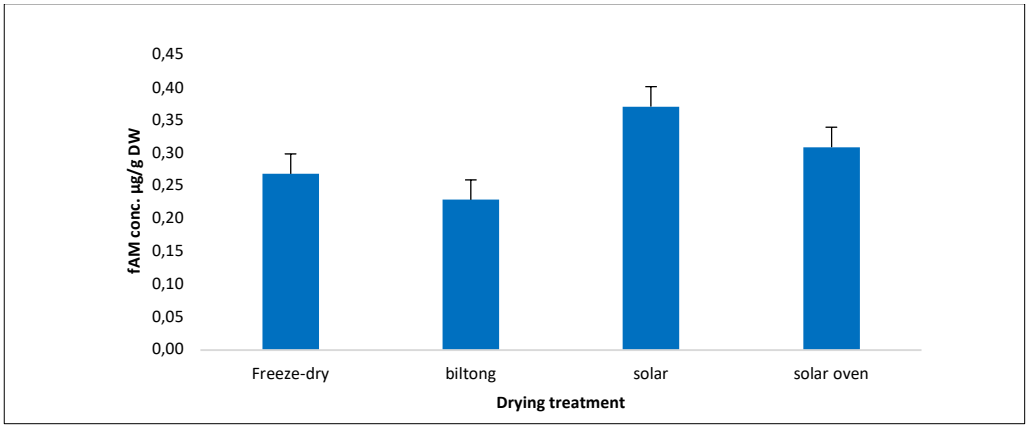
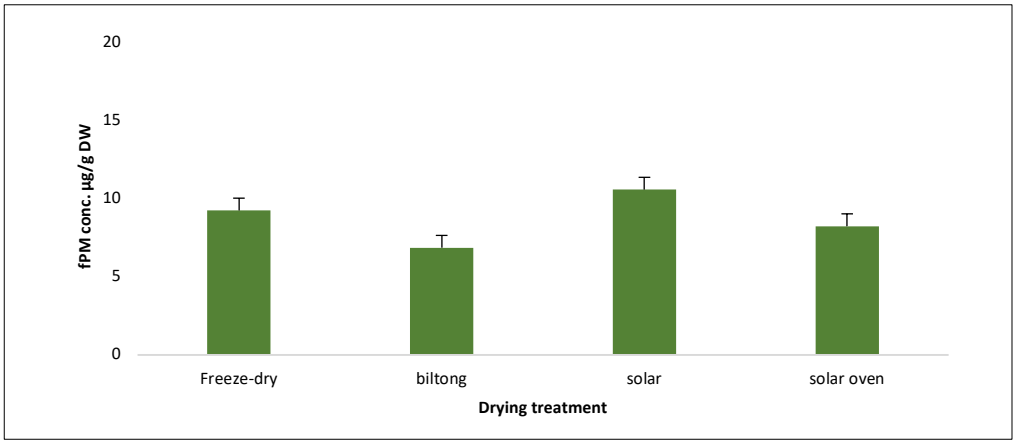


Figure 6: fPM concentrations dehydration experiment



7. *References*

Creel, S., 2001. Social dominance and stress hormones. Trends in Ecology & Evolution 16, 491–497. https://doi.org/10.1016/S0169-5347(01)02227-3

Creel, S., Creel, N.M., 2002. The African wild dog: behaviour, ecology and conservation. Princeton University Press. https://doi.org/10.1016/S0169-5347(02)00039-3

Creel, S., Creel, N.M., Mills, M.G.L., Monfort, S.L., 1997. Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. Behavioural Ecology 8, 298–306.

Crossey, B., Ganswindt, A., Chimimba, C.T. 2018. Faecal glucocorticoid metabolite concentrations and their alteration post-defaecation in African wild dogs (*Lycaon pictus*) from South Africa. Wildlife Bioligy 2018,1.

Fiess, M., Heistermann, M., Hodges, J.K., 1999. Patterns of urinary and faecal steroid excretion during the ovarian cycle and pregnancy in the African elephant (*Loxodonta africana*). General and Comparative Endocrinology 115, 76–89. https://doi.org/10.1006/gcen.1999.7287

Ganswindt, A., Münscher, S., Henley, M., Palme, R., Thompson, P., Bertschinger, H., 2010. Concentrations of faecal glucocorticoid metabolites in physically injured free-ranging African elephants *Loxodonta africana*. Wildlife Biology 16, 323–332. https://doi.org/10.2981/09-081

Millspaugh, J.J., Washburn, B.E., 2004. Use of faecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation. General and Comparative Endocrinology 138, 189–199. https://doi.org/10.1016/j.ygcen.2004.07.002

Monfort, S.L., Wasser, S.K., Mashburn, K.L., Burke, M., Brewer, B.A., Creel, S.R., 1997. Steroid metabolism and validation of noninvasive endocrine monitoring in the African wild dog (*Lycaon pictus*). Zoo Biology 16, 533–548. https://doi.org/10.1002/(SICI)1098-2361(1997)16:6<533::AID-ZOO6>3.0.CO;2-B

Newell-Fugate, A.E., O. Nöthling, J., J. Bertschinger, H., 2012. Seasonal changes in steroid hormone profiles, body weight, semen quality, and the reproductive tract in captive African wild dogs (*Lycaon pictus*) in South Africa. General and Comparative Endocrinology 178, 272–281. https://doi.org/10.1016/j.ygcen.2012.05.008

Van den Berghe, F., Paris, D.B.B.P., Van Soom, A., Rijsselaere, T., Van der Weyde, L., Bertschinger, H.J., Paris, M.C.J., 2012. Reproduction in the endangered African wild dog: Basic physiology, reproductive suppression and possible benefits of artificial insemination. Animal Reproduction Science 133, 1–9. https://doi.org/10.1016/j.anireprosci.2012.06.003

Van der Weyde, L.K., Martin, G.B., Blackberry, M.A., Gruen, V., Harland, A., Paris, M.C.J., 2015. Reproductive hormonal patterns in pregnant, pseudopregnant and acyclic captive African wild dogs (*Lycaon pictus*). Animal Reproduction Science 156, 75–82. https://doi.org/10.1016/j.anireprosci.2015.03.003

Vlamings, B.H.A.C., 2011. Dog appeasing pheromone®: A useful tool to minimize stress and aggression of African wild dogs (Lycaon pictus)? (Prestigious Master: Neuroscience and Cognition). Graduate School of Life Sciences, University of Utrecht, Netherlands.

Washburn, B.E., Millspaugh, J.J., 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. General and Comparative Endocrinology 127, 217–222. https://doi.org/10.1016/S0016-6480(02)00056-4

Webber, J.T., Henley, M.D., Pretorius, Y., Somers, M.J., Ganswindt, A., 2018. Changes in African Elephant (*Loxodonta africana*) faecal steroid concentrations post-defaecation. Bothalia - African Biodiversity & Conservsation. https://doi.org/10.4102/abc.v48i2.2312

Chapter V

Efficacy of Deslorelin (Suprelorin, Virbac) implant as contraceptive measure for free ranging African wild dog

1. *Abstract*

Wild dogs are social canids with complex social structure, forming packs with a nearly obligate cooperative breeding system. Within South Africa, small and isolated populations are managed collectively as a metapopulation, involving reintroductions through the translocation of unrelated opposite-sex groups. The metapopulation project succeeded in their goal of creating a self-sustained metapopulation in South Africa outside Kruger National Park. In order to continue the range expansion of the national metapopulation, the strategy has necessitated the introduction of wild dogs to smaller reserves, where wild dogs have demonstrated a capacity to produce and sustain litters that result in pack sizes which in some cases, put excessive pressure on the prey resources. Due to the limited number of reserves in the metapopulation, contraceptive intervention is considered as a last-resort option to control managed population levels. The GnRH agonists Deslorelin (Suprelorin®, Virbac) has been used as in captivity it was proven to fulfil the temporary and reversible criteria required of a wild dog contraceptive. Despite understanding the potential usefulness of Deslorelin, guidelines are unavailable for managers implementing reintroductions and fertility control in this species under wild conditions. The main goal of the research is to determine the usefulness of reversible hormonal contraception to control the breeding rate of African wild dogs, using historical data collected by the KwaZulu-Natal Wild Dog Advisory Group. We were able to extract sufficient data for 16 Deslorelin implant use over 9 years (2008– 2017). Deslorelin implant success rate corresponded to 87,5%. In at least 6 trials, animals expressed mating

behaviours during the next reproductive season. Results showed that time interval between the implant and the upcoming reproductive season played a role in the efficacy of the contraception. All packs followed after the implant proved to have returned to their natural reproductive cycling and sired offspring, proving that reversibility with accompanying good fertility is ensured when the contraception warns off.

2. *Introduction*

Wild dogs are social canids with complex social structure, forming packs with a nearly obligate cooperative breeding system (Creel and Creel, 2002). Larger packs tend to have higher survival rates due to efficiency in hunting, raising pups and avoiding threats (Davies et al., 2016; Rasmussen and Macdonald, 2012). Within South Africa, small and isolated populations are managed collectively as a metapopulation (Marneweck et al., 2019). The metapopulation strategy involves the reintroduction of wild dogs through the translocation of unrelated opposite-sex groups to a selected release site. Upon arrival on the reintroduction area, wild dogs are kept in a pre-release enclosure to facilitate social integration of the two unrelated, opposite-sex groups, before release into the new area. Following this acclimation period, animals are ultimately released together as a newly formed pack (Gusset and Macdonald, 2010). This approach mimics natural dispersal processes (Creel and Creel, 2002).

The managed metapopulation was developed in South Africa because the lack of large contiguous patches of suitable habitat reduces the dispersal of wild dogs through unprotected areas. This method has been successful in increasing the wild dog population since its implementation in 1998

(Davies-Mostert et al., 2015; Marneweck et al., 2019), improving the status of this endangered species in southern Africa. The project succeeded in their goal of creating a self-sustained metapopulation in South Africa to complement the Kruger National Park population. In order to continue the range expansion of the national metapopulation, the strategy has necessitated the introduction of wild dogs to smaller private and state-owned reserves, where wild dogs have demonstrated a capacity to produce and sustain litters that result in pack sizes which in some cases, put excessive pressure on the prey resources (Marneweck et al., 2019).

To complement the managed metapopulation strategy, the KwaZulu-Natal Wild Dog Advisory Group (KZNWAG) alleviates this pressure by coordinating the translocation of individuals or dispersal groups, when socially and genetically appropriate. Due to the limited number of metapopulation reserves, and their frequently limited capacities to reintroduce or sustain additional wild dogs, such translocations are not always a feasible option. For this reason, contraceptive interventions have been used as a management tool to reduce the likelihood of a host reserve exceeding their acceptable wild dog capacity. Due to the endangered status of wild dogs, contraceptive intervention is considered as a last-resort option to control managed population levels and implemented in a manner that is temporary and reversible to preserve the genetic diversity of this species (KZNWAG personal communication).

The use of contraception in carnivores can provide a useful tool for both for Zoos, and for population management with a temporary and reversible suspension of population growth, to avoid inbreeding or breeding excess (Van den Berghe et al., 2012).

There are four potential methods of reproductive control of captive canid species: surgical methods, immunological, hormonal methods, and under human care, separation of sexes. The feasibility for separation of sexes as a method depends on social behaviour of the species and availability of enclosures in a facility. Some pack animals might be separated without impacting social behaviour whereas in many Canidae species, such as the Africa wild dog, this would disrupt social behaviour (Boutelle and Bertschinger, 2010). The first oral contraceptive for dogs, medroxy-progesterone acetate, became available in 1963 (Bertschinger et al., 2001). Most of the early treatments were based on the use of progestins, which have a negative feedback effect on GnRH pulsatility in the hypothalamus. Long-acting silicon implants impregnated with progestins, have been extensively used to down-regulate female reproduction in lions, tigers, and some wild canid species. Although highly successful as contraceptive agents, their prolonged use resulted in a number of side-effects, some of which were life-threatening (Munson 2002).

Wild dogs are seasonal mono-oestrous breeders with breeding season comprised mostly between February and late April (Boutelle and Bertschinger, 2010; Creel and Creel, 2002; Davies et al., 2016). Occasionally, animals skipping the reproductive season may have a late reproductive cycle, with early summer (October - November) denning (Bertschinger et al., 2001). As the species is a social carnivore with strict social structures, the alpha pair will generally dominate the mating process, but subordinates are also capable of mating (Creel and Creel, 2002; Creel, 2005; Marneweck et al., 2019). All females of a pack might come into

oestrous, and mating is inhibited by behavioural cues (Van den Berghe et al., 2012).

The method of contraception with the most potential for use in wild dogs under natural conditions is reversible hormonal contraception (Bertschinger et al., 2001). There are two main groups of drugs available in South Africa that can be used as the synthetic progestins (Depo-Provera®/MGA implants) and the GnRH agonists Deslorelin (Suprelorin®, Virbac). The synthetic progestins carry a risk of inducing uterine, mammary and hepatic pathologies and have been implicated in the development of cystic endometrial hyperplasia, pseudo-pregnancy and pyometra in canids. These risks may have a devastating effect on the continued reproductive ability of female wild dogs (Bertschinger, 2010) and therefore do not fit the strategy used for the wild dogs' metapopulation. Suprenolin inhibits the release of the follicular stimulating hormone (FSH) and luteinizing hormone (LH) essential for the downstream stimulation of gonads to form viable ova and sperm (Lucas, 2014). This means of contraception can be used both on females and males (Bertschinger et al., 2001; Lucas, 2014) and fulfils the temporary and reversible criteria required of a wild dog contraceptive. Suprenolin has been used in a variety of mammals (Lucas, 2014), including few successful tests on captive wild dogs. The outcome for females appears to be less consistent and shorter in duration than in males, where the treatment seems to have near 100% efficacy (Bertschinger, 2010). There is still some debate regarding the use of the implant on males, as the effect of the downregulation of the reproductive hormones could potentially influence the animal's behaviour, reducing aggression rate, with a potentially destabilizing effect on male hierarchy (Bertschinger et al., 2001).

Despite understanding the potential usefulness of Suprelorin, guidelines are unavailable for managers implementing reintroductions and fertility control in this species under wild conditions. Consequently, many decisions regarding best practice for effective contraception results are based on personal knowledge and experience. To the researchers' best knowledge, there are limited publications describing the use of Suprenolin as a matter of contraception on African wild dogs, and no published studies have been carried out Suprenolin efficacy on wild dogs outside captive institutions. Furthermore, the effect of Suprenolin on wild dog behaviour, hierarchy and the potential impact on home ranges have never been investigated, leaving a knowledge gap that needs to be implemented for the future use of the contraception measure.

The main goal of the research is to determine the usefulness of reversible hormonal contraception to control the breeding rate of African wild dogs, using historical data collected by the Kwazulu-Natal Wild Dog Advisory Group. Historical data will be used to assess the ideal time period to deploy the contraception implant, the effectiveness of this method and the duration of the implant to prevent primary as well as secondary breeding. If female wild dog reproduction is dominated by behavioural suppression, then the chances of multiple female pregnancies or inbreeding is likely to be higher when individuals are unable to disperse, such as in captivity, or when behavioural mechanisms to prevent breeding are less effective. In wild dogs, inbreeding is rare even if individuals remain in their natal pack (Frame et al., 1979; Fuller et al., 1992) Yet the factors preventing this in wild populations appear less effective in captivity, as inbreeding is more common (Cade, 1967; van Heerden and Kuhn, 1985) and this has led to the need to

separate sexes in captivity and to place individuals on contraceptives (Van den Berghe et al., 2012).

3. *Materials and Methods*

Data were collated by the Wild dog Advisory Group (WAG) and the KwaZulu-Natal Wild Dog Advisory Group (KZNWAG). Information were recorded from individual reserve reports regarding breeding and observed animal behaviour. We were able to extract sufficient data for 16 Deslorelin implant use over 9 years; that is, from the first attempted contraception to the most recent in wild conditions (2008– 2017; Table 1). For each contraception event, we recorded date, location, pack composition, number and sex of animal contracepted. During the following period, packs were monitored, and the following data were recorded when possible: pack composition, mating behavior, denning, dispersals. Remote delivery was considered unreliable and all subjects were contracepted following immobilisation. For the purpose of this study, implant consists of a single 4,7 mg dose, administered adjacent to the right shoulder blade. Implants were stored at 4°C prior to use and a cold chain observed.

Historical data collected by the Kwazulu-natal Wild Dogs Advisory Group included data from Somkhanda Game Reserve; Manyoni Private Game Reserve; Zimanga Game Reserve; uMkhuze Game Reserve; Hluhluwe-iMfolozi Park: Sokwezela pack, Dela pack, Bhejie pack and Shengezi pack; Tembe Elephant Park. We defined the reproductive season as mating (February–April), denning (May–July) and non-denning (August–January) (Van den Berghe et al., 2012).

Historical data were analysed to achieve the following aims: 1) Define the effectiveness of Suprelorin as a tool to control wild dog breeding rate (binary: were pups produced during the following 12 months) 2) Assess frequency of late breeding cycles in packs with Suprelorin implants 3) Define Suprelorin implant duration 4) Determine the optimal season for the Suprelorin implant.

4. Results

Deslorelin implant success rate corresponded to 87,5%. Results over N=16 treatments, revealed 14 successful treatments (no pups produced during the following 12 months) and two unsuccessful treatments (pups produced in less than 12 months post implant). The successful treatments included 1 treatment implanted 8 months before the reproductive season, 5 treatments implanted 2 months before the reproductive season, 7 treatments implanted 3 months before the reproductive season, and 1 treatment implanted 1 month before the reproductive season. With the two unsuccessful treatments, animals produced pups during the upcoming denning season (May–July) following the implant (5 and 6 months post implant); in one occasion, all three females become pregnant; in the second, only the alpha female became pregnant. Over the 16 treatments, at least 6 times animals expressed mating behaviours during the next reproductive season (four were observed mating without producing pups during the upcoming denning season, two produced pups). Over the nine years of tests, no late breeding cycle was observed (late breeding = 0, usual breeding =2, no breeding = 15). In five cases, implant effectiveness was tested for two years. Over these five

cases, four packs bred 13 - 17 months post contraception; in one case, the pack did not breed for at least 24 months. It was not possible to obtain further data on this pack. In three cases packs were implanted again after 12 months, including the two cases were the implant did not impede reproduction in the upcoming reproductive season. Packs implanted in late 2017 (N=5) did not produce pups until September 2018, thereafter, results for these cases were included in the category successful but were excluded from the assessment of the implant duration (table 1).

Both unsuccessful cases corresponded to contraception implanted during the month of January. Over the 14 successful cases, 13 were administered between November and December, one was administered in May and one in January.

5. *Discussion*

The results of this study expand upon previous studies focused on the use of the GnRH agonists Deslorelin as a method of contraception for the African wild dogs. Deslorelin was previously found to be an effective contraceptive for African wild dogs, reducing the reproductive rate and avoiding reproduction with failure rate of 10% (Bertschinger et al., 2001). These data are comparable with results presented here, where the success rate (success= non-denning) corresponded to 87,5%, with 12,5%]unsuccessful. The effect of Deslorelin implants in captive African wild dogs was previously described as shorter and more unpredictable compared with that in Cheetah and Leopard, as 13 dog bitches showed signs of oestrus between 3 and 21 months after Deslorelin implantation (Bertschinger, 2010).

Differences in the contraceptive period duration can be attributed to diverse sensitivities of species like the domestic dog and cat to the same dose of Deslorelin (Munson et al., 2001; Trigg et al., 2001). However, African wild dogs are extremely hyperactive compared with cheetahs, indicating a much higher metabolic rate, that could justify the shorter effect in this species (Boutelle and Bertschinger, 2010).

The selection of the Deslorelin dose comes with its own challenges: in the study presented by Bertschinger et al., (2002) a dose of 3 mg was ineffective in suppressing oestrus and mating in a bitch that was already in pro-oestrus at the time of treatment. Two other bitches, one in pro-oestrus and one in anoestrus, also ovulated after treatment with this low dose; in the same study, wild dogs were treated with 6 mg Deslorelin and the effect lasted for 9-14 months. The dose used in the current study was 4,7 mg. Considering the large proportion of success on our results, we speculate that this dose resulted effective and could be sufficient for African wild dog contraception for up to 12 months.

Our results show a consistent effect: over the 16 tests, 15 resulted effective in avoiding denning during the next upcoming reproductive season. Two cases were classified as unsuccessful as denning occurred during the upcoming reproductive season following the implant. Both cases corresponded with reduced time interval between implant and beginning of the reproductive season (1 month), as opposed to the majority of the successful treatments where the implant was applied between 2 and 3 months before the reproductive season (N=12). Deslorelin was implanted in January (1 month before the reproductive season) in three cases, of which 2 were unsuccessful and one resulted successful. It is unclear whether pack

composition differences, inappropriate implant positioning or short interval between implant and reproductive season might justify this condition.

It has been previously observed that, when new packs of dogs are formed by mixing animals from different source packs, oestrus may be induced in all the females of the new pack (Bertschinger et al., 2002). If this was the case, the females of the unsuccessful treatments might have been in early oestrus and therefore, the contraceptive effect could have been mitigated.

The results of the present study indicate that Deslorelin might not affect reproductive behaviours of African wild dogs, as in six over 16 treatments, mating behaviour happened during the mating season. This suggests that Deslorelin does not induce behavioural side effects as it did not suppress reproductive behaviour. Similar results were presented by Bertschinger et al., (2001), where no visible behavioural changes nor hierarchical alterations were noted and scent marking in male wild dogs continued. These authors hypothesize that the latter becomes imprinted earlier and manifest despite the reduced concentrations of testosterone induced by the implant.

Deslorelin implants do not completely suppress ovarian hormones (Van den Berghe et al., 2012) as such, female reproductive behaviour might be preserved. Safety during pregnancy was demonstrated in one wild dog that conceived 4 weeks after deslorelin treatment and later delivered seven live pups (Bertschinger *et al.*, 2001).

Once all the Deslorelin has been released from an implant, the target animal would revert to normal reproductive function (Bertschinger et al., 2002). Reversibility of the implant to fully functional reproductive capacity, without the need for an intervention with immobilisation, and without permanent sterilisation, makes Deslorelin a good option to reduce the rate of

reproduction of African wild dogs without inducing behavioural changes and or by directly interfering with more invasive methods. (Bertschinger, 2010).

6. *Conclusions*

In conclusion, our results showed that the Deslorelin implant offers a reversible method of contraception for African wild dogs under natural conditions, with demonstrated contraceptive effect in 87,5% of cases and for a duration of 12 months. Additional data will be required to clarify the causes behind the unsuccessful events, but we can speculate that the time interval between the implant and the upcoming reproductive season might play a role in the efficacy of the contraception. Therefore, we recommend considering a time interval of 2 – 5 months before the start of the reproductive season to achieve best results. All packs followed after the implant proved to have returned to their natural reproductive cycling and sired offspring, proving that reversibility with accompanying good fertility is ensured when the contraception efficacy disappears. Contraception will help ensure the long term acceptance of wild dogs in the landscape in South Africa, providing invaluable importance for the long term conservation of the species. Further studies are needed to establish any potential effect of contraception on social structure and home ranges of this social carnivore as a consequence of contraception.

7. *References*

Altmann, J., 1974. Observational study of behaviour: sampling methods. Behaviour 49, 227–267.

Bernstein, I.S., 2010. Ethograms, Activity Profiles and Energy Budgets. Enciclopedia of Behaviour, 645-649. Elsevier Ltd.

Bertschinger, H.J., 2010. Controlling wildlife reproduction: reversible suppression of reproductive function or sex-related behaviour in wildlife species. (Ph.D) University of Utrecht, Netherlands.

Bertschinger, H.J., Asa, C.S., Calle, P.P., Long, J.A., Bauman, K., DeMatteo, K., Jöchle, W., Trigg, T.E., Human, A., 2001. Control of reproduction and sex related behaviour in exotic wild carnivores with the GnRH analogue Deslorelin: preliminary observations. Journal of reproduction and fertility. Supplement 57, 275–283.

Bertschinger, H.J., Trigg, T.E., Jochle, W., Human, A., 2002. Induction of contraception in some African wild carnivores by downregulation of LH and FSH secretion using the GnRH analogue Deslorelin. Reproduction, Cambridge Supplement 60, 41–62.

Boutelle, S.M., Bertschinger, H.J., 2010. Reproductive management in captive and wild canids: contraception challenges. International Zoo Yearbook 44, 109–120. https://doi.org/10.1111/j.1748-1090.2009.00107.x

Courchamp, F., Macdonald, D.W., 2001. Crucial importance of pack size in the African wild dog *Lycaon pictus*. Animal Conservation 4, 169–174. https://doi.org/10.1017/S1367943001001196

Cozzi, G., Broekhuis, F., Mcnutt, J.W., Turnbull, L.A., Macdonald, D.W., Schmid, B., 2012. Fear of the dark or dinner by moonlight? Reduced temporal partitioning among Africa's large carnivores. Ecology 93, 2590–2599. https://doi.org/10.1890/12-0017.1

Creel, S., 2005. Dominance, aggression, and glucocorticoid levels in social carnivores. Journal of Mammalogy 86, 255–264.

Creel, S., 1996. Conserving wild dogs. Trends in Ecology & Evolution 11, 337. https://doi.org/10.1016/0169-5347(96)81137-2

Creel, S., Creel, N.M., 2002. The African wild dog: behaviour, ecology and conservation. Princeton University Press. https://doi.org/10.1016/S0169-5347(02)00039-3

Creel, S., Creel, N.M., 1995. Communal hunting and pack size in African wild dogs, *Lycaon pictus*. Animal Behaviour 50, 1325.

Creel, S., Creel, N.M., Creel, A.M., Creel, B.M., 2016. Hunting on a hot day: effects of temperature on interactions between African wild dogs and their prey. Ecology 97, 2910–2916. https://doi.org/10.1002/ecy.1568

Davies, A.B., Marneweck, D.G., Druce, D.J., Asner, G.P., 2016. Den site selection, pack composition, and reproductive success in endangered African wild dogs. Behavioral Ecology 27, 1869–1879. https://doi.org/10.1093/beheco/arw124

Davies-Mostert, H.T., Mills, M.G.L., Macdonald, D.W., 2015. The demography and dynamics of an expanding, managed African wild dog metapopulation. African Journal of Wildlife Research 45, 258–273. https://doi.org/10.3957/056.045.0258

Frame, L.H., Malcolm, J.R., Frame, G.W., van Lawick, H., 1979. Social organization of African wild dogs (*Lycaon pictus*) on the Serengeti Plains, Tanzania 1967–1978. Zeitschrift für Tierpsychologie 50, 225–249.

Gerencsér, L., Vásárhelyi, G., Nagy, M., Vicsek, T., Miklósi, A., 2013. Identification of behaviour in freely moving dogs (*Canis familiaris*) using inertial sensors. PLoS One 8, e77814. https://doi.org/10.1371/journal.pone.0077814

Gusset, M., Macdonald, D.W., 2010. Group size effects in cooperatively breeding African wild dogs. Animal Behaviour 79, 425–428. https://doi.org/10.1016/j.anbehav.2009.11.021

Gusset, M., Slotow, R., Somers, M.J., 2006. Divided we fail: the importance of social integration for the re-introduction of endangered African wild dogs (*Lycaon pictus*). Journal of Zoology 270, 502–511.

Gusset, M., Stewart, G., Bowler, D., Pullin, A., 2010. Wild dog reintroductions in South Africa: A systematic review and cross-validation of an endangered species recovery programme. Journal for Nature Conservation 18, 230-234. https://doi.org/10.1016/j.jnc.2009.11.001

Jackson, C.R., Groom, R.J., Jordan, N.R., McNutt, J.W., 2017. The effect of relatedness and pack size on territory overlap in African wild dogs. Movement Ecology 5,10. https://doi.org/10.1186/s40462-017-0099-8

Knobel, D.L., du Toit, J.T., 2003. The influence of pack social structure on oral rabies vaccination coverage in captive African wild dogs (*Lycaon pictus*). Applied Animal Behaviour Science 80, 61–70. https://doi.org/10.1016/S0168-1591(02)00136-3

Lucas, X., 2014. Clinical use of Deslorelin (GnRH agonist) in aompanion Animals: A review. Reproduction in Domestic Animals 49, 64–71. https://doi.org/10.1111/rda.12388

MacNulty, D.R., Mech, L.D., Smith, D.W., 2007. A proposed ethogram of large-carnivore predatory behavior, exemplified by the wolf. Journal of Mammalogy 88, 595–605. https://doi.org/10.1644/06-MAMM-A-119R1.1

Main, D.C.J., Mullan, S., Atkinson, C., Bond, A., Cooper, M., Fraser, A., Browne, W.J., 2012. Welfare outcomes assessment in laying hen farm assurance schemes. Animal Welfare 21, 389–396.

Majewski, E., Hamulczuk, M., Malak, A., n.d. Cost-effectiveness assessment of improving animal welfare standards in the European Agriculture 22.

Marneweck, C., Becker, P.A., Beverley, G., Davies-Mostert, H.T., du Plessis, C., Forssman, K., Graf, J., Gusset, M., Hofmeyr, M., Kelly, C., Kilian, P.J., Marchal, A.F.J., Marneweck, D.G., Marnewick, K., Owen, C., Postiglione, G., Slotow, R., Somers, M.J., Szykman Gunther, M., van Dyk, G., Whittington-Jones, B., Parker, D.M., 2019. Factors affecting the success of artificial pack formation in an endangered, social carnivore: the African wild dog. Animal Conservation. https://doi.org/10.1111/acv.12490

McCreery, E.K., 2000. Spatial relationships as an indicator of successful pack formation in free-ranging African wild dogs. Behaviour 137, 579–590. https://doi.org/10.1163/156853900502222

McCreery, E.K., 1999. The formation of new reproductive units in free-ranging African Wild Dogs (*Lycaon pictus*).

McDonnell, S., 2003. The equid ethogram: a practical field guide to horse behavior, The Blood-Horse Inc. ed. Hong Kong.

Mills, M.G.L., Gorman, M.L., 1997. Factors affecting the density and distribution of wild dogs in the Kruger National Park. Conservation Biology 11, 1397–1406.

Newell-Fugate, A.E., O. Nöthling, J., J. Bertschinger, H., 2012. Seasonal changes in steroid hormone profiles, body weight, semen quality, and the reproductive tract in captive African wild dogs (*Lycaon pictus*) in South Africa. General and Comparative Endocrinology 178, 272–281. https://doi.org/10.1016/j.ygcen.2012.05.008

Overall, K.L., 2014. The ethogram project. Journal of Veterinary Behavior: Clinical Applications and Research 9, 1–5. http://dx.doi.org/10.1016/j.jveb.2013.12.001

Paquet, P., Darimont, C., 2010. Wildlife conservation and animal welfare: two sides of the same coin? Animal Welfare 19, 177-190.

Potgieter, K.R., Davies-Mostert, H.T., 2012. A simple visual estimation of food consumption in carnivores. PLoS ONE 7. https://doi.org/10.1371/journal.pone.0034543

Potgieter, K.R., O'Riain, M.J., Davies-Mostert, H.T., 2015. Behavioural cues can be used to predict the outcome of artificial pack formation in African wild dogs (*Lycaon pictus*). African Journal of Wildlife Research 45, 215–222. https://doi.org/10.3957/056.045.0215

Rasmussen, G.S.A., Macdonald, D.W., 2012. Masking of the zeitgeber: African wild dogs mitigate persecution by balancing time. Journal of Zoology 286, 232–242. https://doi.org/10.1111/j.1469-7998.2011.00874.x

Reich, A., 1981. The behavior and ecology of the african wild dog (*Lycaon pictus*) in the Kruger National Park (Ph.D.). Yale University,Connecticut, United States.

Robbins, R.L., McCreery, E.K., 2003. Acoustic stimulation as a tool in African wild dog conservation. Biological Conservation 111, 263–267. https://doi.org/10.1016/S0006-3207(02)00294-X

Somers, M.J., Graf, J.A., Szykman, M., Slotow, R., Gusset, M., 2008. Dynamics of a small re-introduced population of wild dogs over 25 years: Allee effects and the implications of sociality for endangered species' recovery. Oecologia 158, 239–47. https://doi.org/10.1007/s00442-008-1134-7

Stanton, L.A., Sullivan, M.S., Fazio, J.M., 2015. A standardized ethogram for the felidae: A tool for behavioural researchers. Applied Animal Behaviour Science 173, 3–16. http://dx.doi.org/10.1016/j.applanim.2015.04.001

Stevenson, M.F., Poole, T.B., 1976. An ethogram of the common marmoset (*Calithrix jacchus jacchus*): general behavioural repertoire. Animal Behaviour 24, 428–433.

Sutherland, W.J., 1998. The importance of behavioural studies in conservation biology 56, 801–809.

Turner, S., Dwyer, C., 2007. Welfare assessment in extensive animal production systems: challenges and opportunities. Animal welfare-potters bar then wheathampstead 16, 189.

Van den Berghe, F., Paris, D.B.B.P., Van Soom, A., Rijsselaere, T., Van der Weyde, L., Bertschinger, H.J., Paris, M.C.J., 2012. Reproduction in the endangered African wild dog: Basic physiology, reproductive suppression

and possible benefits of artificial insemination. Animal Reproduction Science 133, 1–9. https://doi.org/10.1016/j.anireprosci.2012.06.003

Vlamings, B.H.A.C., 2011. Dog appeasing pheromone®: A useful tool to minimize stress and aggression of African wild dogs (*Lycaon pictus*)? (Prestigious Master: Neuroscience and Cognition). Graduate School of Life Sciences, University of Utrecht, the Netherlands.

Walker, R.H., King, A.J., McNutt, J.W., Jordan, N.R., 2017. Sneeze to leave: African wild dogs (*Lycaon pictus*) use variable quorum thresholds facilitated by sneezes in collective decisions. Proceedings of the Royal Society B: Biological Sciences 284. https://doi.org/10.1098/rspb.2017.0347

Woodroffe, R., 2011. Ranging behaviour of African wild dog packs in a human-dominated landscape. Journal of Zoology 283, 88–97. https://doi.org/10.1111/j.1469-7998.2010.00747.x

Woodroffe, R., Sillero-Zubiri, C., 2012. Lycaon pictus (No. e. T12436A16711116). The IUCN red list of threatened species.

Zrzav, J., 2004. Phylogeny of recent Canidae (Mammalia, Carnivora): relative reliability and utility of morphological and molecular datasets. Zoologica Scripta 23.

Chapter VI

Home ranges and territory use of African wild dog in fenced areas

1. Abstract

For the present study, we used T-LoCoH, (Time Local Convex Hull) integrated in the R programming language, a home range construction algorithm that incorporates time into the construction and aggregation of local kernels. This allows for calculation of the amount of time an animal spends in an area, as well as the frequency of revisitation, opening the option to reflect two dimensions of resource value to the animal. Subjects of the study were two packs of wild dogs, location in both cases is a fenced areas. The objectives were 1)assess temporal partitioning of space, 2) to quantify site fidelity 3) define whether wild dogs selected or avoided artificial fences, evaluating visitation and duration rates.

2. *Introduction*

Home ranges often serve as the primary spatial unit for wildlife research and are considered the area to which an animal confines it's normal movement. In order to calculate animal home ranges, researchers have used several methods in geometric topology and spatial smoothing to transform a set of telemetry points into a polygon animal home range (Long and Nelson, 2012). Two methods are most commonly used for computing animal home ranges: the minimum convex polygon (MCP), and the kernel density estimation (KDE) (Laver and Kelly, 2008). Both methods have their advantages and disadvantages: concens regarding the use of MCP are recognised to be sensitivity to sampling intensity and outliers, convex assumption, inclusion of large, unused interior areas; on the other hand, the use of KDE exposes to the potential misleading interpretation of results, which generate difficulty in developing consistency across studies (Laver and Kelly, 2008). During the last 10 years, new approaches to the study of animal land use and home ranges have been developed, taking into consideration not only space and distances, but also time. One of the most common is the potential path area (PPA), a projection of the space-time model onto geographic plane (Long and Nelson, 2012). The PPA represents the set of all accessible locations between two known locations in space and time. As a consequence, the maximum expansion of the PPA is strictly related to the animals maximum velocity.

Significant improvements have been made in developing methods able to assess space-use and behavior, but regardless, these advances have not, in general, been well-integrated. Most home range estimators tend to ignore

time, except for time-intervals, while spatiotemporal and space-state models are often dissociated from a model of space-use (Lyons et al., 2013).

Despite limitations of traditional home range size descriptors, comparative studies have found some general trends that suggest fundamental ecological relationships. For the present study, we used T-LoCoH, (Time Local Convex Hull) integrated in the R programming language, a home range construction algorithm that incorporates time into the construction and aggregation of local kernels. This allows to create a variety of space use models in which internal space is differentiated not only by the intensity of use, but also by behavioral properties such as directionality and time use (Lyons et al., 2013). Among several functions, T-LoCoH allows for time-scaled distance (TSD), transforming the time interval between any two points into a third axis of Euclidean space. The effect of the time-distance axis is to push apart points that are far away in time even though they may be close in two-dimensional space. Hulls produced from neighbours identified by TSD are local not only in terms of space but also time, and their geometric properties may be used to help infer the animal's movement phase.

Between species, as body size increases home ranges sizes also increase (Gittleman and Harvey 1981). This is particularly true for carnivores since animals higher on the trophic scale depend on food sources that exist at lower density in the environment (Gompper and Gittleman 2008). Within a species, home range sizes have been shown to vary depending on local resource availability. African lions (Panthera leo) exhibit similar behavior where prides maintaining home ranges in areas of high prey density have smaller home ranges than prides in prey scarce environments (Loveridge et

al. 2018). Additionally, the amount of time an animal spends in an area, and the frequency of revisitation, reflect two dimensions of resource value to the animal. For example, water points may have a high revisitation index, but each visit may be of relatively short duration (Lyons et al., 2013). Space use also suggests an alternative approach to identifying 'core territory' which classically has been studied exclusively in terms of space use, excluding the variable time. T-LoCoH computes metrics for revisitation and duration of use based upon an inter-visit gap (IVG) parameter provided by the analyst. This might be particularly useful in fenced area to assess the welfare of a species and the potential competitors. In fenced areas, wild dogs can express quantitative and qualitative shifts in prey selection patterns. This is possible due to human-mediated changes in habitat structure with the imposition of fences, inducing fence-impeded and unimpeded kills (e.g. fences allow more kudus to be captured). These shifts provide benefits to wild dogs by increasing hunt efficiency and subsequently reducing the inherent risks associated with hunting (Davies-Mostert et al., 2013).

The current study aims at capturing temporal partitioning of space of two packs of wild dogs in fenced areas, and to assess whether wild dogs selected or avoided particular natural resources and artificial fences, evaluating visitation and duration rates.

3. Materials and Methods

3.1 Study Site

The study was conducted in South East Botswana and North of South Africa. There are three recognized seasons: hot, dry (August-October); hot, wet (November-March); and cool, dry (April-July). Two settings where included: Limpopo Lipadi Private Game and Wilderness Reserve, Tuli Block, Botswana (LL), and Khamab Nature Reserve (KNR), South Africa. LL consists of 22050 he of fully fenced mopani savannah. It is open to private owners for self-drive or guided safari, using up to 10 vehicles. The reserve contains 12 permanent waterholes and 10 natural water ponds where water was present from October 2nd, 2017, to March 2018. Subjects were fee-rooming and had access to the entire area, animals were never feed. The KNR consists of 100000 he, fully fenced Kalahari desert landscape. According to the data disclosure policy, details regarding the reserve (e.g. water distribution and landscape details) will be used exclusively for the necessary data analysis and will not be listed in the present chapter. Both reserves have electric fences with two electric grids at end of the fence, discouraging the AWD from leaving the protected area. Prey availability in both reserves was related to prey distribution and included impala (*Aepyceros melampus*) steenbok (*Raphicerus campestris*) blue wildebeest (*Connochaetes taurinus*), waterbuck (*Kobus ellipsiprymnus*) greater kudu (*Tragelaphus strepsiceros*) and potentially other antelopes and small animals.

3.2 Subjects

A total of N=37 African wild dogs were included in the present study. Of these, N=18 were adults (> 1 year of age), N=7 were juveniles (1 year of age) and N=8 pups (< 1 year of age). All subjects were identified by their unique coat patterns, ear marks, and tail coloration. All subjects were born in the wild. LL subjects included the *Morena* pack, composed of 8 adults: the alpha pair, three males, and three females.

In KNR, subjects of study were 29 animals of the *Botswana* pack (N=10 adults; N=7 juveniles; N=8 pups). All juveniles and pups where born in KNR, while origin of the adults is unknown.

3.3 Field work

GPS collars (Vectronic Aerospace GmbH, Berlin, Germany) specifically designed to record GPS data were fitted to the study animals by a registered veterinarian in compliance with Botswana and South African law. To fit the collars, animals were immobilized. After immobilization, the collared individuals rejoined their group showing no signs of distress. During 2008, we monitored the activity of African wild dogs two packs in two fenced areas. The LL pack was identified as AWD01, and the South African pack hereafter will be identified as AWD05. Throughout the duration of this study, with AWD01 at least two individuals of each pack were collared, but data from no more than one individual per social group where included in the results to avoid data duplication due to the collective movement of packs.

3.4 Data analysis

The activity data used in our analyses were systematically collected by GPS collars. Study animals were followed visually during daytime to ensure that pack composition, positioning and functionality of the collars were consistent. To define home ranges and analyze data, we used Time Local Convex Hull T-LoCoH (Lyons et al., 2013), based upon LoCoH , a non-parametric Lagrangian method for constructing UDs from a set of locations by aggregating local MCPs constructed around each point. For the calculation of isopleths, The k -method was selected. Local convex hulls were constructed around each point and its nearest neighbours, then sorted by density which is proxied by hull area. After sorting, T-LoCoH allows for hulls to be cumulatively merged together by taking their union.

For sampling frequency, threshold was set at 0.2, as a consequence any group of points that are less than 0.2 of the median sampling interval were considered a cluster and thinned down to one location. Time was included in the hull construction allowing examination of rates of re-visitation and the duration of visits to an area. To compute re-visitation and average visit duration, the inter-visit gap period (IVG) was set at 12h (Lyons et al., 2013).

4. Results

Two packs of African wild dogs were succesfully monitored and movements of the collared animals collated (AWD01 isopleth area plotted in figure 1A; AWD05 area plotted in figure 1B). Time-use metrics were computed with an inter-visit gap period of 12 hours based on the known

feeding habits of the species. Isopleth elongation distribution were constructed for AWD01 from 1376 hulls sorted descending by elongation, for AWD05 isopleths were constructed from 938 hulls sorted descending by elongation. Isopleth levels indicate the proportion of total points enclosed. Hulls created using the fixed−k method (k=12, s=0.003, kmin=0, 2 duplicate points offset by 1 map units).

To explore the relationships among the distribution of hulls in time-use space, we produced graphs for visitation rates and duration (figure 3 – AWD01; figure 4 – AWD05). The histogram for AWD01 indicates that the majority of hulls were revisited approx. 5 times by AWD01, and approximately twice for AWD05 (figure 3). Figure 4 shows the differences in duration of visits between AWD5 and AWD1. Visitation rate is plotted on GPS location for both animals in figure 5.

5. Discussion and conclusions

The current study investigates the land use over time of two pack of wild dogs inhabiting two different fenced wild areas. The k -method was chosen to select the k- nearest neighbours around each point.

Isopleths usually reflect a gradient of the proportion of known locations. With home ranges (fig.1) both for AWD01 and AWD05, some points aren't included in any of the isopleths. This is related to the level of largest isopleth (light blue) set at 0.95, meaning that it only encloses 95% of the points. Since these isopleths are sorted according to density, the 5% of the points not covered by the 95% isopleth are the least dense. This value was chosen as 95% is typically considered the home range (Lyons et al., 2013). With figure 2 elongation distribution is presented. Here the areas with the greatest elongation (red areas) include those areas where Two spots was at the periphery of her home range. The holes visible in the core area of the elongation isopleths are there because 1) the biggest isopleth shown is only 0.95, so 5% of the points are not included, and 2) the hulls have been sorted by elongation, not density, so the 5% of points that are not enclosed by these isopleths fall in the 5% least elongated (which tend to be in the core areas where the hulls are more circular). These large hulls potentially over-estimate the external portion of the range.

Home range differences between individuals were expected as the period of collaring time varied. As our sample size was small, it was not possible to conduct any statistical analyses comparing home ranges based on residency status; however, it is clear that the largest home range was that of the

AWD05, despite having the shortest collaring period and the smallest number of fixes. AWD05 visited areas further away from fences most often but showed a marked used of an area close to the fence line. This corresponded to the denning area for the year.

The home range of AWD01 includes most of the fenced area, highlighting the overlap with home ranges of other carnivores such as spotted hyaena (*Crocuta crocuta*) and Lions (*Panthera leo*). This condition is likely due to the relatively small size of the park as well as the fact that the park is fenced, resulting in relatively high carnivore densities (personal observations). Considering that the population of spotted hyaena is not well known in the park, it could be possible that regardless the belief of a high population of these species, the extended range of wild dogs indicate a low population of hyaenas, or a scattered distribution in small clans. If this was the case, we could infer that African wild dogs should not be suffering of kleptoparasitism and thus would not be forced to use a subsection of the fenced area to avoid the competitors. Another explanation might be that the wild dogs avoid lions and hyaenas through the constant adjustments in the range core. This hypothesis is supported by previous research where wild dogs demonstrated temporal avoidance when spatial avoidance was not possible (Darnell et al., 2014). A study by Van Der Meer et al., (2011) found that wild dogs selected habitats based on kleptoparasitism risk, avoiding areas with high densities of hyenas. Another study found little effect of hyenas on wild dogs (Webster et al., 2012), while a recent study shows high degree of temporal overlap among wild dog, lion, hyaena and cheetah species extensive due to the previously undescribed nocturnal activity of wild dogs and cheetahs (Cozzi et al., 2012). Another important

element when dealing with carnivore competition is pack size: large wild dog packs can better defend their kills, and for longer periods of time, than can smaller packs. In the case of AWD01 pack, the small pack size would potentially be an element of risk and thus combining this variable with a potential high concentration of hyaenas clans, a more scattered land use would be expected.

It has been suggested that conservation of high densities of competing carnivores in small, fenced reserves may not be feasible and may lead to the extinction of the smaller competitor. Based on the life-history stage of the pack and the present results, LL subjects appear to have been able to adapt to life in a relatively small fenced reserve with presence of competitors, potentially through a combination of spatial and temporal avoidance, and adjusting their behaviour as necessary.

6. Figures

Figure 1: AWD01- isopleths created from 1376 hulls sorted by area. AWD05 - isopleths created from 938 hulls sorted by area. Isopleths levels indicate the proportion of total points enclosed. Hulls created from the Fixed−k method (k=12, s=0.003, kmin=0, 4 duplicate points offset by 1 map units. Main axes represent GPS coordinates.

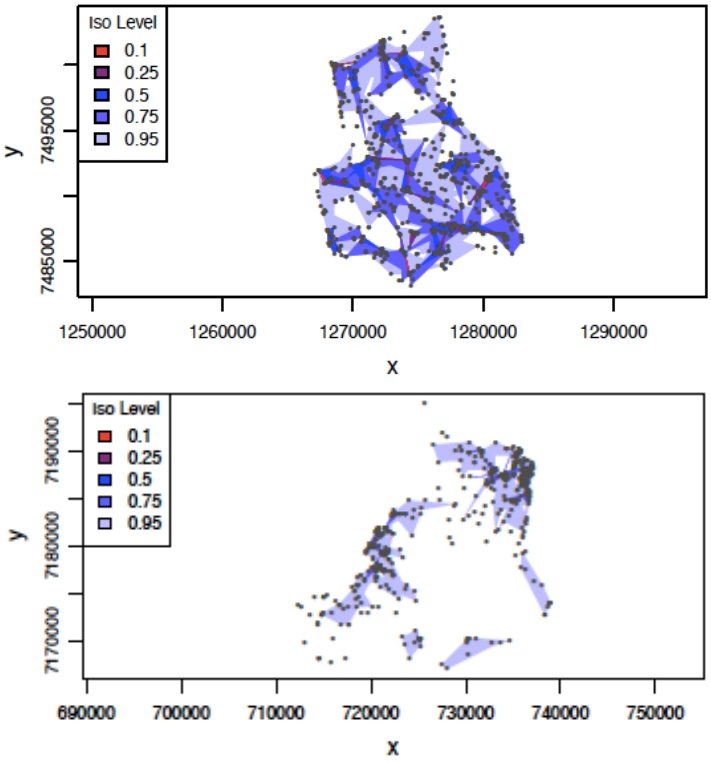


Figure 2: elongation distribution. Isopleth levels indicate the proportion of total points enclosed. Hulls created from 1376 locations of AWD01, and from 938 hulls for AWD05 using the fixed−k method (k=12, s=0.003, kmin=0, 4 duplicate points offset by 1 map units).

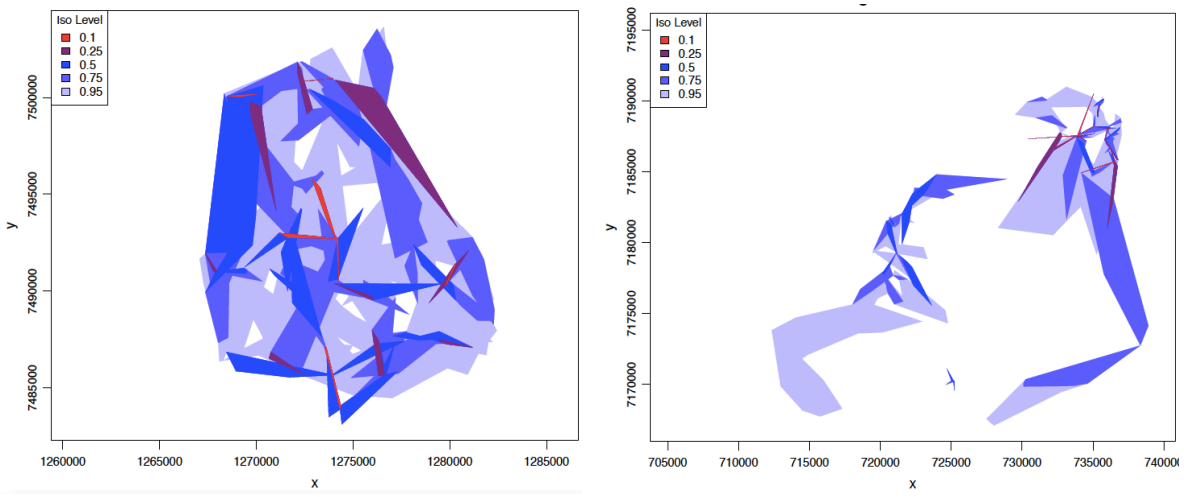


Figure 3: AWD01 frequency and duration of visits

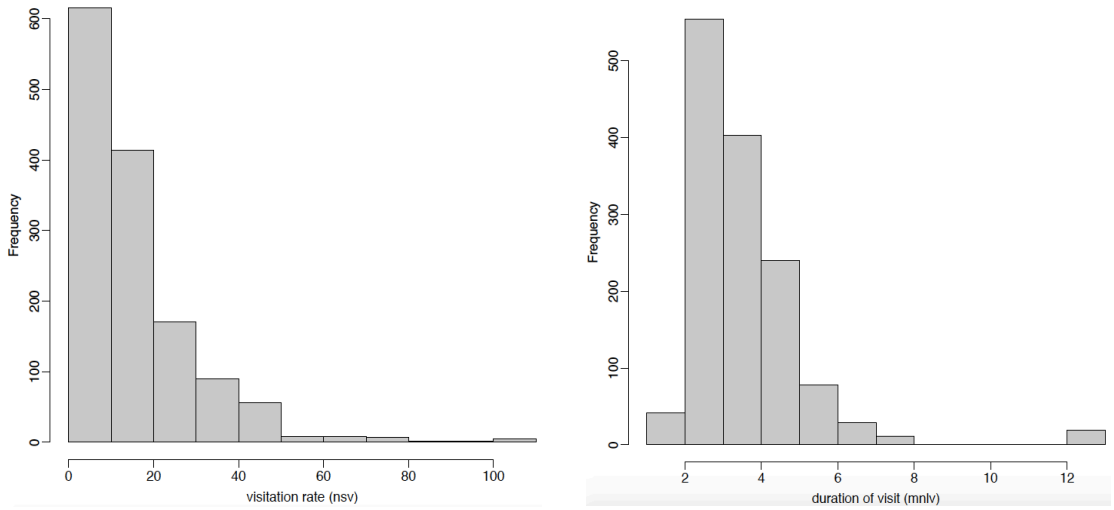


Figure 4: AWD05 frequency and duration of visits

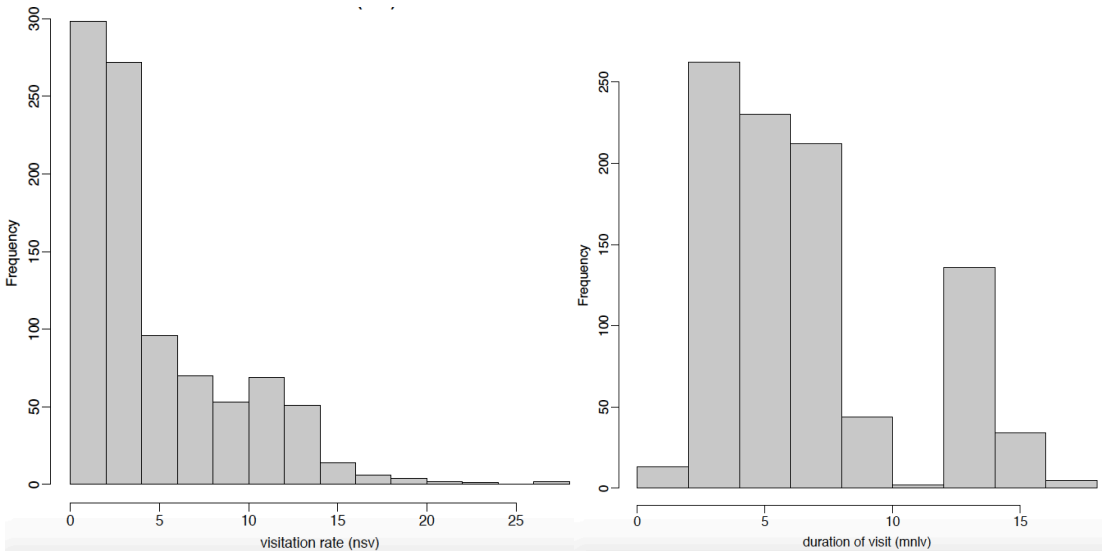


Figure 4: On the x−axis is duration of visit (mean number of locations in the hull per visit). On the y−axis is visitation rate (number of separate visits). Separate visits defined by an inter−visit gap period >= 43200(12hs). x values have been 'jiggled' by 0.05 to better see point density. Y-values have been 'jiggled' by 0.1 to better see point density

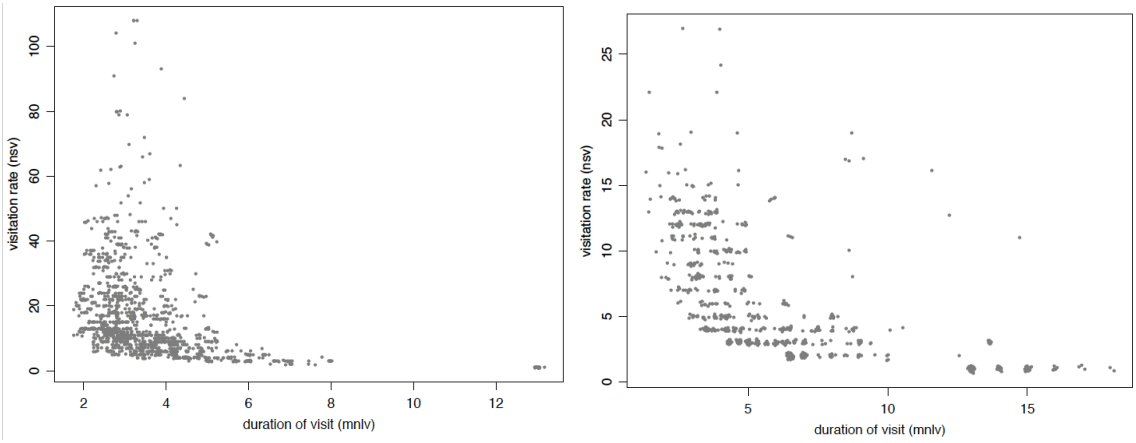


Figure 5: Points colored by visitation rate (nsv). Separate visits defined by an inter−visit gap period >12hs.

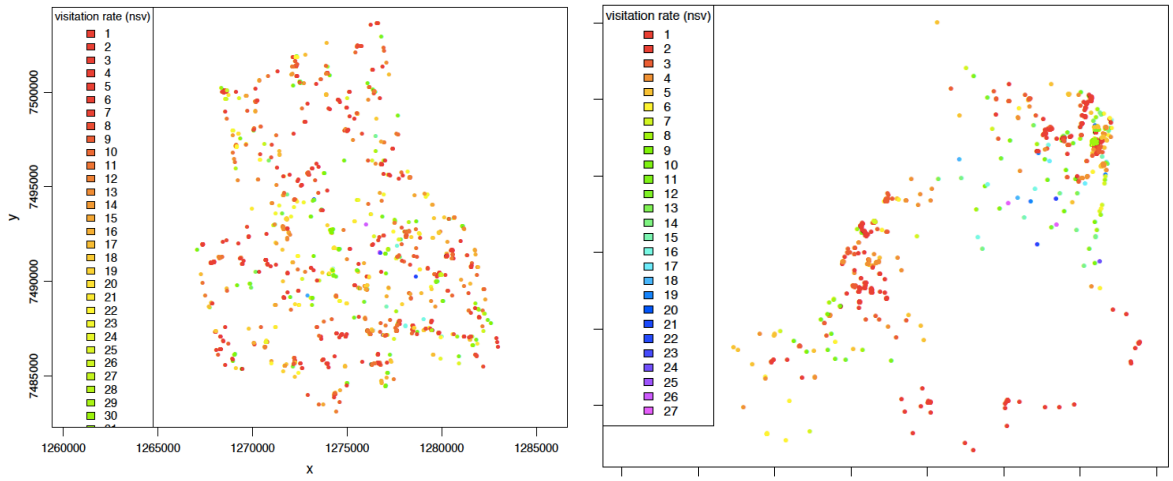


Figure 6: Parent points. Points colored by duration of visit (mnlv). Separate visits defined by an inter−visit gap period >= 43200(12hs). Hulls created from 1376 locations of AWD01 using the Fixed−k method (k=12, s=0.003, kmin=0, 4 duplicate points offset by 1 map units).

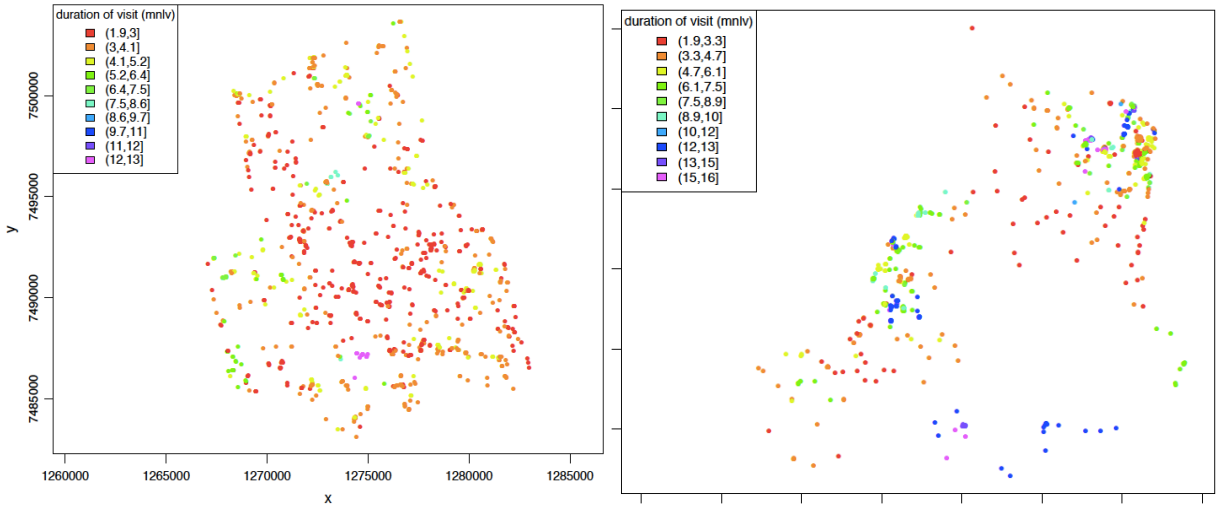
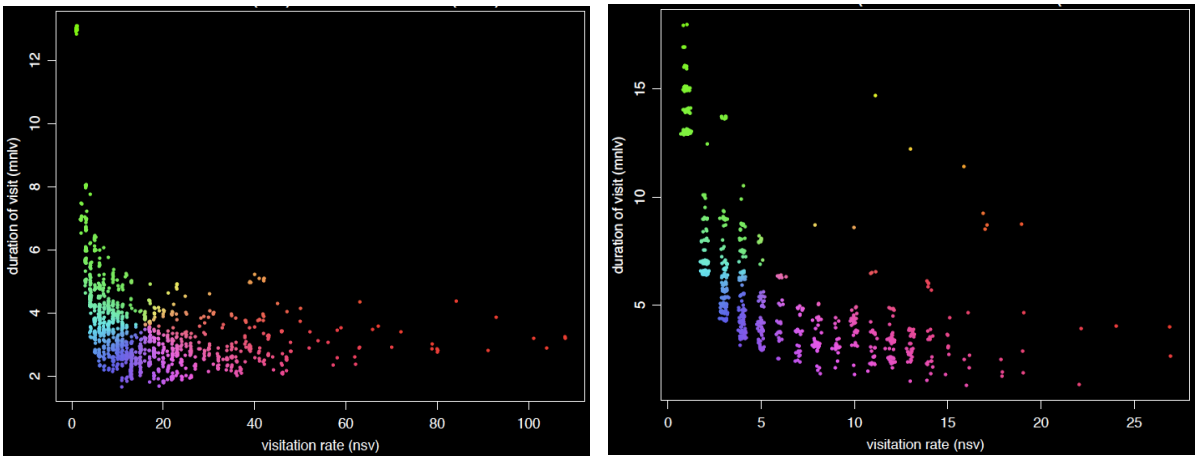


Figure 7: AWD01, visitation rate vs duration of visit. on the x−axis is visitation rate (number of separate visits). On the y−axis is duration of visit (mean number of locations in the hull per visit). Separate visits defined by an inter−visit gap period >= 43200(12hs). x values have been 'jiggled' by 0.1 to better see point density. Y values have been 'jiggled' by 0.05 to better see point density. Mnlv=mean number location per visit



6. *References*

Cozzi, G., Broekhuis, F., Mcnutt, J.W., Turnbull, L.A., Macdonald, D.W., Schmid, B., 2012. Fear of the dark or dinner by moonlight? Reduced temporal partitioning among Africa's large carnivores. Ecology 93, 2590–2599. https://doi.org/10.1890/12-0017.1

Darnell, A.M., Graf, J.A., Somers, M.J., Slotow, R., Gunther, M.S., 2014. Space use of African wild dogs in relation to other large carnivores. PLoS ONE 9. https://doi.org/10.1371/journal.pone.0098846

Davies-Mostert, H.T., Mills, M.G.L., Macdonald, D.W., 2013. Hard boundaries influence African wild dogs' diet and prey selection. Journal of Applied Ecology 50, 1358–1366. https://doi.org/10.1111/1365-2664.12129

Laver, P.N., Kelly, M.J., 2008. A Critical Review of Home Range Studies. Journal of Wildlife Management 72, 290–298. https://doi.org/10.2193/2005-589

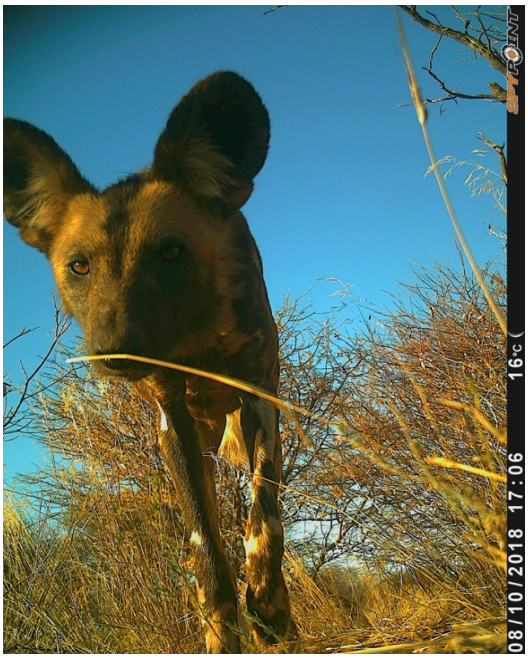
Long, J.A., Nelson, T.A., 2012. Time geography and wildlife home range delineation. The Journal of Wildlife Management 76, 407–413. https://doi.org/10.1002/jwmg.259

Lyons, A., Turner, W.C., Getz, W.M., 2013. Home range plus: A space-time characterization of movement over real landscapes. BMC Movement Ecology 1. https://doi.org/doi:10.1186/2051-3933-1-2.

Van Der Meer, E., Moyo, M., Rasmussen, G.S.A., Fritz, H., 2011. An empirical and experimental test of risk and costs of kleptoparasitism for African wild dogs (*Lycaon pictus*) inside and outside a protected area. Behavioral Ecology 22, 985–992. https://doi.org/10.1093/beheco/arr079

Webster, H., McNutt, J.W., McComb, K., 2012. African wild dogs as a fugitive species: playback experiments investigate how wild dogs respond to their major competitors. Ethology 118, 147–156. https://doi.org/10.1111/j.1439-0310.2011.01992.x

Acknowledgements



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