Linking *in situ* and *ex situ* management: Exploring vaccine immunogenicity and capture stress in the African Painted Dog (*Lycaon pictus*)

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This thesis is submitted in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

in the

College of Humanities, Arts & Social Science
Flinders University
Adelaide, South Australia

November 2018

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Declaration

I, Nicole Anderson, hereby certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Abstract

The African Painted Dog (*Lycaon pictus*) is recognised as one of Africa's most endangered large carnivores with the establishment of global captive populations employed as a precautionary approach to the potential risk of extinction. Effective conservation decisions are needed to mitagate further populations declines in this species with the integration of applied ecology and zoo biology being necessary to improve management outcomes . Health and disease is important for the African Painted Dog as episodic disease events have contributed to severe population declines and localised extinctions of free-ranging populations. Similar risks also extend to captive populations. Vaccination is an integral part of controlling infectious disease in wildlife populations as it decreases disease incidence and lowers transmission rates. Vaccines developed for domestic animals are routinely used to prevent the risk of disease in wildlife species. But, despite widespread use, little is known about how effective these vaccines are for threatened species. This disparity requires investigation and is a focus of this research.

Factors that can influence a vaccine's efficacy relate to the characteristics of the vaccine itself or to the host that is being vaccinated. In this study the latter is examined, with host-specific factors targeting duration of immunity, as well as, the degree of stress individuals' experience when immobilised. This study demonstrates that the inactivated Parvac™ vaccine designed to mitigate the risk of canine parvovirus is safe to use in the African Painted Dog. The duration of immunity is however disproportionately shorter than that for domestic dogs, with a modification of the inoculation schedule recommended to extend protective immunity in the African Painted Dog.

In its assessment of the stress response, the study shows that individual animals were able to adapt to the short term stress imposed by capture and handling. This was determined by the primary stress mediator, cortisol, by invasive and non-invasive means. Through repeated monitoring of cortisol across successive capture events a chronic stress response was also characterised. Social stress is deemed to be a contributing factor in the chronic stress response, with the frequency of stressors rather than their origin being of greater importance. The results demonstrate that where there are consistently high cortisol concentrations protective immunity is reduced.

Examining the immunogenicity of vaccines in protecting species against infectious disease and the effects of stress are both areas that have the potential to refine or develop existing

methodologies and alternative strategies for better animal health and welfare practices. Through its examination of these interlinked themes this study contributes to better disease management practices and improved assessment of the effect that research practices have on animal welfare.

Acknowledgements

First and foremost I would like to thank my husband, Paul. The support and encouragement you have given me over the years has been absolute. Your objective and pragmatic view in discussing unfamiliar topics has been of frequent benefit, in addition to being that 'voice of reason' in those more challenging times. Without saying, your wit and humour about certain situations have often left us in fits of laughter, and then sheer embarrassment on my part after knowing you have shared an embellished version with your work colleagues, who for all accounts are relatively naïve to the discipline of conservation biology. I'm glad my conservation 'antics' have given you a wealth of comedic material. I have to also thank my children, Campbell and Danika, both of whom have only ever known me to be juggling everyday life, work and study. I hope we can enjoy some quality family time together, with me now free of that foreboding feeling that I really should be doing some thesis work. I appreciate the sacrifices you have all made so I could achieve a personal goal.

Thanks must also be given to my university supervisors, Professor Iain Hay and Associate Professor Beverley Clarke, who have been patient throughout my candidature and provided me with direction and constructive feedback in structuring my thesis. To my co-supervisor, Mr. John Lemon, thanks for always being available to talk dogs despite each of us residing in different states. I look forward to strengthening the relationship I have with PDC Inc. and ZCP in working toward better outcomes for free-ranging dogs, with similar sentiments for the captive population here in Australia.

This research was supported by Zoos South Australia and has been overseen and co-supervised by Senior Veterinarian, Dr. Ian Smith. Special thanks Ian for investing the time and resources into this project (and me) and the opportunities that have flowed since. It has been a privilege and one that I don't take lightly. Thanks must also go to past and present staff of the Animal Health Department and keeping staff at Monarto Zoo. These include Beth Pohl, Anna Bennett, Linda Gardiner, Adrian Mifsud, Claire Geister, Gale Akerman, Matthew Brewer, Michelle Lloyd, Jon Allon, Donna Hearn, Lynnette Drabsch, Rebecca Probert and Paula Modra. To those from the carnivore team specifically, I will always look back and smile fondly on the time I spent with you all. The chaos in trying to get markers into subordinate animals, the joy of finding a glittery poo, processing 19 dogs in a day, births in all the other large carnivores and the many laughs we have all shared. By the end of my research we were a well-oiled team and without all of your efforts this project wouldn't have been a success, so thank you. I have valued greatly the opportunity to not

only study the African Painted Dog at Monarto Zoo but have been fortunate to be exposed to the management of Lion, Spotted Hyena and Cheetah. This has allowed me to see the similarity and differences between the species and has given me a broader appreciation of the African carnivore guild and the issues that each of these species face. I have to also thank Emma Still, for your vibrant personality, words of encouragement and go get it attitude – they are a fresh of breath air and an inspiration to many; and to Jerome Kalvas for his amazing artwork, you put us all to shame with such talent.

'The Lab' at Adelaide Zoo has been an environment that has not only developed my laboratory skills but has also been a hub for robust discussion across a broad range of issues. Many thanks to Brian Rich, Peter McCarthy and Wayne Rohrig for being my mentors and providing me with an appreciation of biochemistry, encouraging individual research ideas and dealing with the smell of dried dog faeces for a good proportion of my time in the lab. I couldn't have asked for a better foundation into a discipline that I was not heavily acquainted with. The banter between everyone and the constitutional red wine or beer with cheese and biscuits after a day's work is a unique aspect of the great culture found in the AHD.

Special mention should be given to Dennis Whiteside, Whiteside Constructions Pty Ltd and McMahon Services for upgrading the dog facilities at Monarto Zoo. This was an integral aspect in getting this research project started. The assistance from all involved was greatly appreciated. Financial support was provided by The Sir Mark Mitchell Research Foundation, South Australia with vaccines provided by Pfizer Animal Health Australia (Zoetis). As a recipient of a full scholarship acknowledgement must be given to the Australian Government Research Training Program. Mention should also be given to Paul Burr from Biobest Laboratories, Edinburgh in discussing antibody titre levels in canids; the volunteers that assisted me with the unenvious task of 'poo pick up'; and to the anonymous manuscript reviewers.

List of Acronyms

Abbreviation	Definition of Term
ACTH	Adrenocorticotropic
APVMA	Australian pesticides and veterinary medicines authority
AUC	Area under the curve
AZ	Adelaide zoo
CDV	Canine distemper virus
CITES	Convention on international trade in endangered species
CMS	Convention on the conservation of migratory species of wild animals
CPI	Canine parainfluenza
CPV	Canine parvovirus
CSG	Canid specialist group
DNA	Deoxyribonucleic acid
DOI	Duration of immunity
EIA	Enzyme immunoassay
ELISA	Enzyme-linked immunosorbent assay
FGC	Faecal glucocorticoid concentrations
FPV	Feline panleukopenia virus
GA	General anaesthesia
gMFI	Geometric mean fold increase
gMFR	Geometric mean fold ratio
GMR	Geometric mean ratio
GMT	Geometric mean response of titres
HI	Hemagglutination inhibition
HPA	Hypothalamic-pituitary-adrenocortical
HPLC	High performance liquid chromatography
IM	Intramuscular
IUCN	International union for conservation of nature
KAZA TFCA	Kavango Zambezi transfrontier conservation area
MCV	Minute virus of canines
MZ	Monarto zoo
NP	National park
OD	Optical density
OS	Oxidative stress
PHVA	Population and habitat viability assessment
PVA	Population viability analysis
ROC	Receiver operator characteristic

ROS	Reactive oxygen species
RS	Reactive species
RWCP	Range wide conservation program for Cheetah and African Wild Dogs
SC	Subcutaneous
SD	Standard deviation
SR	Seroprotection rate
TBCA	Selous-Niassa transboundary conservation area
TMB	Tetramethylbenzidine
TWPZ	Taronga western plains zoo
WAZA	World association of zoos and aquariums
WORZ	Werribee open range zoo
ZAA	Zoo Aquarium Association

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CHAPTER 1
INTRODUCTION

1.0 Carnivore conservation

The average extinction rate of vertebrate species has risen over the last century to levels 100 times greater than the background extinction rate (Ceballos *et al.* 2015). Habitat destruction is regarded as the most significant threat with competition from invasive species, over exploitation, persecution and natural stochastic events also having an impact on species abundance (Chapin *et al.* 2000; IUCN 2017b; Morrison *et al.* 2007). The International Union for Conservation of Nature (IUCN) estimates that just over a fifth (22%) of the world's mammals are known to be globally at risk of extinction (IUCN 2017b). Protecting species in their natural habitat is a primary conservation action but where there are severe population declines additional measures are typically required. Maintaining species outside their natural range through *ex situ* (captive) management is one conservation tool used to preserve the survival of threatened species. Fully integrating *ex situ* management into species conservation planning processes can ensure greater support for *in situ* (wild) conservation actions and species recovery. Similarly, better linkage between *ex situ* and *in situ* conservation management can be developed through synergies in targeted research areas. In this thesis the Endangered African Painted Dog (*Lycaon pictus*) is used as a focal species, identifying disease as a common research area for both *in situ* and *ex situ* populations. This research will focus on the complementary use of *ex situ* populations to assist *in situ* conservation efforts. Broadly, it aims to increase the contribution that captive animals have in devising better conservation management strategies.

Animals from the order Carnivora are the most species rich and ecologically diverse group of all mammals (Goswami & Friscia 2010; Viranta 2009). Substantial evidence indicates that large carnivores in particular, are important to ecosystems as they facilitate and maintain ecosystem function (Wilmers *et al.* 2003). In systems where large carnivores are present, it has been observed that they can exert a controlling influence on species below them on the trophic ladder (Berger 1999; Borer *et al.* 2005; Miller *et al.* 2001; Ray *et al.* 2005b). Top-level carnivores act both causatively and non-causatively in the promotion of species richness; can subsidise scavenging species; and change disease dynamics (Ripple *et al.* 2014; Sergio *et al.* 2008). Large carnivores also have wide spread ecosystem effects by influencing prey species behaviour, which has seen changes to carbon sequestration, stream morphology and rates of crop damage (Terborgh & Freeley 2010). A lack of carnivores in

naturally occurring areas is attributed with a reduction in ecosystem integrity, which can subsequently be accompanied by an increase in local extinctions (Sinclair 2003; Terborgh *et al.* 1999).

The future of many large carnivores is uncertain with many species having a declining population trend (IUCN 2017a). An international review of the conservation status of 30 large carnivores from across 5 taxonomic groups indicated that 22 species were cited as being threatened by the IUCN (Fuller 1995). More than half of these species were characterised as being poorly studied, suggesting there is continued scope for research in multiple areas to provide a greater understanding into the conservation of large carnivores. With consideration to only the terrestrial species from Fuller (1995) and those listed in the IUCN Red List, (equating to 27 species) more recent statistics show the majority of threatened large carnivores are distributed across India, China, the Russian Federation, as well as, the USA and Canada (IUCN 2009). The African continent, however, shows not only a consistent number but also a similar composition of species to be at risk with a collective loss of these species having a significant impact on ecosystem function. From this small group of species the Ethiopian Wolf (*Canis simensis*), African Painted Dog, Cheetah (*Acinonyx jubatus*) and Lion (*Panthera leo*) were identified as being in crisis (Ray *et al.* 2005a; Ripple *et al.* 2014; Winterbach *et al.* 2012).

The wide-area requirements of many large carnivores have posed significant challenges in the conservation of these species. Maintaining reserve networks large enough to sustain viable populations of top-level carnivores has emerged as an overarching conservation objective (Soulé & Terborgh 1999). Many carnivores are not only vulnerable to extinction and require single species management, but also fit into alternative conservation schemes that reflect different conservation initiatives, e.g. umbrella or flagship species concepts (Gittleman *et al.* 2001). Regardless of the strategy applied to protect these species, carnivores must be permitted to exist at ecologically effective levels to fulfil their biological role (Clark 1999; Sinclair 2003; Terborgh *et al.* 1999).

Conservation through *in situ* approaches should be the primary means of maintaining carnivore populations and the ecological services they provide. In today's world, however, this is becoming increasingly difficult with rising human populations requiring greater space and imposing additional pressure on a full range of species. Conserving carnivores *in situ* by protecting important habitat patches, augmenting existing populations by releasing additional animals, or through the reintroduction of species to areas from which they have

been extirpated are all conservation strategies employed to maintain species persistence. However, for these approaches to be effective an understanding of the given species population viability involving demographic, genetic, behavioural, life history characteristics and social acceptance are needed to increase the likelihood of success (Sarrazin & Barbault 1996). Collectively there must be a general shift in conservation thinking from viewing top-level carnivores as isolated parts of ecosystem management, to viewing the maintenance of viable populations of these species as essential components of an integrated system of sustainable ecosystem management.

Reintroduction biology inherently links *in situ* and *ex situ* management through the use of integrated science-based research. This scientific discipline has grown exponentially since the 1990s and provides opportunities to develop and improve conservation outcomes for free-ranging animals (Armstrong & Seddon 2008). Beyond establishing and supplementing extant populations to maintain viability, *ex situ* conservation provides opportunities to educate the public on relevant conservation issues and gather detailed information on life history and behavioural traits (Jones & Merton 2012). Captive management can also allow for the fine tuning of intensive management procedures (e.g. capture, breeding) or can facilitate the development of alternative management strategies (e.g. minimising disease risk). In some situations such information can be challenging to obtain from wild animals. The IUCN (2002) suggest that effective integration between *in situ* and *ex situ* approaches should be sought wherever possible. The World Association of Zoos and Aquariums (WAZA) supports this, recommending that conservation actions be viewed along a continuum of management intensity to increase the contribution of zoos (and aquariums) to global biodiversity conservation (WAZA 2013).

1.1 Project outline

In the wild the African Painted Dog lives at low densities and is a likely end point for disease transmission from other species (Fanshawe *et al.* 1997). For this carnivore infectious disease has been responsible for both dramatic die-offs and localised extinctions (Woodroffe *et al.* 2004b). This has resulted in disease being categorised as a significant conservation area with its management not only important for small naturally occurring populations but also for those participating in conservation programs such as translocation, captive management and reintroduction (Mills *et al.* 1998). Disease can also present a significant risk in animal movement protocols and the transportation of individuals between zoos and nature reserves (Miller 2007). Control and prevention of all significant

diseases (e.g. rabies, distemper, parvovirus) has been identified as one priority issue for the African Painted Dog (Mills *et al.* 1998).

For captive populations two priority research areas were identified within the Population and Habitat Viability Assessment (PHVA, Mills *et al.* 1998), being drug and vaccine testing. Vaccination is a primary method used to increase survival rates during major disease outbreaks and is frequently employed to minimise the effects of infectious disease on an individual (Walsh 2013). Increasing the understanding of canine vaccines that protect against diseases that exist both within the African Painted Dogs' natural range and those that are endemic to locations where animals are held (e.g. captive facilities) are increasingly important for developing effective mitigation strategies. However, for vaccination programs to be a cost-effective conservation tool, consideration needs to be given to the efficacy of a vaccine and the proportion of the population that needs to be inoculated to decrease disease transmission rates (Plumb *et al.* 2007). Even with major advances in vaccine technology there is still a need to gather basic knowledge on immune responses and effectiveness of vaccine delivery methods.

Disease studies targeting the African Painted Dog have focused primarily on highly pathogenic diseases such as rabies and distemper (CDV), with minimal research conducted on other viral diseases. One such disease is canine parvovirus (CPV) which is an emerging and re-emerging pathogen that can have significant impact on small or recovering wild populations, e.g. Grey Wolves *Canis lupus* (Barker & Parrish 2008; Mech 1995). Young animals are most susceptible posing a major issue to recruitment rates and general pack survival. From field studies more generally however, there has been very little information obtained on the potential impact that this infectious disease has on wild canid populations. To expand on existing knowledge relating to the African Painted Dog and CPV, this study will explore the immunogenicity of one vaccine used as a prophylaxis. As most vaccines administered to endangered species are used outside of their registered purposes (i.e. off-label with respect to target species), applied research through medical trials can only assist conservation scientists with more informed options when implementing programs to minimise disease risk.

While there are significant benefits to implementing a vaccination program there can also be a number of drawbacks (Ewen *et al.* 2012). These include, for example, the choice of vaccine and the risk of injury or stress on an individual during administration and capture, if required. While it has been debated, there is a belief that stress induced by research

activities (e.g. deploying radio collars, vaccination) has contributed to the historical decline of African Painted Dogs in some parts of Africa (see Burrows 1992; 2011b; 1995b; East *et al.* 1997). This is in contrast to other researchers who do not accept this viewpoint (refer to Creel et al. 1996a; 1997b; De Villiers et al. 1995; 1997; Macdonald 1993; Woodroffe 2001). Stress related research in this species has focused on a small number of areas with there being a lack of information on how acute physiological changes can lead to a chronic stress response. The inability of an individual to return to a normal functioning level has the potential to lower immuno-competence and therefore increase an animal's susceptibility to a disease. It is this maladaptive response which is central to the handling-stress hypothesis proposed by Burrows (1992). Understanding how individuals respond and then cope (or not cope) after being repeatedly exposed to stressful situations can be important for assessing and redeveloping more appropriate management practices.

To assess capture stress adequately in the African Painted Dog greater information is required both at the time of a procedure, as well as, through non-invasive means. Non-invasive techniques are commonly utilised in wildlife species including the African Painted Dog (Creel 2001; Schwarzenberger 2007). However, the correlation between the stress hormones measured by invasive and non-invasive methods has not been investigated in this canid. This research will not only seek to address this gap in knowledge but will also expand on basic information on the feedback mechanisms in the primary physiological system (i.e. hypothalamic-pituitary-adrenal axis) that is responsible for the production of stress hormones. Such investigations collectively give a more holistic view of how dogs respond to capture and the possible relationship that this has in broader management activities like vaccination.

1.1.1 Research aim and objectives

The primary aim of this study is to investigate the immunogenicity of a vaccine administered to captive bred African Painted Dogs. A subsequent aim will be to examine the degree of stress individuals experience when immobilised. Increasing the knowledge of factors that can influence the management or success of a conservation program is critical in threatened species management.

The key objectives of the thesis are to:

1. Review background information on the natural history of the African Painted Dog
2. Document the use of commercially available vaccines in the African Painted Dog and the associated serological responses
3. Assess the immunogenicity of a vaccine protocol designed for use in domestic dogs to determine whether this regime provides an adequate level of protection in African Painted Dogs
4. Examine whether the delivery method utilised in the vaccination trial affects the seroprotection rate
5. Investigate the concept of stress in the African Painted Dog and the effect it has had on population persistence
6. Monitor the stress response of captive held dogs during single and repeated handling events by invasive and non-invasive means
7. Discuss possible management implications for captive and free ranging populations

1.2 Thesis structure

This thesis is organised as both a review of existing information combined with a number of experimental investigations. The review chapters will provide an understanding and appreciation of known information and assists in formulating each of the experiments. Each experimental chapter includes the following sections: Introduction, Material and methods, Results, Discussion, and Conclusion.

Chapter 2 introduces the life history characteristics of *Lycaon pictus* and its uniqueness among other members of the canid clade. The ecology in terms of habitat and distribution is described along with foraging and breeding behaviours. Reasons for population decline are further discussed in association with the conservation management of this species.

Chapter 3 presents an overview of the literature that focuses on the major threatening processes, infectious disease, for endangered canid populations. In Australia canine parvovirus is the greatest viral threat to dogs. The aetiology of the canine parvovirus pathogen and its prevalence both in Australia, as well as, within free-ranging African Painted Dog populations will be described, thus having relevance to both *in situ* and *ex situ* populations. This chapter also consolidates research on the use and effectiveness of different vaccines utilised in *L. pictus*.

Chapter 4 describes the experimental results of a vaccination trial targeting the African Painted Dog and the inactivated Parvac™ (Pfizer 2010) vaccine. The immunogenicity of the vaccine and its effectiveness in providing an adequate level of protection in the target species is assessed. Alternative administration routes are compared to establish possible differences in delivery methods.

Chapter 5 details the role that stress plays in conservation and brings together a body of research that associates the localised decline of African Painted Dog to handling. Studies involving African Painted Dogs and glucocorticoids are examined highlighting the specific metabolite used and the stressor targeted.

Chapter 6 is an experimental chapter that investigates capture physiology in a group of captive bred African Painted Dogs. This chapter examines the stress response of animals that participated in the vaccination trial, as well as, those that undergo routine management activities. The primary glucocorticoid metabolite, cortisol, is targeted and monitored through both invasive and non-invasive means.

Chapter 7 brings together the body of research presented in this thesis. The implications for management are discussed as a result of the findings made in the experimental chapters. This is accompanied by the limitations found in each approach and directions for future research.

2.0 Introduction

There are approximately 36 species of canids globally with the African Painted Dog (*Lycaon pictus*, Temminck 1820) regarded as the second most endangered canid on the African continent, after the Ethiopian Wolf (Carlson *et al.* 2004; Macdonald & Sillero-Zubiri 2004). Like most wide-ranging low density species, this canid is highly susceptible to habitat loss and fragmentation with dramatic declines in populations occurring in the last 100 years (Woodroffe *et al.* 1997; Woodroffe & Sillero-Zubiri 2012). The IUCN Red List of Threatened Species reassessed the conservation status of this canid in 2012 and reaffirmed it as Endangered, C2a(i) (Woodroffe & Sillero-Zubiri 2012). The African Painted Dog is not currently listed on CITES, the Convention on International Trade in Endangered Species despite some concern over legal and illegal trade (CITES 2007). In 2009 the African Painted Dog was listed in the Convention on the Conservation of Migratory Species of Wild Animals (CMS), Appendix II with a resolution in 2014 designating 'Concerted Action' is needed to conserve this species (CMS 2016). The following sections aim to describe the natural history of *Lycaon pictus* while also discussing reasons for decline and current management. This overview addresses the first of the study objectives.

2.1 Taxonomy and phylogeny

The naming and classification of *Lycaon pictus* has evolved over time with Temminck (1820) naming a Mozambique specimen as *Hyaena picta* (Reich 1981). The use of *Hyaena* as the genus name exemplified the immaturity in classifying carnivores during that period (Reich 1981). In later decades and with the availability of large fossil collections, a system in keeping with Linnaeus' (1758) hierarchy was established and refined so inferences could be made between carnivorous species according to morphological similarities in dentition (Wozencraft 1989). This development later led to a changing of the scientific name for this species from *Hyaena* to the Greek derived word *Lycaon* referring to 'wolf' (Mivart 1890). The species name, *pictus*, originates from Latin and translated means 'painted'. Collectively both terms refer to this species as the 'Painted Wolf' (Creel & Creel 2002). Other common names frequently used in the English language for this canid are Cape Hunting Dog, Hyena Dog and African Wild Dog (Mivart 1890; Sillero-Zubiri *et al.* 2004). Breuer (2002) describes alternative non-English names as Cynhyène, Afrikanischer Hyänenhund, Wildehond, Mbwa mwitu and Safandou originating from French, German, Afrikaans, Swahili and Foufoulbe respectively. No subspecies are formally recognised (Girman *et al.* 2001; Girman & Wayne

1997) but Kingdon (1997) suggests there are five subspecies including *Lycaon pictus lupinus, L. p. managuensis, L. p. pictus, L. p. sharicus* and *L. p. somalieus.*

Anthropological studies in association with archaeological excavations have revealed a rich fossil record of wolf-like canids, with a vast majority being millions of years old (Nowak & Federoff 1998). Wang *et al.* (2008) describe the rapid evolution of *Canis* during the Pleistocene (approx 1.8 Ma) as the Wolf Event, being associated with significant changes in species distribution resulting from extreme continental glaciations. Some of the larger *Canis*-like lineages subsequently became specialised through the development of different food behaviour strategies. Species included in the *Canis falconeri* group evolved eventually giving rise to the widely dispersed hypercarnivore *Xenocyon* in Eurasia (Martínez-Navarro & Rook 2003; Wang *et al.* 2008). Morphologically the genus *Lycaon* and *Cuon* (i.e. Dhole) are considered descendants of this lineage and are monophyletic to *Canis* (Martínez-Navarro & Rook 2003; Wang *et al.* 2008). Fossils categorised as belonging to the *Lycaon* genus date back as early as the Middle Pleistocene (Elandsfontein, South Africa) with recorded occurrences in Europe providing a link between other wolf-life canids and the modern African Painted Dog (Wang *et al.* 2008; Wayne 1989). The evolution of canids specifically on the African continent is, however, considered to be poorly understood (Wang *et al.* 2004; Wang *et al.* 2008).

In the past there has been some ambiguity in the phylogenic positioning of the African Painted Dog (Girman *et al.* 1993). This species is the only representation of its genus and is classified within the wolf-like canid clade, to which *Canis* and *Cuon* also belong (Girman *et al.* 1993; Marsden *et al.* 2009; Wayne 1989). *Lycaon pictus* was formally placed in a separate sub-family *Simocyoninae* but this family sub-division has since dissolved and is no longer recognised (Woodroffe *et al.* 1997; Wozencraft 1989). A more recent taxonomic classification positions *Lycaon pictus* in the Subtribe Canina, Fischer De Waldheim, 1817 along with Wolves and Dholes (Wang *et al.* 2008).

Wayne *et al.* (1997) conducted a molecular analysis of 2001 base pairs from 26 canid species which supports an independent origin for *Lycaon*, with *Cuon* and *Canis* being more closely related. The African Painted Dog is considered basal to both *Cuon* and *Canis* sharing a common ancestor with the South American canid clade (i.e. Bush dog, Maned Wolf). This group is regarded as the sister taxa group to the wolf-like canids (Wang *et al.* 2004) with respective phylogenic relationships shown in Figure 2.1(a). Sequencing data indicated a

divergence of 11.3-13.7% for African Painted Dogs comparative to other canid-like species (Creel & Creel 2002).

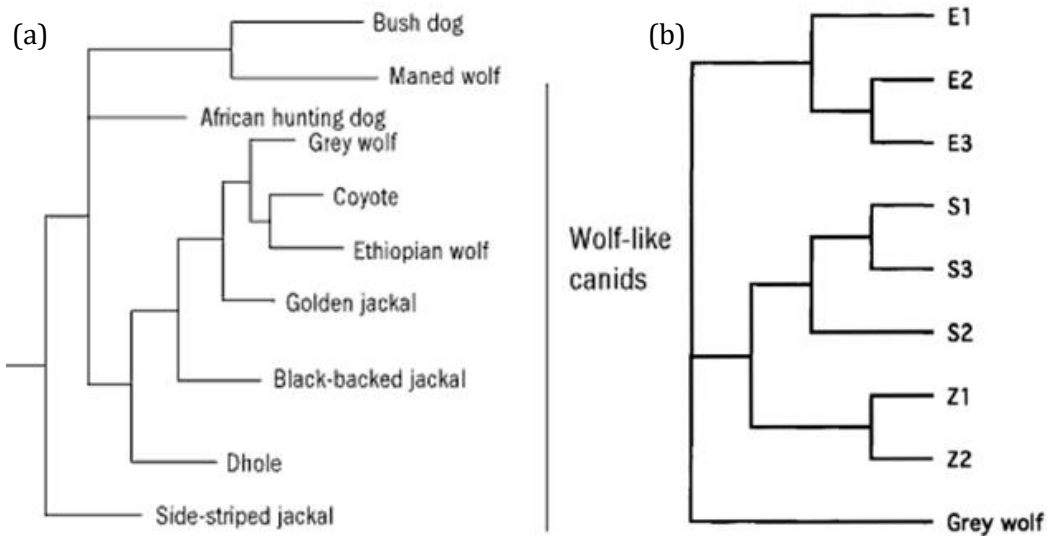


Figure 2.1. Molecular relationship of *L. pictus*
(a) Phylogenic relationship based on molecular analysis of multiple canid species conducted by Wayne *et al*. (1997). Source: Wang *et al*. (2004).
(b) Subdivision in *Lycaon pictus*: genotypes E1 and E3 are found in the east African populations, whereas S1, S2, Z1 and Z2 genotypes are recorded in only southern African populations. Source: Girman & Wayne (1997).

For *Lycaon pictus* itself, morphological and genetic differences have indicated that there is some independence between the southern, western and eastern populations of Africa (Girman *et al.* 1993; Marsden *et al.* 2012). Girman *et al.* (2001) analysed the molecular variance in microsatellite data and mitochondrial DNA (mtDNA) to assess the hierarchy of population subdivision and patterns of gene flow between sampled geographically isolated locations (Kruger, Hwange, Serengeti Nat. Park). The most parsimonious analysis showed two distinct clades (Figure 2.1(b)) resulting from two independent haplotypes (Girman & Wayne 1997). The divergence in the sequences was calculated at 1% providing grounds for a subspecies classification (Creel & Creel 2002; Girman *et al.* 1993). This uniqueness was particularly evident at the respective ends of this species geographic range (Kruger and Serengeti Nat. Park) with a recent mixing of the different clades in the intermediate populations (Girman *et al.* 2001; Girman & Wayne 1997; Marsden *et al.* 2012).

2.2 Morphological and phenotypical characteristics

The African Painted Dog is a large but lightly built canid with long slim legs and large rounded ears. *Lycaon pictus*, unlike other canids, have only four digits on their paws instead of five with the front paws usually larger and broader than those of the hind paws (Woodroffe *et al.* 2004b). The pelage of this species is distinctive and is typically comprised

of a tri-coloured mosaic pattern consisting of black, yellowy-tan and white organised in irregular patches that vary from individual to individual (Reich 1981; Creel & Creel 2002). Refer to (Figure 2.2).

Figure 2.2. Lycaon pictus with its distinctive tri-coloured pelage

Skinner and Chimimba (2005) suggest that the hair on the shoulders of an African Painted Dog is approximately 40mm long with a slight increase in length down the mid-back area and around the collar. The hair extending from the ears and the sheath of the penis of male dogs are the only other distinguishable areas that are noted to have long course hairs, with both resulting from the presence of sebaceous glands in the skin of the respective areas (Skinner & Chimimba 2005). In converse, hair along the limbs is considerably shorter in length and more sparse with limited or no undercoat noted on the main body and pants (Skinner & Chimimba 2005).

Due to the wide variation in coat pattern, individuals are often easily identified by their unique markings (Creel & Creel 2002). Sillero-Zubiri *et al.* (2004) suggest that animals found in Southern Africa tend to have a brighter coat colour compared with individuals found in the east of the continent. Across the species more generally, however, African Painted Dogs tend to have some consistent phenotypical characteristics in the tail and the head. These typically include a yellow-tan head with a black muzzle that extends into a line along the sagittal crest (Breuer 2002; Kingdon 1997). The large ears that help this animal stay cool and improve hearing, are generally dark with the rim, as seen by an anterior view, fringed in black. This is coupled with noticeable white tufts of hair that emanate from the

lower portion of the outer ear (Skinner & Chimimba 2005). Most dogs are also noted to have a distinctive black band around the tail delineating the white tip from the remaining tail or hindquarters. Creel & Creel (2002) indicate that tail markings can be broken down into a number of classes that can aid in the easy identification of individuals.

The longevity of most wild individuals is between 6-7 years with few living beyond 10 years (NNF 2009), while captive individuals have been known to live an average of 15 years with a maximum of 17 years recorded (Nowak 1999; Weigl 2005). The typical weight range for this species is between 18 and 28kgs with males being slightly larger than females (de Magalhaes & Costa 2009; Skinner & Chimimba 2005). Creel & Creel (2002) provide general body measurements for this canid from a study conducted in Selous GR, Tanzania with the mean back and toe to shoulder lengths of male dogs being 81.4cm and 72cm respectively. Whilst, the tail lengths can range between 30-40cm (Stuart & Stuart 2001).

It has been reported that this canid is sexually monomorphic (Girman *et al.* 1993), but Creel & Creel (2002) found that females had a statistical difference of approximately 4% in corresponding skeletal size when compared to their male counterparts. Similarly, the size of the skull had slight variation between male individuals with the average length being 25.6cm. For female dogs the recorded skull length was smaller with a divergences of up to 5% (Creel & Creel 2002). Studies conducted in Southern Africa have also recorded characteristic differences in the apparent size of each of the sexes (e.g. NNF 2009, Sillero-Zubiri *et al.* 2004).

African Painted Dogs have 42 teeth organised as $I\frac{3}{3}\ C\frac{1}{1}\ P\frac{4}{4}\ M\frac{2}{3}$ (Skinner & Chimimba 2005). Martínez-Navarro & Rook (2003) state that in association with some of the other *Canis*-like lineages the *Lycaon* genus has specialised towards obligate carnivory. Dentition of this canid has evolved similarly to *Cuon* and *Speothos* in that the inner cusp of the talonid is absent. Wang *et al.* (2004) indicate that typically this part of the tooth creates a basin but in African Painted Dogs, Dholes and *Speothos* this section of the tooth forms an alternative subsidiary blade. This adaption has been related to animals that have a greater emphasis on holding and slicing their food rather than grinding, indicating that the diet of these species is highly predacious (Ewer 1973).

The shape of the cranium varies markedly in canids reflecting the diversity of food strategies within the group. Morphologically the skull of *L. pictus* (Figure 2.3) is described as being somewhat shorter and thicker set when compared to other canid-like species

(Mivart 1890). As shown by the dorsal view the sagittal crest is well developed in African Painted Dogs, where it then joins the supra-occipital crest and branches into two heavy ridges terminating at the postorbital bars (Skinner & Chimimba 2005).

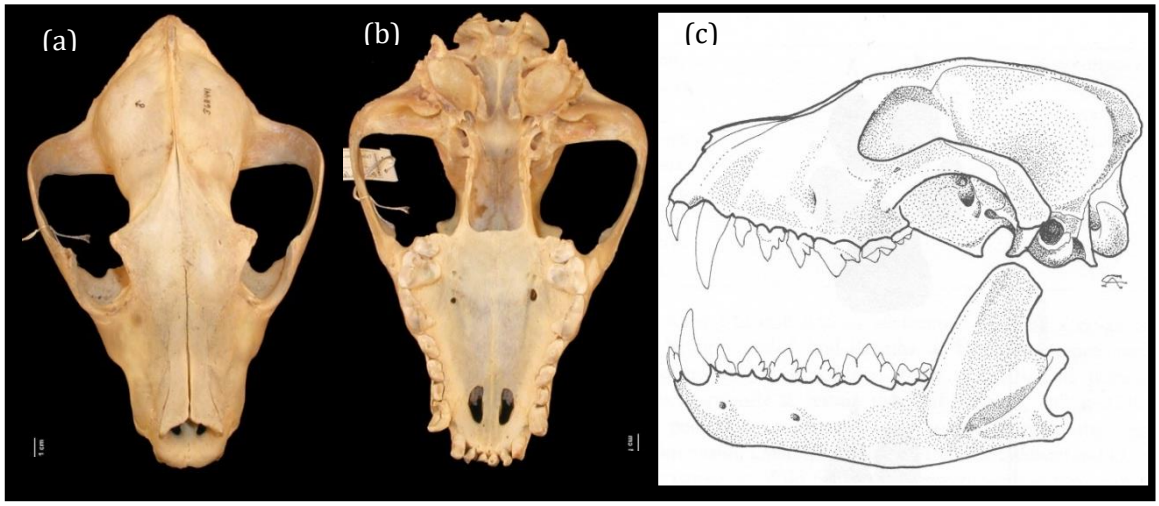


Figure 2.3. Morphological characteristics of a Lycaon pictus skull: (a) dorsal, (b) ventral, (c) lateral. Source: van Valkenburgh (2006) and Skinner & Chimimba (2005).

The zygomatic arches are also thickset and extend broadly from the skull, allowing for the attachment of well-developed temporalis and masseter muscles (Skinner & Chimimba 2005). Wroe *et al*. (2005) suggest that skull width is a reliable predictor of bite force in carnivores. This study showed that the measure, bite force quotient, was greatest for obligate carnivores that typically prey upon animals with a larger body mass than themselves. Of the 31 extant species (across 6 family groups) examined, the African Painted Dog was found to have the highest bite force quotient for all placental mammals, preceded by two marsupials i.e. the Tasmania Devil (*Sarcophilus harrisii*) and Spotted Quoll (*Dasyurus maculatus*).

2.3 Ecology

2.3.1 Habitat

Fanshawe *et al.* (1991) describe the widespread geographic range of *L. pictus* being across most habitat types that also had a high prey abundance. African Painted Dogs are therefore considered habitat generalists historically existing in all ecosystems but the Congo Basin rainforests and true deserts (Mills *et al.* 1998; Woodroffe *et al.* 1997). NNF (2009) indicate that this canid has been observed above the snow-lined slopes of Mt. Kilimanjaro, as well as, the desert plains of Namibia after periods of good rain. Populations seem unable to maintain residency in areas with less than 200mm annual rainfall Woodroffe *et al.* 2004b).

Accounts that are more recent suggest that this carnivore still exists in a diverse array of habitats types, which include semi-deserts, grassy plains, savannahs, upland forests, and to a lesser degree montane habitats and higher alpine areas (Creel & Creel 2002; IUCN/SSC 2007b; Malcolm & Sillero-Zubiri 2001; Woodroffe *et al.* 2004b). Hayward *et al.* (2006) consider the preferred habitat of African Painted Dogs are savannah-woodlands.

2.3.2 Distribution and population trends

Lycaon pictus is believed to have had a widespread historical distribution with approximately 250,000 dogs once estimated to occur throughout the African continent (Fanshawe *et al.* 1991; NNF 2009). Previously recorded in 39 African countries throughout the sub-Sahara, there has been a considerable retraction in this species range (Sillero-Zubiri *et al.* 2004; Woodroffe *et al.* 1997). It is now believed that African Painted Dogs occupy just 7% of their historical range with population estimates being around ~6,600 individuals with only ~1,400 being mature individuals (Marsden *et al.* 2012; Woodroffe & Sillero-Zubiri 2012). African Painted Dogs naturally occur at low densities, comparative to other carnivores, and range more widely to avoid larger carnivores such as Lions and Hyenas (Mills & Gorman 1997). This species' historical distribution coincides with vegetation types whose significant zonal distribution is heavily influenced by precipitation patterns (Okitsu 2010; Wang 2000). Figure 2.4 reflects both the historical and current distribution of *L. pictus* at a continental scale.

Population declines were first noted during the 1970s prompting Fanshawe *et al.* (1991) to conduct a pan-African survey (1985-88) to determine the species' continental status (Ginsberg 1993). Conclusive evidence showed the decline of this carnivore was significant as considerable absences and reductions in geographic range had been documented (Ginsberg 1993). Reflecting on the increased threat to the persistence of this species, the

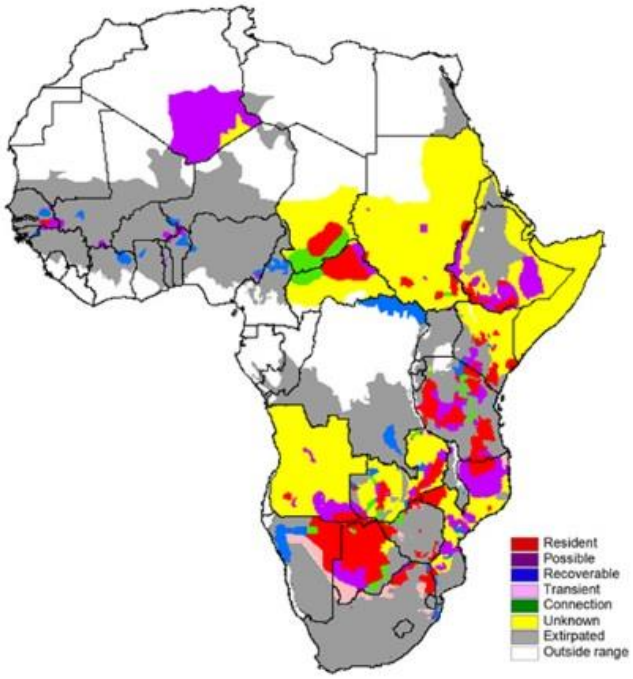


Figure 2.4. Geographic distribution of African Painted Dogs across the African continent, updated 2016 (RWCP 2017b).

IUCN revised the conservation status of the African Painted Dog in 1990 from Vulnerable to Endangered (Ginsberg 1994b; McNutt *et al.* 2008). Later surveys, as summarised by Fanshawe *et al.* (1997) and during the IUCN SSC range wide conservation process (IUCN/SSC 2007a; b; 2012), assessed the remaining populations as only 18 of the 39 countries once inhabited with a declining population trend. In North and West Africa Painted Dogs have been seemingly eradicated with significant reductions also recorded in Central and North-east Africa. Countries where dogs are confirmed residents include Angola, Benin, Botswana, Burkina Faso, Central African Republic, Chad, Ethiopia, Kenya, Malawi, Mozambique, Namibia, Sengal, South Africa, South Sudan, Sudan, Tanzania, Zambia and Zimbabwe (NNF 2009; RWCP 2016; Skinner & Chimimba 2005; Woodroffe *et al.* 1997; Woodroffe & Sillero-Zubiri 2012). Woodroffe and Sillero-Zubiri (2012) indicate that there are two hotspots of abundance with the largest populations found in Southern Africa (northern Botswana, western Zimbabwe, eastern Namibia, and western Zambia) and the southern area of East Africa (i.e. Tanzania and northern Mozambique).

2.3.3 Home range

The general term ' home range', which here encompasses the concepts of home range and territories, is defined as areas that meet the energy requirements of an individual or group and are where animals conduct their normal daily activities, including foraging, selecting mates and raising young (Andreka *et al.* 1999). Compared to other canid species, African Painted Dog home ranges are considered extremely large and non-defensible (Gittleman *et al.* 2001). The annual pack range size can vary substantially from 150-3,800km^2 depending on the density and temporal distribution of prey and the structure of the habitat (Fuller *et al.* 1992; Mills & Gorman 1997). Schaller (1972) states that the average annual range size used by this species is typically around 650km^2, with this area likely to change depending on the environmental and anthropogenic influences being exerted on habitats. African Painted Dogs found in ecosystems that are predominantly wooded (e.g. Selous National Park (NP), Kruger NP) and whose prey species are more sedentary are considered to have a smaller than average home ranges (Mills & Gorman 1997). This is starkly different from semi-arid habitats such as the north-east of Namibia where home ranges can exceed 3,000 km2 (NNF 2009). In habitats where the migration of ungulates is more pronounced (e.g. Serengeti NP) home ranges are slightly larger than average being around ~665km^2 (Schaller 1972; Woodroffe *et al.* 2004b). Research into African Painted Dog movement and habitat preference has indicated that grassy plains are less preferred than other more densely or wooded habitat types (Creel & Creel 2002).

During breeding season (approx. 3-5 months of the year) the pack will hunt in a more restricted area around their den site (Burrows 2011a). Population densities also vary depending on habitat type with animals that occupy grassy plains (i.e. Serengeti NP) having mean densities of 1 adult per 208km^2 (Burrows *et al.* 1994), while in woodland habitats (i.e. Selous Game Reserve) densities have been recorded as three to nine times higher with one adult per 25km^2 (Creel & Creel 1996; Creel *et al.* 1997c). As shown by the Northern Selous population, packs do not visit all parts of their territory equally preferring to spend more time in the centre of their range rather than at the edges (Creel & Creel 2002). Home range overlap between packs in this geographic region averaged between 25-35% and was relatively consistent with other populations in Kruger NP and the Serengeti (Creel & Creel 2002; Reich 1981; Schaller 1972). African Painted Dogs typically have larger home ranges and range more widely than required to fulfil their energetic requirements to increase avoidance and limit interspecific competition with other carnivores (e.g. lions).

2.3.4 Diet and foraging

In the wild African Painted Dogs feed almost exclusively on mammalian prey that they have hunted and killed and rarely scavenge (Childes 1988; Mills & Biggs 1993). This obligate carnivore is a crepuscular hunter generally pursuing prey on a daily basis during the twilight hours of 0500-0900hrs and 1730-1930hrs (Creel & Creel 1995; Malcolm 1999). Creel & Creel (2002) indicate that the size range of prey species varies considerably with recorded predation occurring on small species weighing 1 to 2kg. Such species include the Bat-eared Fox (*Otocyon megalotis*, Rasmussen 1996) and various species of Hares (*Lepus spp, Pedetes capensis*, Fuller & Kat 1993), which make only a very small contribution to their diet. At the other end of the spectrum, Creel & Creel (2002) indicate that African Painted Dogs can predate much larger herbivore species with body masses approximating 200kg; being adult Zebra (*Equus burchelli*), juvenile Buffalo (*Syncerus caffer*) or Eland (*Taurotragus oryx*). More typically African Painted Dogs tend to predate ungulates within a weight range of approximately 10-120kg (Creel & Creel 2002). Research has shown that as the number of adults within a pack increases so does the degree of hunting success, the mass of the prey target, probability of multiple kills, and subsequently the net gain benefit (Creel 1997a; Creel & Creel 1995; Fanshawe & Fitzgibbon 1993; Hayward *et al.* 2006). Larger packs are also better able to defend kills from other carnivores such as kleptoparasitic Hyenas (Fanshawe & Fitzgibbon 1993).

Hayward *et al.* (2006) analysed 18 studies encompassing 5 countries within the extant African Painted Dog populations to assess their preferred prey. This study identified that dogs had a preference for species with a mean weight range of 50.8 (+/- 28.3kg), from moderately sized herds that did not impose significant threat of injury (Hayward *et al.* 2006). These favoured prey species include Thomson's Gazelle (*Gazella thomsonii*), Greater Kudu (*Tragelaphus strepsiceros*), Impala (*Aepyceros melampus*) and Bushbuck (*Tragelaphus scriptus*). Reich (1981) indicates that three of these ungulates have an increased preference for thicker vegetative areas associated with woodland savannah habitats. This suggests that African Painted Dogs require more effort to discover these species but less time to chase down and capture their prey (Hayward *et al.* 2006; Reich 1981). Consequently, African Painted Dogs do not only rely on their sight to identify the direction in which their prey travels, but also use hearing and smell to locate their prey (Creel & Creel 1995). Hayward *et al.* (2006) also suggest that sight is their most important sense.

The foraging behaviour of African Painted Dogs usually begins with a 'social rally' (Creel & Creel 1995). This involves a range of actions including a frenzy of nosing, lip-licking, tail

wagging and circling simultaneously increasing the state of excitement of individuals and making the pack more alert for a co-ordinated hunt (Feldhamer *et al.* 2007). After these intense social interactions the hunt immediately begins with a kill typically occurring within 30 minutes (Boinski & Garber 2000). Once a prey species has been sighted, the pack will chase the ungulate herd testing individuals for their relative fitness before one animal is selected and chased by many dogs. Despite this event being a co-ordinated attack, the pack can opportunistically split targeting more than one animal. Depending on the preys' size, one dog will try and target the nose of its quarry while other dogs bite the hind legs and abdomen region to bring it down, killing the prey through disembowelment. African Painted Dogs can reach speeds of up to 60km/hr during a chase and are specially adapted to conserve water whilst in pursuit, thus extending the length of time they can run before they have to rest or seek water (Sillero-Zubiri *et al.* 2004; Taylor *et al.* 1971).

Visee (2001) describes the social etiquette and behaviour of free ranging African Painted Dogs when feeding at a kill, with pups and the alpha female having priority. Pups use a variety of vocalisations and behavioural postures to keep other dogs from encroaching on the carcass. While the alpha pair socially reinforces this access, they, along with other adult dogs stand guard and drive away potential scavengers. After the pups and alpha female are satiated, animals that are considered yearlings (aged 12-24months) move into feed followed by the alpha male and the remaining subordinate adults which consume what is remaining. All pack members not only regurgitate food for pups but also for those individuals which are old, sick or injured (Estes & Goddard 1967; Malcolm & Marten 1982).

2.3.5 Social organisation and reproduction

Kin selection is an evolutionarily stable strategy that is present in highly social canids like the African Painted Dog, with dominant and submissive behaviours dictating the establishment of social order (Abrantes 2005). *Lycaon* is an obligate cooperative breeder with two defined hierarchies in a pack, one amongst the males and the other among the females (Burrows 2003; Creel & Creel 2002). Being dominant in a pack typically involves more aggressive interactions to maintain rank with these behaviours intensifying during breeding season. Female dogs are seasonally monoestrous with January to April considered to be their primary breeding season followed by a secondary period of receptivity in June and July (Newell-Fugate *et al.* 2012). A rise in testosterone in male dogs occurs just prior to the primary breeding season and remains elevated primarily during the first month, i.e. January (Newell-Fugate *et al.* 2012). The timing of the breeding season can shift slightly

depending on the geographic latitude and availability of resources within a population (Newell-Fugate *et al.* 2012; Van den Berghe *et al.* 2012).

In general, the alpha pair are usually the only animals that reproduce, with behavioural interactions being the most likely cause of reproductive suppression in subordinates (Van den Berghe *et al.* 2012; Van der Weyde *et al.* 2015). Genetic investigations have shown that subordinate males do regularly mate with dominant females as multiple paternities have been observed in some litters (Girman *et al.* 1997; Spiering *et al.* 2010). In contrast, lower ranked females breed intermittently (6-10% annually) with offspring typically born after the dominant females' litter (Creel *et al.* 1998; Girman *et al.* 1997). There is high mortality in pups born to a subordinate female with many not surviving to 1 year. Despite this, adoption of pups by a dominant female can occur and is suggested as a potentially important management option to augment existing packs (McNutt 1996a). The success in raising a litter of pups by a solitary female is rare in this species with packs rather than individuals typically used as the unit of measurement to monitor the populations (MET 2013; Woodroffe *et al.* 2009).

Gestation in the African Painted Dog ranges between 69-72 days (Monfort *et al.* 1997; van Heerden & Kuhn 1985). Breeding females produce large litters relative to body size, occasionally numbering as many as 22 pups with 8 being the average (McNutt 1996a; van Heerden & Kuhn 1985). African Painted Dogs exhibit an adaptive bias in the sex ratio of litters with primiparous females having a male bias and multiparous females having a female bias (Creel *et al.* 1998; McNutt & Silk 2008). Pups are born blind and deaf with black and white fur. The patterning of the white pelage is distinctive to the individual and is retained into adulthood. The black coloured fur can develop into a variety of colours including brown, tan and gold. Lactation lasts between 56-70 days and eyes and ears open from 10 days (Malcolm & Marten 1982; Woodroffe *et al.* 2009). Pups emerge from the den at the age of 3-4 weeks about which time they start to feed on regurgitated meat (Creel *et al.* 1997c; van Heerden & Kuhn 1985). All other pack members provision both the lactating female and pups with food and provide protection, while some individuals take on more of a babysitting or helper role (Creel & Creel 1995; Girman *et al.* 1997; Malcolm & Marten 1982). The denning period lasting approximately 3 months after which pups start to accompany the pack on hunts (Creel & Creel 1995). Pup mortality can be as high as 70% annually with survival to the age of 1 being dependent on pack size and the breeding females' age (Buettner *et al.* 2007; Creel *et al.* 1997c; McNutt & Silk 2008).

Sexual maturity is believed to occur around the age of 2 years (Frame *et al.* 1979; van Heerden & Kuhn 1985). The average age of dispersing African Painted Dogs has been recorded at 2.64 years, or during the first year of sexual maturity, with the probability of male dogs dispersing significantly declining after this age (Creel & Creel 2002). Dispersal events are triggered predominantly to avoid inbreeding or competition and is one strategy used by sub adult individuals to obtain a breeding position in a newly formed pack (Creel & Creel 2002; McNutt 1996b; Wang *et al.* 2008). Females are 1.5 times more likely than males to disperse from their natal pack and tend to have greater spatial philopatry when establishing a home range (McNutt 1996b). In comparison, male dogs generally delay emigration and typically travel much further distances than females, with this behaviour being indicative of inbreeding avoidance (McNutt 1996b). The vast dispersal distances travelled by this species has contributed to their colonisation success and continental-wide distribution (Wang *et al.* 2008). A critical threshold of five individuals has been established as the minimum number of animals to form a pack, thereby allowing adequate trade-off between breeding and hunting success (Courchamp & Macdonald 2001).

2.4 Reasons for decline

Mankind has been the greatest influence in the continuing population decline of the African Painted Dog. Extinction for this species is predicted within a few decades if the declining population trend is not stopped. While eradication programs in more recent decades have ceased, persecution has continued due to increased contact with humans and their domesticated animals (Fanshawe *et al.* 1991). Accidental mortality along road networks and indiscriminate captures in snares used to harvest bush meat have also contributed to localised population declines (KAZA 2014). McNutt and Silk (2008) suggest that the African Painted Dogs' current distribution is not limited by a specific ecosystem but rather by human activities. Sensitivity to anthropogenic mortality is thought to increase as a result of being an obligate cooperative breeder (Courchamp & Macdonald 2001).

Conservation of the African Painted Dog requires mitigation of threats over an extensive spatial scale (IUCN/SSC 2007b). Habitat loss is a major threatening process with this species being particularly susceptible to fragmentation due to their extensive home ranges and low population densities (Fanshawe *et al.* 1991; IUCN/SSC 2012). A reduction in habitat has also contributed to declines in preferred prey species including, Impala, Greater Kudu and Bushbuck (Hayward *et al.* 2006; Ripple *et al.* 2014). Competition with other large carnivores (e.g. Lion and Hyena) which overlap in range, represent another important cause of mortality in adult and juvenile dogs (Woodroffe *et al.* 2007). Declining populations can

be further affected by demographic stochasticity, inbreeding depression, and genetic drift (Caughley 1994; Hayward *et al.* 2006). As highlighted in Chapter 1, infectious disease is highly episodic causing localised extinction and contributing to the failure of reintroduction attempts (Gascoyne *et al.* 1993a; Hofmeyr *et al.* 2000; Kat *et al.* 1995; Scheepers & Venzke 1995). Conservation management of disease threats is complex as common sympatric canid species, e.g. Jackals and domestic dogs, have a shared susceptibility and act as disease reservoirs (Alexander *et al.* 2010). The influence of environmental stochasticity, specifically high ambient temperatures is considered to have long term impacts on behaviour and fecundity (Woodroffe *et al.* 2017). Modelling of climate change across the range of African Painted Dog populations indicated that for every 1˚C rise in mean daily maximum temperature, there was a 25-day increase in the inter-birth interval with poorer pup production and reduced pack activity (Woodroffe *et al.* 2017). The cumulative effect of these threatening processes has the capacity to drive this canid towards extinction.

2.5 Conservation management

2.5.1 *In situ*

All countries within the African Painted Dogs' current distribution have given this canid either partial or total protection (McNutt *et al*. 2008). Conserving the remaining populations of this species is dependent on greater international co-operation, limiting further fragmentation of habitats and, where possible, improving well connected habitats across an expansive landscape scale (Woodroffe *et al.* 2005). Recognising the conservation challenges in protecting this species and the Cheetah (that shares similar ecological traits, e.g. wide ranging behaviour), the Cat and Canid Specialist Groups of the IUCN/SSC, in collaboration with the Wildlife Conservation Society and the Zoological Society of London formed the Range Wide Conservation Program for Cheetah and African Wild Dogs (RWCP). This initiative has established coordinated conservation actions for both species creating three Regional Conservation Strategies and assisting in the development of seventeen National Conservation Action Plans (RWCP 2017a). Collectively these plans cover >85% of the African Painted Dogs' natural range.

In Southern Africa, transboundary populations account for nearly 90% of the regional dog population with protection considered critical for long term persistence (KAZA 2014). Following the framework of the Regional Strategy and National Action Plans, a Transfrontier Strategy was developed incorporating more than20 Protected Areas across 5 countries (KAZA 2014). The Kavango Zambezi Transfrontier Conservation Area (KAZA TFCA) is estimated to protect approximately 24% of the total remaining dog population.

Similar initiatives have been developed in East Africa with approximately 40% of the regional population occupying transboundary areas (IUCN/SSC 2007a). The Selous-Niassa Transboundary Conservation Area (TBCA), which connects habitat between Tanzania and Mozambique, has been identified as a significant biological link within this region. It is believed to conserve approximately 12.5% of the total remaining dog population (Baldus & Hahn 2009). Countries like Zambia act as an important dispersal link between the southern and eastern regional populations (WWF 2011; ZCP 2011). Transboundary collaboration and management of this species has also been assisted by the CMS, which provides a framework that encourages the development of transboundary partnerships, Memorandums of Understanding and strategic plans. A recent proposal made by the CMS and CITES to pool resources and expertise is expected to deliver targeted conservation actions and guide policy for better long term outcomes (CMS 2017).

The Southern African regional population is unique in comparison to other regional populations in that there are both free-ranging populations and those that are managed more intensely (Mills *et al.* 1998). For instance, Kruger National Park holds the only viable free-ranging dog population in South Africa. Following recommendations in the PHVA, however, a second viable population was established within the KwaZulu-Natal, Limpopo and North West provinces by managing smaller geographically isolated reserves as a collective meta-population (Davies-Mostert *et al.* 2009; Mills *et al.* 1998). Haywood *et al.* (2007) suggest that reserves <1000 km^2 are too small to adequately maintain a self-sustaining dog population. To increase the viability of these populations animals are periodically translocated between subpopulations as a means of mimicking the natural process of immigration and emigration, while also decreasing the risk of localised extinction (Davies-Mostert *et al.* 2009; Gusset *et al.* 2010; Mills *et al.* 1998).

Davies-Mostert *et al.* (2009) critically assessed the meta-population management strategy used in South Africa finding that this conservation approach was a functional solution providing the commitment of stakeholders was ongoing. This review recommended the meta-population management program continue and expand by identifying and restoring landscapes and connecting habitats (Davies-Mostert *et al.* 2009). Increasing landscape connectivity can positively influence accessibility to favourable resources and contributes towards the colonisation of empty habitat (Crooks & Sanjayan 2006). A long term prospect for South Africa is to restore connected landscapes through large scale wildlife conservancies and the development of trans-frontier wildlife areas to facilitate natural population dynamics (Davies-Mostert *et al.* 2009; Lindsey *et al.* 2005b).

The protection of large tracts of land to preserve dogs and other ecologically similar species is unlikely to occur without the inclusion of smaller reserve networks, buffer zones around protected areas and private and communal lands (Gittleman *et al.* 2001). This necessitates the participation of local communities with well thought out education programs (e.g. conflict mitigation, land use) that not only facilitate community involvement but also enable land holders to have a degree of project ownership (Saunders 1990). For researchers and wildlife managers there is an increasing emphasis on resolving conflicts between people and predators to lessen the number of indirect or direct kills sustained from humans (Gittleman *et al.* 2001; Gusset *et al.* 2009; Hayward & Somers 2009; Treves & Karanth 2003; Treves & Naughton-Treves 1999). Allowing community involvement is essential for any carnivore-focused conservation program as the significance of human attitudes can dramatically influence a positive or negative conservation outcome. The greatest opportunity for positive conservation gains and the continued existence of this threatened species in the wild is from countries in Eastern and Southern Africa (NNF 2009; Woodroffe *et al.* 1997).

2.5.2 *Ex situ*

African Painted Dogs were first recorded in zoos in 1948 with it likely that they were held in captivity prior to this (ARAZPA 2008). Currently, there are just over 600 dogs in captivity in 99 zoos and safari parks (ZIMS 2017) with an unknown number of additional animals held in private collections. Many of these zoological organisations contribute to the long term maintenance of global captive stocks, which can be considered an important insurance population against species extinction (ARAZPA 2008). The PHVA recommends that institutions holding African Painted Dogs in captivity develop appropriate management and organisational plans (Mills *et al.* 1998). Some organisations have also developed on-going partnerships with *in situ* conservation programs to provide technical, financial or logistical support. Documenting husbandry techniques and conducting educational programs are additional strategies utilised by organisations holding *ex situ* populations to improve existing conservation efforts for free-ranging animals.

Founders for the Australasian captive collection initially arrived in Australian zoos in 1964 with a second founding lineage imported from South Africa in 2002-2003 (ARAZPA 2008). This species has been identified as a priority species for display (ARAZPA 2009) with the primary purpose of the Australian Species Management Program to generate genetically representative, socially competent individuals that, if required, will be made available for a reintroduction program (ARAZPA 2009). One of the aims of the regional captive

management plan is to support the development and documentation of husbandry techniques to assist *in situ* conservation efforts (ARAZPA 2008).

Historically the African Painted Dog has bred quite successfully in captivity (Frantzen *et al.* 2001; Ginsberg 1994a). However, with space constraints within organisations in addition to the challenges of managing social groups, the dog population in Australasia has experienced inconsistent growth over the long term with widely varying annual growth rates (AZA 2003; ZAA 2017). The Australasian region now has an ageing population with a heavy male bias (ZAA 2017). Pups within this region are predominantly born between March and June with a smaller proportion in November-December (ARAZPA 2008). In the last several years however, there has been poor reproductive success with many neonatal deaths being attributed to cannibalism within the first few days of life. Comparative to *in situ* populations, captive bred dogs below the age of 12 months have a much lower survival rate (Lamglait *et al.* 2015). The Australasian captive collection is deemed to be in a non-growth state and requires importation of dogs to enable greater population sustainability (ZIMS 2017).

The PHVA does outline that the use of captive bred animals for reintroduction should not be precluded as an occasional conservation action when deemed necessary (Mills *et al.* 1998). This recommendation is given under the proviso that sufficient structural definition has been established for source populations, in terms of genetic diversity and origin (Mills *et al.* 1998; Woodroffe *et al.* 1997). While there are no geographically distinct subspecies, there is substantial genetic variation across the species range for this to be an important consideration during the species recovery process (Creel & Creel 2002). DNA analysis by Girman *et al.* (2001) showed that captive dogs had lower microsatellite variation, cautioning that out breeding wild caught animals may be a more preferable breeding strategy. Integrating studbook information with a genetic assessment of the European captive dog population by Marsden *et al.* (2013) found a high incidence of inbreeding and very skewed founder contributions. It was also noted that considerable genetic diversity from wild populations was conserved in the captive population, being attributed to recent imports of new animals. Dogs in Australasia are yet to be genetically tested but with future imports of new animals and existing uncertainties in pedigrees, one would expect this analysis to be a leading recommendation in the next revision of the captive management plan. Implementing genetic management plans for captive held animals assists wildlife managers to identify strategies in which to minimise the loss of genetic diversity and reduces inbreeding.

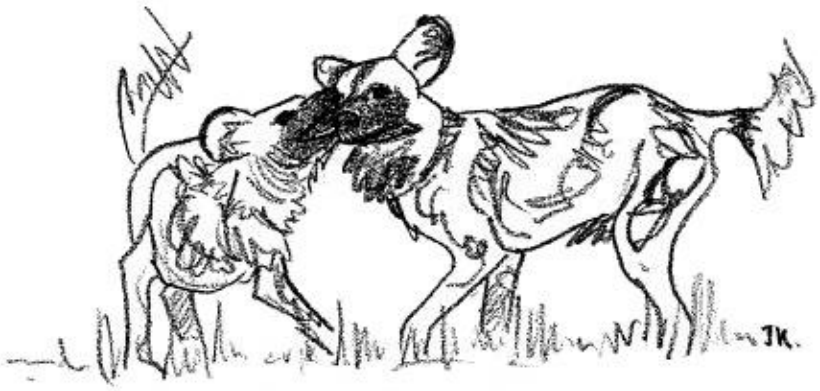
Previous attempts at reintroducing African Painted Dogs have had mixed results (Woodroffe & Ginsberg 1999; Woodroffe *et al.* 1997). A high proportion of unsuccessful reintroduction attempts have predominantly utilised only captive-bred individuals with repeated failures identifying several problems (Woodroffe *et al.* 1997). These included: the dependence of captive individuals on humans for food and subsequently having under developed hunting skills, their naive attitude to larger predators, the composition of the release pack, and the management of disease (Frantzen *et al.* 2001; Mills & Gorman 1997; Sillero-Zubiri *et al.* 2004; Woodroffe & Ginsberg 1999). To address some of these issues subsequent reintroduction attempts have combined both wild caught and captive individuals (e.g. Hluhluwe-Umfolozi Park and Madikwe Game Reserve, South Africa) or have translocated dogs (Woodroffe *et al.* 1997).

Captive bred dogs are managed using a meta-population approach which is a congruent strategy used in some free-ranging populations. This provides some scope for greater collaboration between *in situ* and *ex situ* management. An integrated meta-population strategy has been developed in other endangered species (e.g. Greater Bilby, *Macrotis lagotis*, Tasmanian Devil, *Saracophilus harrisii*) where captive populations are considered one sub-population within the meta-population of free-ranging animals (Hogg *et al.* 2016; Lees *et al.* 2016). Including captive bred dogs within a similar framework would facilitate better genetic management, assist in the cross pollination of ideas, identify emerging research areas, and adapt alternative management practices. Such integrated conservation management is progressive and can reduce the impression that *in situ* and *ex situ* populations should be managed as separate entities.

2.6 Summary

The African Painted Dog is a highly social member of the canid family that has experienced significant decline over recent decades due to an inability to inhabit human dominated landscapes (Fanshawe *et al.* 1991; McNutt *et al.* 2008). To prevent extinction of this species substantial conservation effort has been required across all management levels (Carlson *et al.* 2004; Davies-Mostert *et al.* 2009; Gusset *et al.* 2010; KAZA 2014; Lindsey *et al.* 2005a; NNF 2006). Establishing very large protected areas and conservancies on both communal and private land, restoring dogs in habitat networks to maintain meta-populations and the establishment of *ex situ* populations are all tools being utilised to conserve this species (Sillero-Zubiri *et al.* 2004; McNutt *et al*. 2008). Continued research into the proximate threats experienced by this species is essential to not only limit further declines but to also allow for effective adaptive management. In Chapter 3 one of the main threatening

processes for this species, infectious disease, will be discussed further. This will highlight the serious effect that this threat has had on species persistence and preventative approaches used to effectively combat disease.



3.0 Introduction

Disease is regarded as a priority issue for African Painted Dog conservation with management of such risk being important for both free-ranging and captive populations (CMS 2016; Mills *et al.* 1998). The major pathogens of concern for this carnivore are known to infect a broad number of species in the associated mammalian subclass, with these regarded as generalist pathogens (Laurenson *et al.* 2004). The most significant of these include the rabies virus, canine distemper virus and canine parvovirus. This chapter outlines the effect that infectious disease has had on endangered canid populations along with one of the main mitigation tools, vaccination. The aetiology and prevalence of the canine parvovirus in Australia and separately that of free-ranging African Painted Dog populations will be discussed. To address the second study objective, a review of the use of commercially available vaccines administered to the African Painted Dog is conducted covering a range of canine pathogens.

3.1 Infectious disease and vaccination

Endangered canids are described as having an increased susceptibility to disease outbreaks resulting from their trophic position, sociality and exposure to other sympatric species including the domestic dog, *Canis familiaris* (Gascoyne *et al.* 1993b; Woodroffe *et al.* 2004a). Domesticated dogs are ideal reservoir hosts with evidence now suggesting they have actively contributed to increased rates of disease transmission, particularly in the Ethiopian Wolf (Sillero Zubiri *et al.* 1996), Bat-eared Fox (Sabeta *et al*. 2007) and the African Painted Dog (Woodroffe *et al.* 2012). The effect of disease can increase mortality and suppress population growth rates making populations that are already experiencing some degree of threat (e.g. habitat loss, anthropogenic effects) more vulnerable to extinction (Breed *et al.* 2009; de Almeida Curi *et al.* 2010). The threat of disease on population persistence is however dependent on a disease's pathogenicity (Murray *et al.* 1999). Highly pathogenic infections (e.g. rabies or CDV) tend to threaten small to medium sized populations and are not generally sustained in wildlife populations (Montali *et al.* 1987; Sillero-Zubiri *et al.* 2004), while less pathogenic infections like CPV are known to affect small or recovering populations, which may include animals that are reintroduced or translocated (Haydon *et al.* 2002; Woodroffe 1999). For example, Mech *et al.* (2008; 1993) found limited recruitment in a free ranging Grey Wolf population after the establishment of CPV. Smaller populations can also suffer from lower genetic variability and inbreeding, which can reduce

immunocompetence and increase the susceptibility to infection and disease severity (Pedersen *et al.* 2007; Spielman *et al.* 2004). Many threatened species including the African Painted Dog can be considered 'spill over' hosts that become infected by more abundant host species, e.g. Jackal (Woodroffe *et al.* 2004a). Additional viral pathogens that have had an impact on wild canid populations globally include, infectious canine hepatitis, oral papillomatosis and canine coronavirus (Banyard et al. 2012).

In conserving endangered canids a range of management options have been described to control diseases. These include options to reduce disease in reservoir species, reduce disease in target species, prevent contact between reservoir and target species or no intervention (Woodroffe *et al.* 2004a). Programs that are designed to limit contact between hosts and reservoirs or lower disease transmission through vaccination have the capacity to significantly reduce the risk of disease related mortality in these populations (Acevedo-Whitehouse 2009). In situations where animals are to be introduced from wild or captive sources the risk of disease is minimised by a number of factors including, reviewing animal health histories from source populations, examining the incidence and prevalence of local or regional enzootic animal diseases, a period of quarantine, health screening and, if appropriate, pre-release treatment (Woodford 2000; Woodford & Kock 1991).

Vaccination is one of the most widely used mitigation strategies employed to prevent animal diseases (Plumb *et al.* 2007; Flacke *et al.* 2013). Haydon *et al.* (2006) distinguish the roles that this approach has, with one focus being the elimination of a disease in a population. Vaccination programs designed for endangered species are aimed at reducing the effects that large outbreaks have on populations by increasing an individual's ability to resist or cope with a disease's harmful effects (Banyard *et al.* 2012; Cleaveland *et al.* 2006; Wobeser 2007). The low vaccination coverage concept utilised in conserving endangered species can also protect a core group of individuals during a disease outbreak (Prager *et al.* 2011; Vial *et al.* 2006). The prophylactic use of vaccines can increase a population's resistance to a disease, especially when herd immunity has been attained (Aguirre *et al.* 2002; Brzeski *et al.* 2015; Fine 1993). Inoculating animals can also be utilised as a remedial treatment for individuals that have been exposed to a particular pathogen, see Hofmeyr *et al.* (2000).

The success of vaccination programs is significantly influenced by the proportion of the population that requires to be inoculated to achieve herd immunity and the efficacy of the utilised vaccine (Haydon *et al.* 2006). In the Action Plan for African Painted Dogs attention is drawn to the latter and the protocols surrounding vaccine use, stating that they should be

explored in captive bred animals or those that are unsuitable for release, as there are a number of plausible reasons as to why vaccination failure may occur (Macdonald *et al.* 1992; Woodroffe *et al.* 1997). Reducing transmission rates or controlling disease in wildlife populations is often limited by the availability of specific vaccines which are mostly untested for use in wildlife species (Ndeereh *et al.* 2012). While the delivery of such preventative measures in field settings is surrounded by logistic, financial, and ethical considerations (Miller & Fowler 2012).

3.2 The canine parvovirus pathogen

Parvoviruses have been observed in species from six carnivore families (Felidae, Canidae, Procyonidae, Mustelidae, Ursidae and Viveridae) with all described as having very similar clinical signs (Steinel *et al.* 2001). These autonomous diseases belong to the subfamily *Parvovirinae* and are an unenveloped single-stranded deoxyribonucleic acid (DNA) genome of approximately 5,000 base pairs (Barker & Parrish 2008; Casal *et al.* 1995). The first canine parvovirus was identified in 1967 from dog faeces and named the minute virus of canines (MVC) because of its size (Binn *et al.* 1970; Smith-Carr *et al.* 1997). MVC infections are not usually fatal instead causing mild to severe respiratory and gastrointestinal disease (Carmichael *et al.* 1991). The second and more commonly known canine parvovirus (CPV-2) exists in three forms and was first detected in wild wolves (*Canis lupus*) in northeast Minnesota during 1973 (Mech *et al.* 2008). The earliest record of CPV in domestic dogs was in Greece in 1974 and later in Belgium, the Netherlands, Australia, United States and Japan (Kelly 1978; Koptopoulos *et al.* 1986; Parrish *et al.* 1988). This infectious disease spread rapidly within a short period of time initially killing thousands of unprotected dogs worldwide (Steinel *et al.* 2000). CPV is found in most wild and domestic populations in all continents except Antarctica and is considered a global pathogen (Barker & Parrish 2008).

CPV-2 is believed to have originated as a host range variant from the feline panleukopenia virus (FPV) with Barker & Parrish (2008) providing a detailed description of the close relationship found between FPV, mink viral enteritis and CPV-2. CPV has evolved substantially in the last 30 years with the original strain replaced by a more virulent form (CPV-2a) between 1979 and 1982 (Koptopoulos *et al.* 1986; Truyen 2006). In subsequent years two additional strains have been recognized, CPV-2b was identified in 1984 and CPV-2c detected in 2000, with amino acid changes in the capsid protein characterising each of the variants (Shackelton *et al.* 2005). The antigenic variant found predominantly in Australia is CPV-2a, which differs from the more common strains of CPV-2b and CPV-2c found in Europe and the United States (Meers *et al.* 2007) Woolford *et al.* (2017) recently

confirm, however, that the CPV-2c variant is present in Australia (i.e. Adelaide) prompting further research into its prevalence.

CPV affects dogs of all ages with high morbidity and mortality observed predominantly in young puppies and naïve adult dogs (Decaro *et al.* 2007). Young animals between 1-6 months of age are particularly prone to this disease as virus replication occurs only in dividing cells, which are prolific in individuals of this age (Tsao *et al.* 1991). Age can also be a factor in how this pathogen manifests with two medical syndromes identified (Frölich *et al.* 2005). In individuals that are less than 4 months old there has been a greater number of cases involving myocarditis, while animals of all ages are equally likely to exhibit haemorrhagic gastroenteritis (Greene 2012; McCandlish *et al.* 1981). Other symptoms observed during infections include severe dehydration, lethargy, vomiting, lymphopenia or leukopenia (Steinel *et al.* 2001).

The incubation period for CPV ranges between 4-10 days with the enteric form having a shorter incubation time than that which manifests as myocarditis (Appel & Parrish 1987; EAZWV 2002). Transmission can occur through infected individuals via the faecal-oral route but is not required directly for efficient transmission as this pathogen can also spread through fomites (i.e. an object which is likely to carry an infectious agent) or contact with people (Steinel *et al.* 2001; Tsao *et al.* 1991). The survival rate of CPV in domestic dogs is as low as 9.1% in the absence of treatment and 64% or higher where aggressive medical treatment is provided (Goddard & Leisewitz 2010). As there is no medical cure for parvovirus, supportive treatment such as fluid therapy is suggested to maintain hydration and electrolyte balance (Macintire & Smith-Carr 1997). The best prophylaxis for CPV infections is the adoption of an effective immunisation protocol (Appel & Parrish 1987).

In pups, protective immunity is initially provided through the ingestion of the colostrum with titres believed to approximate that of the breeding female (Pollock & Coyne 1993). The availability of colostrum is also inversely proportional to litter size (Goddard & Leisewitz 2010). The half-life of maternally derived antibodies is between 8-10 days with protective levels in pups diminishing between 4 and 16 weeks of age (Pollock & Coyne 1993). This decay in immunity is dependent on the bitches' titre levels with susceptibility to infection increasing once hemagglutination inhibition (HI) titre levels fall below 1:80 (Pollock & Carmichael 1983). Vaccinating animals provides the basis for preventing the enzootic spread of parvovirus. Maternal antibodies can suppress a vaccination response in young animals and is considered a major issue for immunisation failures for these animals

(Goddard & Leisewitz 2010). To minimise exposure risk inoculations should begin at 6-8 weeks of age with revaccination occurring before 20 weeks, while for adult dogs one or two inoculations depending on vaccine type is considered to confer protection (AAZV 2011; Pollock & Coyne 1993).

CPV is extremely stable in the environment and once shed through faeces it can stay infectious for weeks or months (Gordon & Angrick 1986; Hoelzer & Parrish 2010; Tsao *et al.* 1991). This pathogen is extremely resistant to pH and temperature changes as well as most disinfectants (Appel & Parrish 1987). Viral inactivation is found to occur with ultra violet radiation and chemical agents such as formalin or commercial hypochlorite solutions (Scott 1980).

In Australia canine parvovirus is deemed a core infectious disease. An animal health surveillance system was launched in 2010 by Virbac Animal Health Disease to provide information on the occurrence, transmission and risk factors relating to CPV and other infectious diseases known to exist in domestic dogs and cats. This national surveillance project estimates case fatality rates for CPV related diseases and associates them to a range of risk factors, e.g. age, sex, vaccination status, breed, reproductive status, season and state of residence (Ling *et al.* 2012). During the 2010 calendar year there were 1,451 individual cases of CPV with the median age of dogs in reported clinical cases being between 4½ and 7½ months (Ling *et al.* 2012). Dogs that had either a partial or complete vaccination history constituted 20.3% of clinical cases, whereas 58.3% of dogs suffering CPV were unvaccinated. In all other cases a vaccination history was unknown. Seasonality was highly associated with mortality with instances of CPV 3.1 times more likely to occur in summer. A comparison between states of residence also showed that the highest proportions of euthanasia resulting from CPV infections occurred in Queensland (37.2%) and collectively Tasmania, South Australia and Northern Territory (39.5%). A crude population based estimate of case fatality from naturally acquired CPV was 43.2% with this rate being higher than similar studies conducted in New Zealand, United States and Europe which range between 26% and 36% (Ling *et al.* 2012).

There are strict statutory conditions imposed on zoos in Australia that enable them to display exotic species like the African Painted Dog. These restrictions can also insulate species from a possible disease outbreak but can never completely eliminate the risk. As CPV is easily transmitted and long lived in the environment, understanding the effectiveness

of a preventative disease program for *ex situ* populations is vital to guard against future episodic events and to safe guard the viability of the insurance population.

3.3 Parvovirus and free-ranging African Painted Dog populations

A number of serological studies have explored the prevalence of CPV in free-ranging African Painted Dog populations. A survey of the Maasai Mara, Kenya (1,510 km^2) by Alexander *et al.* (1993) and later Kat *et al.* (1996) found that both domestic dogs and African Painted Dogs had been exposed to CPV-2 with domestic dogs 4.8 times more likely to test positive. Domestic dogs have been identified as reservoir hosts for many canine pathogens (Cleaveland *et al.* 2000; Laurenson *et al.* 1997). The African Painted Dog population in this region have experienced recurrent disease episodes demonstrating that viral diseases can contribute, in association with other exacerbating factors (e.g. modified habitat), to significant population decline and local extinction (Alexander & Appel 1994; Creel *et al.* 2004; Gascoyne *et al.* 1993b; Goller *et al.* 2010).

In northern Kenya (Laikipia District, 4,500 km^2), Woodroffe *et al.* (2012) compared the risk of exposure to conspecifics, other packs and domestic dogs during 2001-09. Results indicated that African Painted Dogs had an increased exposure risk to four pathogens, which included CPV, when contact with domestic dogs was observed. The proportion of African Painted Dogs that were seropositive to parvovirus was greatest in small packs who had increased opportunity for contact with *C. familaris* (Woodroffe *et al.* 2012). Interactions between African Painted Dog packs did not reveal any increased risk to pathogen exposure.

Creel *et al.* (1997c) collectively analysed demographic and serological data from the African Painted Dog population in Selous Game Reserve, Tanzania (43,000 km^2, 1992-95). Sympatric carnivores included Lions, Spotted Hyena, Black-backed and Side-striped Jackals with the reserve considered to be free of domestic dogs. Jackals are another major vector for canine disease with antibodies to rabies, CDV and CPV detected in a number of studies (Alexander & Appel 1994; Spencer *et al.* 1999). Inter-specific aggression and predation between African Painted Dogs and Jackals is believed to be widespread providing ideal opportunity for disease transmission (Butler *et al.* 2004; Kamler *et al.* 2007). Results from the collected serological samples showed that 68% of the African Painted Dogs in Selous were seropositive to CPV-2 being distributed across 10 of the 11 surveyed packs (Creel *et al.* 1997c). This indicated that CPV was quite widespread. Pups from this study were occasionally observed to have episodes of diarrhoea and lethargy with a reduction in the mean litter size coinciding with an increase in CPV-2 titre levels.

In contrast a more recent serological survey in communities surrounding two major Zambian ecosystems (South Luangwa NP, 13,775 km^2 and Liuwa Plains NP, 3,660 km^2) showed a low prevalence of CPV in comparison to other regions of Sub-Saharan Africa (Berentsen *et al.* 2013). This baseline study found that none of the African Painted Dogs sampled tested positively (n=12) while 10% and 4.8% of Hyenas and Lions respectively had been exposed to CPV. In the South African province of KwaZulu-Natal a similarly low prevalence was recorded with only 4.2% of dogs testing positive to CPV (Flacke *et al.* 2013). Within this regional area disease surveillance has been in an important factor in the reintroduction and conservation of this species due to significant population declines during 2004-06.

Based on other wolf-like canid populations the effect of CPV on population density can range from minimal yearly differences to annual population increases being inversely related to the number of animals testing positive to CPV (Mech 1995; Mech *et al.* 2008; Mech & Goyal 1993). For stable, high density populations, the effect of this disease is not considered a major factor in limiting population persistence (Creel *et al.* 1997c; Mech *et al.* 2008). A review of disease threats in the Ethiopian Wolf by Laurenson *et al.* (1998), suggest that where CPV is endemic with mortality generally seen only in young animals, while for animals in naïve populations or those that have very low exposure, individuals of all ages are susceptible.

Landscape heterogeneity is another factor that can limit or facilitate direct transmission of multi-host pathogens. In a study conducted by Alexander *et al.* (2010), a disease outbreak in the Okavango Delta, Botswana (2,600 km^2, 1992-1992) was found to be spatially limited to areas of vegetation change. The extent of the outbreak for this African Painted Dog population occurred only in the high wildlife density flood plain area with no cases of disease found beyond the expansive water courses or into the contiguous packs range which occupied low wildlife density areas (i.e. Mopane woodland). Unlike more homogenous landscapes (e.g. Serengeti grasslands) mixed habitat types afford a greater number of transition areas between vegetation types, which can directly influence community structure, density and distribution of host species, contact rates and pathogen transmission (Alexander *et al.* 2010).

The studies discussed in this section highlight some of the broad conditions in which CPV can persist in free ranging populations. This pathogen is an important cause of juvenile mortality and contributes to the maintenance of small populations in fragmented

landscapes. Wild canids that live alongside unvaccinated domestic dogs have increased susceptibility to canine pathogens and are more likely to test positive to infectious disease (Woodroffe & Donnelly 2011). Managing disease in domestic animal or sympatric species reservoirs is complex and requires a broad range of factors to be considered. The circumstance of each scenario can have important implications for managing the recovery of endangered species and those sub-populations within a meta-population system (Gog *et al.* 2002).

3.4 Vaccine use

Active immunisation has played a significant role in the control of infectious diseases in a broad range of species, as it stimulates a specific immune response to a respective pathogen(s) (Cleaveland 2009). Traditional viral vaccines are based on the living state of an antigen with there being two main types, live attenuated or inactivated where the virus is dead (Joost 2010). There are legitimate concerns for using either type of vaccine in African Painted Dogs as they are not a highly immunogenic species and the vaccines themselves can induce clinical disease (Woodroffe *et al.* 2004a). Ethical considerations also need to be weighed up when devising potential vaccination protocols, especially given the conservation status of *L. pictus*, the small population sizes found in free ranging populations and the proximity of wild canid species to each other while in captivity.

The characteristics of these vaccine types are in many ways quite different, with each having certain advantages and disadvantages. The main advantages for an attenuated vaccine is that a single dose stimulates a rapid cell mediated and humoral response with long term protection (Popelin 2010). The intervals between subsequent booster doses can often be wider than inactivated vaccines as attenuated vaccines induce good immunological memory. Despite this some live vaccines have reverted to virulence subsequently manifesting into the disease for which the vaccine is designed to protect against. The disadvantages of attenuated vaccines are parallel to the advantages of inactivated vaccines. The latter requires larger doses, with the duration of protection considered to be shorter than that of live vaccines (Meeusen *et al.* 2007). While the inclusion of adjuvants are used to increase the speed or magnitude of the body's response to the vaccine they also have little effect on secondary immune responses (Tizard 2013). Inactivated vaccines are commonly utilised in animals that may be immunocompromised, pregnant or in the management of highly endangered species such as the African Painted Dog or Ethiopian Wolf. Despite the increased safety that inactivated vaccines offer endangered species, their use can be redundant if they are ineffective in protecting against the designated infectious disease.

To ascertain the number of serological studies and the use of vaccines in African Painted Dogs a search of several well-known scientific databases holding citations to peer reviewed journals and books was conducted ranging from 1965 to 2015. The keywords included in each search consisted of either the scientific name, *Lycaon pictus*, or the frequently used common name *African Wild Dog*. These were independently combined with four other keywords being; *serology, vaccination, antibody* and *parvovirus* with all date ranges considered in each database. The returned results were reviewed with any duplicated records removed. Collectively these results were broadly classified into 6 categories (Table 3.1) reflecting vaccine use, serological surveys, epidemiology, disease management and relevance. The first two categories in Table 3.1 are comprised of publications specific to African Painted Dogs. All other publications were grouped into the remaining categories which included pathological studies from other species that have some relevance to this study. This review identified 17 studies that reported findings on the effectiveness of different vaccines under various management situations. These included studies on inactivated and attenuated, as well as, monovalent and multivalent vaccines for all the major canine pathogens. Studies that had conducted serological surveys were distributed in six African countries being, Namibia, Botswana, South Africa, Zambia, Kenya and Tanzania.

Table 3.1. Literature search pertaining to vaccine use and serological investigations in the African Painted Dog (1965-2015).

Database	No. of returned records	Category					
		Vaccine use	Serological surveys	Epidemiology	Conservation/ management	Other species	Not relevant
Biological Abstracts	36	14	7	2	13	1	1
Cambridge Journals Online	66	1	-	6	10	25	24
Current Content	56	5	1	14	14	12	10
Expanded Academic ASAP (Gale)	17	-	1	4	5	2	5
Medline	25	11	5	4	5	-	-
Oxford University Press	7	-	-	-	3	2	2
Proquest	61	1	-	13	14	21	12
PubMed	51	14	7	11	7	7	8
SAGE Journals Online	5	1	-	-	-	2	2
Scopus	64	13	5	11	17	7	11
Web of Knowledge	90	15	7	19	17	25	12
Publication no.	332	5.1%	3.3%	14.8%	21.1%	29.8%	25.9%

Examining the cited literature from publications in the vaccine use category and a broader search of the internet to include reports generated from non-government organisations revealed a further four studies that had investigated the use of vaccines. Two of these articles were peer reviewed but were poorly indexed or did not contain any of the search terms. A summary of these collective studies are shown in Appendix 1 along with information pertaining to the type, brand, delivery approach and vaccination protocol. Studies that have targeted the parvovirus pathogen have been bolded in the appendix. As expected most of the literature focuses on rabies and CDV. Not all studies reported an antibody response for all vaccine valencies, while others simply described the use of a vaccine after clinical signs of disease had been observed, see Hofmeyr *et al.* (2000); Van de Bildt *et al.* (2002). There is clear support to suggest that vaccinating the African Painted Dog has resulted in vaccine induced disease, with multiple studies reporting clinical disease and mortalities after being inoculated mainly with distemper vaccines (see Durchfeld *et al.* 1990; McCormick 1983; van Heerden *et al.* 1989)

There are only three studies that reported an antibody response for CPV with all circumstances combining this pathogen in a bi or multivalent vaccine. van Heerden *et al.* (2002) assessed antibody titre levels for CPV with pre vaccination titres levels ranging from 1:512 to 1:2048 suggesting that they these animals had previously been exposed to this pathogen. These dogs were vaccinated twice, a month apart and then a year later, with the live attenuated Vanguard Puppy 5 (Pfizer) vaccine. All individuals had a significant increase in titre levels after the first vaccination (up to 1:8192) with little change after the second inoculation. After a year the titre levels in all animals remained high (>1:256). In domestic dogs antibody titre levels greater than 1:80 as measured by the hemagglutination inhibition method are considered to be protective (Maia & Gouveia 2001; van Heerden *et al.* 2002). It was concluded that this vaccine was protective despite there being no challenge experiment.

Spencer and Burroughs (1990) also used a live attenuated vaccine, Vanguard DA2P + CPV (Smith Kline Animal Health) on seven dogs, with antibody titre levels measured one month after vaccination. Five dogs showed seroconversion at this time with two others being bled after a further two weeks. These animals did not seroconvert but were found to have increased titre levels after the third blood sample. The George Adamson Trust (1996) conversely used an inactivated vaccine, Dohyvac I-LP (Solvay Duphar) to prevent parvovirus and leptospirosis infections in 25 African Painted Dogs that were aged five months. Animals were again inoculated twice a month apart with repeated blood sampling

occurring two weeks after the second booster. All dogs were found to have adequate protective immunity with an annual booster recommended. The long term duration of immunity (DOI) was not established in either study.

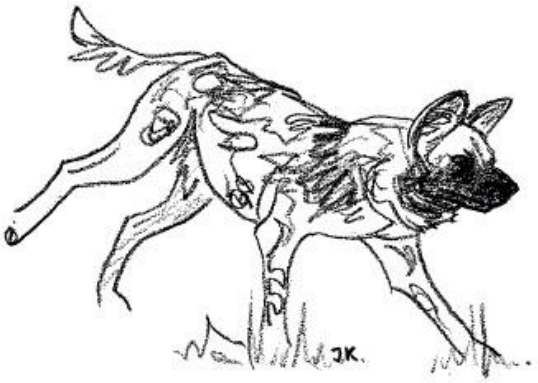
To draw on the experiences in *ex situ* populations, Popelin (2010) surveyed a broad range of zoos and wildlife parks in Europe to establish which procedures and vaccines were used widely and considered safe. Vaccination histories of a diverse group of non-domesticated canids including *L. pictus* were assessed. This study identified 17 of the 25 (68%) parks surveyed vaccinated their animals with the remaining citing availability of appropriate vaccines and practical difficulties in administration as the primary causes for non-vaccination. From the responding institutions, there were 12 commercially available and 1 non-commercial vaccine utilised to protect against a host of pathogens. These products included Nobivac (Intervet); Duramine (Fort Dodge); Hexadog, Eurican P, Eurican L, Leptodog and Rabisin by Merial; Vanguard 5+, Vanguard CPV Lepto, Parvac™ by Pfizer (Zoetis); Canigen (Virbac) and CDV-ISCOM (Erasmus Medical Centre, Rotterdam).

Vaccines administered to captive held African Painted Dogs in Popelin's review predominantly involved younger animals with remote delivery being the most favoured approach. Popelin (2010) further recommended that where a live attenuated vaccine is to be used it is preferable to use an inactivated vaccine such as, CDV-ISCOM (Schering-PloughAnimal Health) for CDV or Parvac™ (Zoetis) for CPV, as a primer to initiate an immune response prior to the administration of the attenuated vaccine.

In Australia, the Australian Pesticides and Veterinary Medicines Authority (APVMA) provide a centralised repository for all registered agricultural and veterinary products in the Australian marketplace (APVMA 2012). Most vaccines used to increase protection to CPV in domestic dogs are multivalent live attenuated vaccines (see Appendix 2). The exception is Parvac™ produced by Pfizer Animal Health (Zoetis), which is the only monovalent inactivation vaccine available in this country and commonly used in captive collections to protect against parvovirus infections. Both the live attenuated and inactivated vaccines are derived from either the CPV-2 or 2b antigen with it assumed that protection is homogenous across all variants i.e. CPV-2a, 2b and 2c (Chalmers 2006).

3.5 Summary

For *L. pictus* some attenuated vaccines commercially produced for use in domestic dogs have been shown to revert to virulence. Given the risk of vaccine induced disease and the poor immunological response found in this canid, vaccine efficacy (or immunogenicity) should initially be tested on healthy adults free from other infections (van Heerden *et al.* 2002). CPV is not considered a highly pathogenic disease in comparison to rabies and CDV, but still has the capacity to have serious effects on small and recovering populations. As shown, there are few studies that have assessed the efficacy of a particular vaccine in this species. Having a safe and reliable vaccine is the foundation of an effective disease mitigation strategy. Chapter 4 examines the issue of reliability in one canine vaccine by undertaking a vaccination trial targeting the African Painted Dog and the canine parvovirus pathogen.



4.0 Introduction

Wildlife health research is essential for endangered species as it provides important information about disease trends and risk factors, outcomes of treatments or interventions, logistics and costs (Nass 2009). The different approaches employed to investigate such issues are often complementary, yielding useful information for disease mitigation. Of these, clinical trials provide vital evidence about the immunogenicity and adverse effects of medical interventions by controlling certain variables that could influence the results of a study (Nauta 2010). They are also crucial for comparing and improving the use of drugs, vaccines, medical devices, and diagnostics. This chapter addresses the third and fourth objectives of this study by presenting the results of a vaccination trial involving the African Painted Dog and the only inactivated CPV vaccine available in Australia.

4.1 Canine Parvovirus

Research relating to CPV and managing its potential risk through surveillance and possible treatment protocols is limited in African Painted Dog populations. This is not assisted by the small number of studies reporting a post vaccination antibody response. From those in the literature all have combined this pathogen in a bi- or multivalent vaccine (George Adamson Trust 1996; Spencer & Burroughs 1990; van Heerden *et al.* 2002) with there being no study that has assessed the immune response of an inactivated monovalent vaccine (i.e. a single strain of a single killed antigen). Expanding basic research on vaccine use in such species as the African Painted Dog has the potential to help establish more appropriate vaccination schedules, particularly as the use of inactivated vaccines are a valuable option in managing disease risk in endangered species (Miller 1996). It can also assist in determining which preventative medicine approaches are more likely to succeed under field conditions.

This experimental approach examines the immunogenicity of the inactivated monovalent vaccine Parvac™ (Pfizer) in *Lycaon pictus*. This vaccine is commercially available for use in domestic dogs (*Canis familiaris*) and is known to be safe for use in pregnant bitches and those individuals that are at high risk or immunodeficient (Pfizer 2010). This vaccine is also frequently used in *ex situ* populations to guard against CPV infections in a variety of endangered canid species. However, information pertaining to duration of immunity is

unknown. The objective of this chapter is to (i) assess immunogenicity and the duration of immunity and (ii) evaluate the effects of mode of delivery.

4.2 Materials and methods

4.2.1 Experimental animals

The study site was Monarto Zoo (MZ) situated approximately 64kms from Adelaide, South Australia (35.1021° S, 139.1424° E). Climatic conditions at Monarto are characterised as Mediterranean with mild wet winters and dry summers (Woods *et al.* 2001). Maximum temperatures average 15°C in the winter to 31°C across the summer months with an average rainfall of 325mm annually (BOM 2012). MZ was first opened in 1993 with African Painted Dogs being part of the collection since 2002. This captive facility is an open-range wildlife park occupying approximately 1,500 hectares and at the commencement of the study housed (separately) the largest number of African Painted Dogs in the Australasian region (19 males and 5 females) (ZoosSA 2009).

Monarto Zoo has been an active participant in the Zoo Aquarium Association (ZAA) regional breeding program, producing a number of litters in the past decade. Eighteen male dogs from four cohorts aged respectively as 5, 6, 7 or 11 year olds were used in the vaccination trial. All individuals from the younger cohorts were progeny of successful breeding seasons at MZ with the oldest individual being part of the second import of dogs into the region, originating from De Wildt Cheetah and Wildlife Centre, South Africa. This bachelor group was displayed long term in the main African Painted Dog exhibit but were separated into two groups after 9 months of the study due to conspecific aggression. The increase in aggression between cohorts coincided with the breeding season and resulted in one fatality of the study group (*A69114*). The youngest cohort and main aggressors were subsequently held in off-limit enclosures. Coat markings for this species are highly variably and unique allowing identification of specific individuals to be made by trained observers.

Generally dogs held at MZ were not vaccinated against any infectious disease due to its remote location and lower disease transmission risk. At the beginning of the study all animals were regarded as being healthy with experimental procedures performed in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Ethics approval was gained through the Flinders University Animal Ethics Committee.

4.2.2 Vaccine administration and schedule

At the commencement of the study all dogs (n=18) were divided randomly into three treatment groups each consisting of six individuals. Groups included a control where animals were initially given a placebo (water for injection), while dogs in the second and third groups were vaccinated by hand injection (i.e. in a squeeze cage) and remote delivery (i.e. darted) respectively using the inactivated Parvac™ (CPV-2) vaccine (Pfizer 2005). The recommended dosage rate for this vaccine is 1ml for dogs of all ages with the administration route being either subcutaneous or intramuscular (Pfizer 2005). The use of a blow pipe (length 105cms) discharged at approximately 1m was utilised for all animals in the remote delivery group except for the annual vaccination of *A69115* where a Dan-Inject (model JM) dart rifle was employed. This animal was darted within 20ms using 5-7 Bar of pressure. In both situations 3ml syringes (Telinject USA Inc) were used with 15/20 needles.

The frequency of immunisations followed the recommended protocol for naïve animals as outlined by Welbourn *et al.* (2011). These canine vaccination guidelines state that there should initially be two inoculations approximately one month apart followed by an annual booster at 12 months. Table 4.1 shows the schedule of administration with monitoring of antibody titre levels occurring for all groups during the intervening months, as well as after the annual booster. Dogs *A59172* and *A59175* received a third booster to explore what influence an extra dose had on the vaccines immunogenicity.

Table 4.1. Vaccination schedule and program for monitoring antibody titre levels. (Animal identifications listed in the footnote).

Day	Group 1	Group 2	Group 3
0	Placebo	Parvac delivered by hand	Parvac delivered remotely
33	Monitoring only	Parvac delivered by hand	Parvac delivered remotely
68	Monitoring only	Monitoring only	Parvac delivered to A59172 and A59175 only
131	Monitoring only	Monitoring only	Monitoring only
278	Monitoring only	Monitoring only	Monitoring only
369	Monitoring only	Parvac delivered by hand	Parvac delivered remotely
404	Monitoring only	Monitoring only	Monitoring only
446	Monitoring only	Monitoring only	Monitoring only
777	-	Monitoring only	Monitoring only

Group 1: A29038, A59176, A59171, A69114, A69117, A69120;
Group 2: A49134, A49135, A59174, A69116, A69118, A69119;
Group 3: A49160, A59172, A59173, A59175, A69113, A69115.

4.2.3 Blood sample collection

Antibody monitoring occurred nine times across a 25 month period. Bloods taken on day 0 determined baseline titre levels prior to vaccination while samples collected on day 777 were done opportunistically as 7 vaccinated dogs were being transferred to another zoo and required pre-shipment health checks. All animals were brought through a purpose-built raceway and immobilised in a squeeze cage to facilitate blood collection. Sampling was performed by venepuncture from either the cephalic or saphenous vein with a minimum of 2.5ml of whole blood collected and placed into a sterile serology tube. In instances where vaccination was to occur, blood collection was performed just prior to the administration of the vaccine. All samples were centrifuged on the day of collection and placed into a screw top container and stored at -80˚C until required for laboratory analysis.

4.2.4 Determination of parvovirus Ab titres

A hemagglutination inhibition assay was performed by a commercial laboratory service (Biobest, UK) to determine the antibody response for collected samples. This classic laboratory procedure has been the main method used to investigate and quantify immune responses. Technicians at Biobest were blinded as the only information provided to them was the animals identification number. The employed test used the CPV-2b antigen with HA activity of the virus being 4HA units. A two-fold serial dilution was performed on test samples to determine the amount of antibodies present. In domestic dogs antibody titre levels greater than 1:80 are considered to be protective (Maia & Gouveia 2001; van Heerden *et al.* 2002). The HI titre value was determined by the reciprocal of the highest dilution of serum that completely inhibited hemagglutination for dogs in each corresponding session. For computational purposes, titres for example of <1:20 (or similar) were assigned the value for the preceding dilution e.g. 1:10. Seroprotection was defined as pre-vaccination HI antibody titres ≤ 1:10 and a post vaccination titre of ≥1:80, or when the post-vaccination titre had a four-fold increase.

4.2.5 Statistical methods

For vaccine immunogenicity trials the end point is a measure of humoral or cellular immunity which is considered to be a correlate for protecting against infectious disease (Edwards 2001; Nauta 2010). To assess immunogenicity three primary end points were examined including the geometric mean response of titres (GMT), geometric mean fold increase (gMFI) and seroprotection rate (SR). As HI antibody titres were reciprocals the lowest tested dilution factor was incorporated in a $\log_2$ transformation (Nauta 2010).

Immunogenicity values were analysed using Excel (Excel 2010) and Graph Pad Prism (Graph Pad Software Inc. 2013) with statistical significance defined as P values <0.05.

To evaluate whether the HI titres of the vaccinated animals differed significantly from those in the control group the Mann-Whitney *U* test was used on data collected one month after the initial inoculation to two months following the annual booster (days 33 to 446). The Friedman test with Dunn's multiple comparison was applied to the same time period focusing only on vaccinated animals to examine the differences in antibody titres across time. A Wilcoxon matched pair's signed rank test assessed GMTs at specific time intervals to compare against baseline values within each treatment group.

To obtain whether mode of delivery influenced the number of dogs being seroprotected the pooled vaccination results were separated into two treatment groups (subcutaneous hand injection, SC: remote delivery, intramuscular, IM). Immunological values were calculated for each time period (day 33 to 777) along with a Fisher's exact test. It was hypothesised that the ratio of seropositive to seronegative dogs should be the same at each sampling event for animals vaccinated by hand injection and those vaccinated by dart. A Reverse Cumulative Distribution Plot was constructed using all time intervals to show the percentage of dogs that obtained a specific immunological value. These were partitioned into the two treatment groups and compared against control animals.

4.3 Results

After the capture and restraint of the study group, dogs were monitored daily for changes in behaviour and health. Initial base line antibody titre values were examined identifying only one dog that did not have a HI titre value of <1:10. This animal (*A49160*) had a protective titre value of 1:80 leading to a baseline imbalance and was subsequently excluded from the statistical analysis.

4.3.1 Immune response to inoculation

Comparisons in immunogenicity values for control and vaccinated dogs were made from samples collected during days 33-446. Based on an exact significance value the Mann-Whitney *U* test showed vaccinated dogs had a significantly higher immune response than control animals, $U\ (114) = 129.5$, $p = <0.0001$. Figure 4.1 shows the geometric mean (bars) and 95% confidence intervals (error bars) calculated for control and vaccinated animals being 6.13 ($CI_{95\%}$ 5.43 to 7.07) and 59.01 ($CI_{95\%}$ 43.86 to 81.85) respectively.

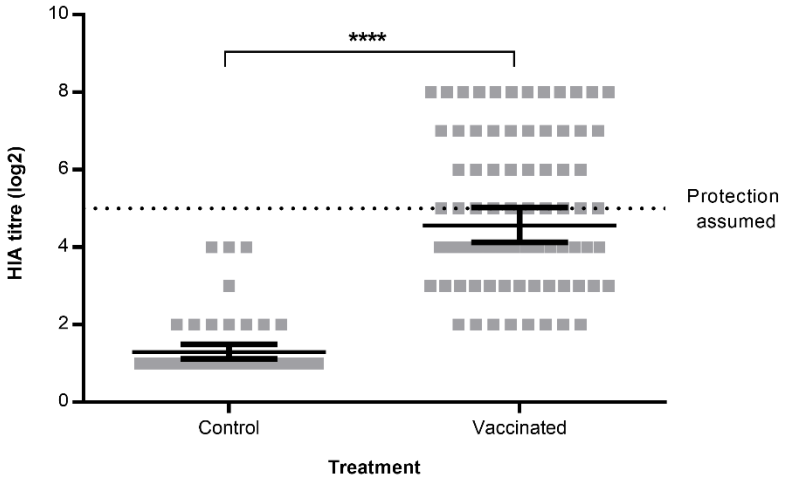


Figure 4.1. HI titre values from all control animals and dogs vaccinated with the Parvac™ vaccine over days 33-446.

Between sampling events the GMTs for dogs in the control group showed little variability with titres ranging from 5 to 14 ($CI_{95\%}$ 5.2 to 38.6). The largest fold change in the GMT for this group was observed on days 131 and 278 (Table 4.2) with titre values of the latter being 2.8 times the baseline value. A Wilcoxon matched pairs signed rank test indicated only moderate differences in titre values at days 0 and 278 ($\underline{z} = 1.5$, $p = 0.125$). No dog from this treatment group was considered to have protective immunity throughout the study.

Dogs that were vaccinated had GMTs that ranged from 17.6 ($CI_{95\%}$ 11.7 to 26.5) to 363 ($CI_{95\%}$ 183 to 720). The Friedman test evaluated the response of antibody titres across days 33 to 446 (Figure 4.2) finding significant differences over time ($F_{r\,(6)} = 50.02$, $p < 0.05$). A 6.6 fold increase in immunological values was evident one month following the initial inoculation with 18% of animals considered to be seroprotected. At day 68 geometric mean titres peaked with 91% of the dogs having antibody titres above 1:80. The geometric mean ratio (GMR) between treatment groups showed a fold increase of 72 in the titres of vaccinated dogs compared to control animals.

Table 4.2. Immunogenicity values for control and vaccinated dogs.

Treatment	Statistic	Day							
		0	33	68	131	278	369	404	446
Control	N	6	6	6	6	6	5	5	5
	GMT (SD)	5.0 (1.0)	5.0 (1.0)	5.0 (1.0)	7.94 (1.4)	14.14 (2.6)	5.0 (1.0)	6.6 (1.46)	7.6 (2.53)
	gMFI*	-	1.0	1.0	1.6	1.8	0.4	1.3	1.1
Vaccinated	GMT (SD)	5.0 (1.0)	33.1 (1.87)	363.0 (2.77)	45.4 (2.25)	31.9 (1.90)	17.6 (1.83)	340.8 (1.78)	181.5 (2.38)
n=11	SR (%)	-	18	91	45	18	0	100	91
	gMFI*	-	6.6	11.0	0.13	0.69	0.57	19.3	0.5
Geomean ratio (GMR)		1.0	6.6	72.6	5.7	2.2	3.5	51.7	23.9
Geomean fold ratio (gMFR)		-	6.6	11.0	0.08	0.4	1.8	15.3	0.5

* Fold change across time intervals

The geometric mean (gMFI) decreased by 87% at day 131 with the seroprotection rate also falling to 45%. This declining trend in the seroprotection rate and GMT continued till the annual booster where no animal was found to have protective immunity. A Wilcoxon matched pairs signed rank test indicated however that titre levels at day 369 were still significantly different to baseline immunological values ($\underline{z} = 2.0$, $p = 0.001$). Within the first 12 months protective immunity was primarily induced over a period of 98 days (days 33-131).

The second significant rise in antibodies was observed in the month following the annual booster with the largest geometric mean fold increase of 19.3 recorded. GMTs at day 404 reached 340.8 ($CI_{95\%}$ 231.4 to 501.9) with all vaccinated animals considered to be seroprotected. There was a geometric mean fold decrease of 50% in titres 42 days later with all but one individual (*A59175, IM*) sustaining a titre level deemed to be protective (91%). Evaluating the peaks of immunity (days 68 and 404) saw a 6% difference in GMTs with there being a greater response at day 68. This was in converse to the gMFI which found day 404 as having the highest mean fold change in titres.

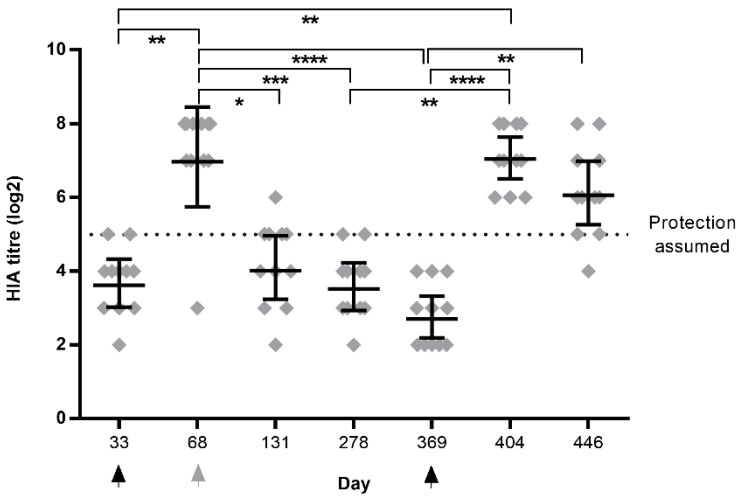


Figure 4.2. Change in antibody titres for vaccinated animals over time.
Arrows indicate booster vaccinations (black: all dogs; grey: only two dogs)

4.3.2 Comparing mode of delivery

Pooled titre results were separated into two groups reflecting the vaccines mode of delivery, being hand injected or remotely delivered via a dart. One month on from the initial inoculation saw similar fold increases in titres with the geometric mean titre of hand injected animals and darted dogs respectively being 35.6 ($CI_{95\%}$ 15.2 to 83.4) and 30.3 ($CI_{95\%}$ 18.9 to 48.6). A nominal change in the GMR was observed at day 68 with there being a 2.1 fold increase in titres for hand injected animals compared to those vaccinated remotely (Table 4.3). Both the GMR and gMFR fell below 1 at day 131 indicating that not only were the antibodies titres in the darted group higher than those animals vaccinated by hand injection but also the fold change was less sizable. Differences in the maximum seroprotection rate were observed only in the first year with just one dog (*A69113)* from the remotely delivered group not achieving a protective titre.

Dogs vaccinated by hand had the highest fold increase in the geometric mean after the annual booster with GMT being 359.2 ($CI_{95\%}$ 207.7 to 621.1) and 320 ($CI_{95\%}$ 135.3 to 756.7) for dogs in the remotely delivered group. The following sampling period showed the greatest declines in antibody titres were from the remote delivery group with the GMT waning by 60%. Opportunistic sampling more than a year after the annual booster (day 777) showed 50% of individuals (n=6) still had protective immunity. In these animals duration of immunity in the second year was sustained for a minimum of 373 days (777 - 404).

Table 4.3. Immunological values for differing modes of delivery

Treatment	Statistic	Day								
		0	33	68	131	278	369	404	446	777
Hand	N	6	6	6	6	6	6	6	6	3
Injected	GMT (SD)	5.0 (1.0)	35.6 (2.25)	508.0 (1.43)	40.0 (1.86)	28.3 (1.79)	15.9 (1.76)	359.2 (1.68)	226.3 (2.07)	100.8 (2.88)
	SR (%)	-	33	100	33	17	0	100	100	67
	gMFI*	-	7.1	14.3	0.1	0.7	0.6	22.6	0.6	0.1
Remote	N	5	5	5	5	5	5	5	5	3
Delivery	GMT (SD)	5.0 (1.0)	30.3 (1.46)	242.5 (4.2)	52.8 (2.86)	34.8 (2.14)	20.0 (2.0)	320.0 (2.0)	139.3 (2.80)	25.2 (2.90)
	SR (%)	-	0	80	60	20	0	100	80	33
	gMFI*	-	6.1	8.0	0.2	0.7	0.6	16.0	0.4	0.1
Geomean ratio (GMR)		1.0	1.2	2.1	0.8	0.8	0.8	1.1	1.6	4.0
Geomean fold ratio (gMFR)		-	1.2	1.8	0.4	1.0	1.0	1.4	1.4	1.0
Fisher's Exact Test†		-	0.455	0.455	0.567	1.000	1.000	1.000	0.455	0.400
Rate Difference (%)		-	3.9	1.3	0.6	0.8	nil	1.0	1.3	5.0
Rate Ratio		-	6.1	4.3	0.3	0.8	0.8	1.8	4.3	11.7

* Fold change across time intervals

† $\alpha = 0.05$

Dogs vaccinated by hand were at this time found to have titres four times higher than animals in the remote delivery group. Conducting the Fisher's exact test showed no significant differences in the ratio of seropositive or seronegative animals in each of the treatment groups across each time period. Figure 4.3 shows the change in GMTs across the study period for all treatment groups.

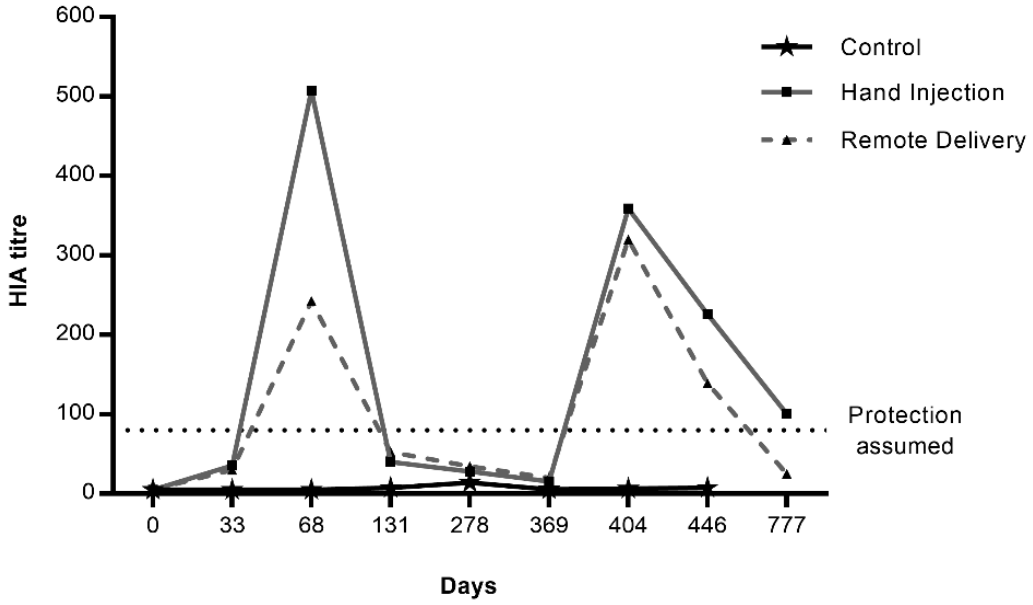


Figure 4.3. Geometric mean response for respective treatment groups

In Figure 4.4 the Reverse Cumulative Distribution Plot shows the spread of immunogenicity values between the control and vaccinated animals. The distribution curve of the immunogenicity values as shown in the control group is indicative of the small variation found around the mean (Table 4.2) and the consistently low titre values found across all blood sampling periods. In comparison, dogs vaccinated by hand all sustained similarly high immunogenicity values resulting in the distribution being skewed to the right followed by a sharp decline. The less steep curve seen in animals that were darted illustrates the greater degree of variability found in recorded immunological values and differences in sample size.

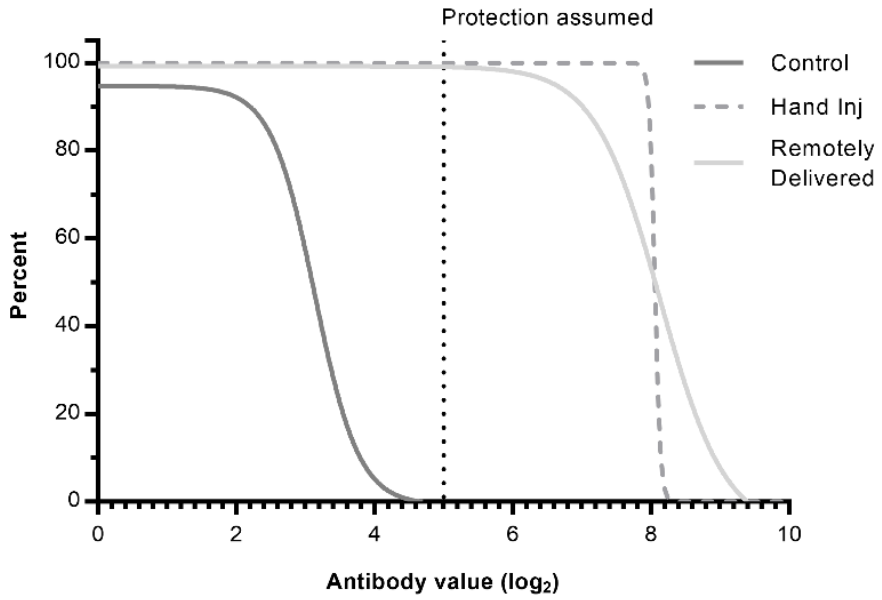


Figure 4.4. Reverse cumulative distribution plot

4.4 Discussion

The findings from this study indicate that the commercially available inactivated Parvac™ vaccine did stimulate an adequate humoral response in *Lycaon*, which was considered to be protective. No negative post vaccination reactions were observed throughout the study implying that this vaccine is safe for use in this canid. Data pertaining to duration of immunity in inactivated vaccines is generally quite limited with even less information for 'off-label' species (Schultz 2006). The primary response to the vaccine allowed immunity to persist for approximately 90 days, given the rise and fall of titres. From day 131 to the annual booster at day 369 immunity fell below the protective level. A similar result has been observed in Bush Dogs (*Speothos venaticus*) that were repeatedly vaccinated with an inactivated panleukopenia virus with titres being undetectable after 3 months (Mann *et al.* 1980).

Following the annual booster, there was a rapid anamnestic response with all dogs becoming seropositive in the subsequent month. Protected immunity persisted in the majority of animals for at least 77 days. With the reduced lag time in the production of antibodies it reflects a proliferation of memory B cells (Tizard 2013), which extended the period of protective immunity in a number of dogs. Opportunistic sampling more than a year after revaccination showed half of the sampled animals had sustained a protective titre. Two dogs were on the threshold of protection (1:80) with the remaining individual having an antibody titre of 1:320. This suggests that subsequent immune responses are able to provide protective immunity for a longer time before declining to a level where an

individual is susceptible to the disease. Tizard (2013) noted that some felines inoculated with the inactivated panleukopenia virus could continue to produce low levels of antibodies for many years.

Serology results showed no statistical differences in the seroprotection rate when comparing the two modes of delivery. While all inoculations performed in this study were deemed to be successful, the complete dose of injectable vaccines delivered by dart cannot always be confirmed (Miller & Fowler 2012). Goddard *et al.* (1990) established that the immune response of an inactivated CPV vaccine can be affected by administration route resulting from a lower dosage rate. Results of this study showed there was greater variability observed in the GMT between treatment groups, potentially indicating that delivery approach may influence long term protection. Comparative to live vaccines, inactivated vaccines typically require larger dosages when administered via a parenteral route and more frequent inoculations with smaller interval times between each (Sharma & Adlakha 2009).

Individual antibody titres of *A69115* were examined for potential differences in the response after being inoculated with the Dan-Inject dart rifle. The observed trend in waxing and waning of titres was not dissimilar from other vaccinated individuals from the remote delivery group. Alternatively, dogs *A59172* and *A59175* who received a third booster at day 68 had a less pronounced decline in antibody titres between days 131 and 278. As the sample size was small these results were only reviewed qualitatively. Natural exposure cannot be discounted in this study as serological results for *A49160* revealed a baseline imbalance in antibody titres at the commencement of the trial. Positive cut-off values used by laboratories to detect exposure to CPV have been reported as 1:10 (Fiorello *et al.* 2004), while Woodroffe *et al.* (2012) used ≥1:20 to indicate prior exposure to this pathogen in free ranging dog populations. This would suggest that the increase in titres found at day 278 in the control group could potentially be attributed to natural exposure despite no clinical signs being observed. In half of the control animals titres of individual dogs had either a 4 or 8-fold change compared to baseline levels. Blood collection at day 278 occurred mid-autumn where temperatures in the preceding months were above average (BOM 2012). Seasonality has been noted to increase the risk of CPV with a greater incidence observed during warmer months (Goddard & Leisewitz 2010; Houston *et al.* 1996).

Commercial vaccines commonly target the original CPV-2 antigen which has since been completely replaced by three alternative antigenic types; 2a, 2b and 2c (Decaro &

Buonavoglia 2012). In Australia the new CPV-2a is the predominant antigen in dog populations (Meers *et al.* 2007), while in southern Africa the CPV-2b variant is most widespread (Parrish *et al.* 1991; Steinel *et al.* 1998). Serological analysis has found that there are substantial differences in the cross-neutralizing activity between the various heterologous virus types (Pratelli *et al.* 2001). For example, dogs inoculated with a CPV-2 had higher neutralizing antibodies to this variant than the heterologous CPV-2b variant. Utilising a CPV-2 vaccine can lower and shorten immunity against the alternative variants with only sub-optimal protection achieved (Pratelli *et al.* 2001). In field situations the differences in protective immunity between homologous and heterologous variants is not clearly understood (Truyen 2006). There is some suggestion however that the use of certain vaccines that target specific antigen types could better complement that which is present in a given environment or that a polyvalent vaccine should be developed (Cavalli *et al.* 2008; Truyen 2006).

Monitoring post vaccination responses in exotic canids is important as there is not an expansive amount of information relating to antibody production, ability to seroconvert and sustained immunity. This process further assists in determining the safety of vaccines as some diseases have induced infection resulting in morbidity and mortality. Evaluating a serology response against a measure of protection, such as that for domestic dogs, can minimise the use of challenge studies. In the case of this vaccine there was no publicly available information pertaining to the DOI in domestic dogs for comparison purposes. With the African Painted Dog being an endangered species a direct challenge of the study group with the targeted pathogen would have been unethical. This puts greater reliance on immunological values to assess vaccine efficacy. Erring on the side of caution due to the absence of a challenge Böhm *et al.* (2004) increased the cut-off titre value which assumed protective immunity. This approach has merit when vaccinating endangered canid species as Spencer and Burroughs (1990) noted that there are potential differences in immune responses between members of the Canidae family with reference to CPV. It was further indicated that protocols should be developed specifically for individual species and for respective vaccines.

The discontinuous periods of protection immunity during the trial suggests that the given vaccination schedule was inadequate. To maintain a longer interval of protective immunity, particularly in the first year, an additional booster should be given at around 4 months with biannual revaccination occurring thereafter. The George Adamson Trust (1996) advocates the use of multiple boosters in a given year for this endangered species, especially when

utilising inactivated vaccines. Administration of Parvac™ could also be used in an attempt to prevent more animals from becoming infected during periods of high risk, thereby limiting the geographic spread of CPV through a barrier or suppressive vaccination strategy (DEFRA 2010). Results of this research also support Popelin's (2010) view who recommends using Parvac™ as a primer for attenuated vaccines, thereby reducing the chance that it may revert to virulence and cause clinical disease.

Managing infectious disease in free ranging populations generally focuses on populations but with rare and endangered species that emphasis can shift to the individual (Wobeser 2007). The success of a disease management approach for wildlife species can be challenged by factors relating to the practicality of some control methods due to cost, confirmation of a vaccine's efficacy, and effective surveillance and monitoring programs. Resources involved in immobilizing animals and the logistics in locating them can impose additional financial costs. Hence, the administration of the Parvac™ vaccine is more practicable in captive populations but less likely for free ranging packs due to the dogs' ranging behaviour and the recommendation for biannual vaccination.

As the integrity of habitats change, thereby imposing an alternative set of environmental stressors on wildlife populations, there is a greater need to better understand the dynamics of pathogenic diseases. CPV infections in other large wolf-like canid populations are known to have varied effects on population density, from very little to annual population increases being inversely related to the number of animals testing positive to CPV (Mech 1995; Mech *et al.* 2008; Mech & Goyal 1993). In African Painted Dog populations, there is little known about the regulatory role that CPV plays in population persistence. With an awareness of the preventative medicine approaches that are likely to succeed in a given situation it helps to improve the available options to wildlife conservationists.

4.5 Conclusion

Canine parvovirus occurs globally and is an emerging and re-emerging pathogen that has a broad range of host species. To decrease the risk of infectious disease, particularly in endangered wildlife species, validating a vaccine's immunogenicity is needed. As demonstrated the Parvac™ vaccine did elicit an adequate humoral response which constituted protective immunity. The duration of immunity was however limited but with an adapted vaccination schedule there is scope for protective immunity to be extended. The seroprotection rate was not affected by mode of delivery though monitoring of individual titres over a longer period of time could better assist with this determination. Despite the

limited knowledge about the efficacy of available vaccines in non-domesticated carnivores, there are greater advantages than disadvantages to vaccinating individuals should the need arise. Further research into the use of this vaccine in other canid species could assist in determining species specific differences in immune response, while examining factors like stress could help minimise negative vaccination outcomes. The latter issue of stress will be the focus for the following chapters.

5.0 Introduction

The efficacy of a vaccine is influenced not only by a variety of vaccine related factors but also by factors that pertain to the individual host. Management practices in both free ranging populations and captive environments have the capacity to expose animals to stressful situations. In some circumstances if an individual is unable to respond appropriately to such an event immunocompetence could become compromised (Apanius 1998). This chapter will outline the so-called 'handling debate' associated with the decline of *L. pictus* in various ecosystems across Africa and the potential role that stress had in this process. This is followed by an overview into what the term 'stress' means and how an individual responds physiologically when adapting to a changing situation. Indices used to measure stress both in African Painted Dogs and other endangered species will also be discussed. The information presented in this chapter provides context to the experimental approaches undertaken in Chapter 6 and addresses the fifth study objective.

5.1 The handling debate

The historical account of the decline and subsequent local extinction of African Painted Dogs in the Serengeti NP and the Masai Mara, Kenya has been discussed broadly in the literature with polarising views. Burrows (1992) hypothesised that the regional demise of this species in the early 1990s was disease-driven and linked to the effects of stress associated with intervention or handling (e.g. vaccination, immobilisation, blood collection or radio collaring). These animals were vaccinated using the inactivated rabies vaccines, Rabisin (Rhone-Merieux), Imbrab (Rhone-Merieux) and Madivak, Hoescht (Macdonald *et al.* 1992). It was postulated that the survival of handled dogs was reduced compared to unhandled individuals with this explanation dubbed the handling-stress hypothesis. Burrows *et al.* (1994) claimed several possible reasons for lower survival including: (i) packs may have contained carriers and individuals with latent pathogens, (ii) acute stress caused by intervention or chronic stress exacerbated by intervention, could have induced excretion of the pathogen by carriers, reactivated latent pathogens or minimised incubation time and, (iii) increased incidence of clinical disease or transmission rates within a pack.

Macdonald *et al.* (1992) regarded this hypothesis to be improbable, with Creel (1992) indicating that there was limited evidence to support handling as being dangerously stressful or that latency in some pathogens (e.g. rabies) existed. Kat *et al.* (1995) also

refuted Burrows' claim about latency in rabies suggesting it was purely speculative and should not be confused with an extended incubation time.

Ginsberg (1994b) acknowledged that the dramatic decline of this species was correlated to changes in research practices (i.e. intervention) but it was also consistent with a catastrophic disease event (e.g. rabies, CDV). Using gathered demographic data a Population Viability Analysis (PVA) for the Serengeti dog population determined that the localised extinction was unlikely to be due to chance alone (Burrows 1992; Ginsberg *et al.* 1995a). This strengthened Burrows' belief in the hypothesis. Modelling handling and survivorship of dogs in five ecosystems by Ginsberg *et al.* (1995a) however, showed that there was no effect on African Painted Dog occupancy, with different mortality factors at play within each ecosystem. It was considered that the mortality patterns observed in the Serengeti demographic data were consistent with a disease epidemic independent of any handling (Creel 1992; Ginsberg *et al.* 1995a; Macdonald *et al.* 1992). Burrows (2011c) reviewed the PVA indicating it was unrealistic as only demographic data collected during the believed population crash in 1975-76 was used to build the model and, for the other populations the data utilised was incomplete.

De Villiers *et al.* (1995) weighed into the debate by assessing the cortisol response of both captive and free-ranging dogs to a single immobilisation event and whether handling (capture/vaccination) caused mortality. This study showed that the cortisol response in dogs were not dissimilar to other immobilised carnivores (e.g. Spotted Hyena) and were consistent with an adaptive acute stress response. Handling was also shown not to be a causal link to reduced longevity in African Painted Dogs. De Villiers *et al.* (1995) stated that these findings did not support Burrows' (1992) hypothesis and suggested instead that chronic stress and the long term effects of social stressors were more relevant. De Villiers *et al.* (1995) criticised the classification of 'handled' (Burrows 1994), as mortality was shown to be significantly higher for vaccinated animals compared to those that were just immobilised (including radio collaring) and hence should have been analysed separately. Subsequent research by De Villiers *et al.* (1997) investigated the relationship between stress, rank, age and social skilfulness finding that there were age related differences in cortisol profiles that could be reflective of the dominance style employed by individuals to assume rank.

Citing research which had investigated the effects of routine stressful events on immunocompetence, Burrows *et al.* (1995a) rejected the claim that a single immobilisation

event does not cause chronic stress. They went on to further criticise the study done by De Villiers *et al.* (1995) suggesting the work had not demonstrated seroconversion after inoculation or monitored antibody titre levels over the research period.

Creel *et al.* (1997b) later separated the effect of handling from dogs that were radio collared and those that were unhandled, by opportunistically collecting faecal samples over a two year period in Selous Game Reserve to examine corticosterone levels. This study's objective was to test the impact of handling under typical field conditions for free ranging dogs rather than for vaccine use. Their results showed that compared to unhandled animals, collared dogs did not experience a negative stressor: no immunosuppressive response was detected. East *et al.* (1997) criticised a number factors in this study including, their choice to test for corticosterone rather than cortisol, the bias in selecting healthy animals to radio collar, and that there was no relevance to the impact of vaccine use on immunocompetence and survivorship, which related directly to the handling-stress hypothesis. In response, Creel (1997b) justified the use of corticosterone by stating that regardless of the hormone being tested, both corticosterone and cortisol share a synthetic pathway in the adrenal cortex with the same physiological mechanisms applying to each. Creel (1997b) also removed animals deemed hurt, sick or aged 5 years or older from the analysis to represent only healthy animals and still found no significant effects relating to handling.

Woodroffe (2001; 1997) reviewed the evidence associated with the disappearance of African Painted Dogs from the Serengeti ecosystem and the handling-stress hypothesis. Her findings were published in the Action Plan (1997) stating that the most likely reason for the dogs' localised extinction was because of a disease outbreak, and the failure of the vaccination program to provide effective immunity. It was argued that mortality was not confined to just the study packs or those that were vaccinated but also to unhandled non-study packs. Her work questioned whether dogs harboured latent viruses and the three mechanisms of reactivation proposed by Burrows *et al.* (1994; 1995a). These being, (i) that the stress of immobilisation for radio collaring (or blood sampling) might have reactivated an infection; (ii) drugs used for immobilisation might have suppressed their immune system making dogs more susceptible to infectious disease; and (iii) the utilised vaccines might have an immunosuppressive effect. While no causal link was established between handling and mortality it was acknowledged that immobilisation could not be proven to be harmless (Woodroffe 2001), particularly as there had been limited publishable research into repeated handling events and the role of chronic stressors. The Canid Specialist Group (CSG) did accept that there was a statistically significant correlation between handling and

reduced longevity. Woodroffe (2001) concluded by saying that the handling-stress hypothesis was not decisive in explaining the loss of the study animals in the Serengeti-Mara ecosystems.

After the disappearance of the African Painted Dog and the subsequent moratorium on handling from 1992, African Painted Dogs started to reappear in the Serengeti ecosystem by 2000. In 2007 invasive research techniques resumed with a whole pack dying later that year from CDV, confirmed by laboratory analysis (Goller *et al.* 2010). This was only the third clinically confirmed case of CDV in a free ranging African Painted Dog population with the other two episodes recorded in Chobe NP, Botswana in 1994 (Alexander *et al.* 1996) and in the Serengeti in 1968 (Schaller 1972). This extinct packs' range coincided with an area considered to be '*cordon sanitaire'* around the north-eastern boundary of the park where domestic dogs were routinely vaccinated against CDV, CPV and rabies. Despite these dogs not being vaccinated directly, their loss is consistent with the theory of the handling-stress hypothesis and incorporates at least two of its mechanisms.

Burrows (2011c) compared the Serengeti extinction event to the effects that invasive research methods have had on population abundance in other wild populations, e.g. Kruger NP, Moremi GR. Like the Serengeti, these populations have been studied intensively for extended periods and have also suffered rapid declines. Ginsberg *et al.* (1995b), for example, reported the survival of radio collared and blood sampled adult dogs in Kruger NP (1990-93) to be less than 12 months with 34% and 21% respectively of individuals not surviving. During 1995 to 2005 there was a recorded decline of 60% for individuals and 30% for packs in this ecosystem (Burrows 2011c). Pup survival was also shown to decrease steadily from 71% in 1975-78 (Reich 1981), to 56% in 1989, 30% during 1990-93 (van Heerden *et al.* 1995) and 61% to 34% in 1995-2000 (Burrows n.d). After 2005 greater emphasis was placed on photographic evidence to estimate population abundance with routine invasive research scaled back. Wildlife biologists studying this population could not explain the decline from a once stable population, with the only suggestion made being continuing years of above average rainfall (Mills 2002).

Similarly for Moremi GR, Botswana 25% and 33% of radio collared and blood sampled dogs respectively survived less than one year following handling (Burrows 2011c). At both study sites, animals with an alpha status were targeted predominantly and hence anaesthetised for radio collaring or blood sampling (Girman *et al.* 2001; McNutt 1996b). Burrows (2011c) claimed that there was little consideration given to the reproductive success of packs or to

the causes of death (i.e. disease related or accidental) post capture. He also suggested that the large mortality in adult dogs, particularly in Kruger, is predictive of the handling-stress hypothesis, regardless of the mechanism. Pack longevity would also be compromised by the strict social hierarchy held in packs as the death of either member of an alpha pair typically results in pack dissolution (Burrows 2011d).

Burrows (2011c) describes the sporadic loss of packs and the observed pattern of mortality to be significantly different after the prophylactic use of vaccines and the handling of African Painted Dog. This opinion stands in contrast to a number of well-known conservation biologists who provide limited acceptance of the handling-stress hypothesis as the most likely reason for the localised extinction of the Serengeti dogs and the decline of dogs elsewhere. The potential for detrimental effects to occur as a result of different handling practices cannot be discounted however and should be explored further to gain a better understanding into the short and long term effects these research methods have on this species.

5.2 Stress

Stress and stressful experiences have had a long association with the aetiology and pathophysiology of many health conditions in all animals, with there being notable knowledge gaps in the health consequences of chronic or repeated stress (McEwen & Gianaros 2011; McEwen & Stellar 1993). Stress responses can be categorised into three typical stages and are based on an evaluation by the brain to a perceived threat or stressor. The first two stages are adaptive and include (i) a change in physiology, behaviour and cognition (i.e. fight or flight) to enhance survival, and (ii) a resistance stage where physiological systems are restored to stable levels to minimise the deleterious effects of the stressor (Mendoza *et al.* 2000). The third stage is described as the stage of exhaustion which is initiated when the stage of resistance is not successfully completed by the return of stress related parameters to basal levels or that they remain at persistently high levels, thereby characterising chronic stress (Mendoza et al. 2000; Selye 1946).

It is the maladaptive aspect of the third stage which is central to the concept of allostatic load/overload. *Allostasis* refers to the process whereby an organism maintains physiological stability by altering internal systems to appropriately meet a host of environmental demands (Sterling & Eyer 1988). This is distinct from *homeostasis* which holds the body's internal environment in a constant through a series of self-correcting (negative feedback) actions within specific organs (Juster *et al.* 2010).

An allostatic load model helps to describe the process of adaptive functioning when repeated allostatic responses are activated by stressful stimuli. Biomarkers that represent primary mediators of an allostatic load include a range of stress hormones (e.g. cortisol) and pro and anti-inflammatory cytokines (Juster *et al.* 2010). These can have direct effects on cellular activity with other biological systems required to compensate for the over or under production of these primary mediators. Hence, the interactive effects of these mediators (Figure 5.1) are very complex and non-linear in behaviour, resulting from the compensatory changes both in magnitude and timing between each of the associated mediators (McEwen 2008). Secondary outcomes of allostasis are caused from a shift in mediators being linked to a range of metabolic, cardiovascular and immune parameters reaching suboptimal limits which can then lead to a sub-clinical state (Juster *et al.* 2010). This physical culmination of dysregulation is regarded as allostatic overload (or a tertiary outcome) and can lead to a range of endpoints including physical disorders, disease and in extreme situations senescence (Costantini *et al.* 2009).

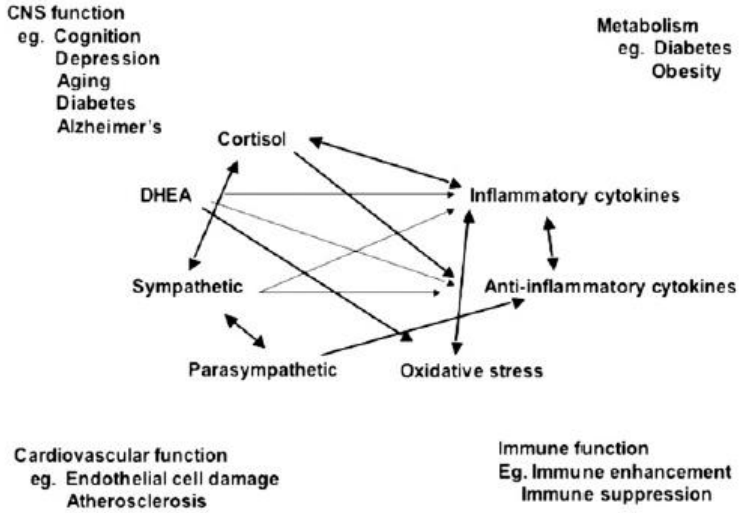


Figure 5.1. Non-linear network of allostatic mediators involved in a stress response. Arrows depict which system regulates the others, taken from McEwen (2008)

By contrast, meeting the demands imposed by stressful experiences can promote resilience and good health (McEwen & Gianaros 2011). For threatened and endangered species, having the capacity to adapt and deal with stressful experiences is essential for healthy populations and continued persistence. Stressors are varied in captivity and can stem from a range of environmental factors (e.g. lighting, audio and, olfactory stimuli, temperature, substrate) and conditions associated with being confined (e.g. restricted movement, an inability to retreat, abnormal social groupings, forced proximity to humans, transportation, reintroduction, Morgan & Tromborg 2007). In free-ranging populations stressors may include the inability to acquire adequate nutrition, changed climatic conditions, the social

dynamics of a species or the presence of other competitors or predators (Creel 2001; Parsons 1995; Robbins *et al.* 2004; Van Meter *et al.* 2009). More frequently these are also coupled with anthropomorphic stressors such as human-caused landscape change, indirect or direct harassment or disturbance, impacts from pollution, tourism, or in some situations the effects of war and civil unrest (Ament *et al.* 2008; Ditchkoff 2006; Lindsell 2011; Macbeth *et al.* 2010).

Factors that can further influence a stress response include an animal's life history stage, developmental history or reproductive condition. All can play a role in the degree of variation seen not only between individuals of the same species but also over the lifetime of the same individual (Reeder 2005). Variability between individuals has posed the greatest problem in assessing stress responses particularly in free ranging animals as there is a high degree of environmental, social and genetic plasticity (Moberg 2000).

Principally, monitoring stress is achieved by measuring glucocorticoid concentrations in various body fluids (Mostl & Palme 2002), with the main biological materials sampled including blood plasma, saliva, hair or faeces (Macbeth *et al.* 2010; Montanha *et al.* 2009; Young *et al.* 2004). The glucocorticoid, cortisol, is the primary steroid hormone monitored in mammals and is influential in regulating reproduction, digestion, immunity and growth (Novak *et al.* 2013; Sheriff *et al.* 2011a). In addition to stress hormones, monitoring oxidative stress parameters have recently been recognised as a complimentary approach to further understand the proximate mechanisms of how populations respond to environmental change (Costantini *et al.* 2011).

5.2.1 Glucocorticoid metabolite studies in African Painted Dogs

Studies investigating glucocorticoid metabolites in this species have focused mainly on reproduction and adrenal function, social dynamics and stress, validation of laboratory techniques and the response to various olfactory stimuli (Table 5.1). Prior to the handling-stress hypothesis there was limited research into the chemical release of hormones in African Painted Dogs and how they control and regulate the activity of certain cells or organs. van Heerden and Kuhn (1985) initially quantified progesterone, oestradiol-17β and cortisol concentrations prior to and after oestrus for sexually mature captive held females. They found that the dominant females in this group had highly elevated peripheral serum cortisol concentrations coinciding also with high progesterone and oestradiol levels. These results were in converse to the subordinate females who experienced a minimal rise in progesterone. It was suggested that the psycho-social stress exhibited in female dogs of this

species initiate a temporal change in circulating sex-steroid hormones, which has been described as a main mechanism for reproductive suppression in subordinate animals (Sapolsky 1985).

In free-ranging packs from Selous GR and Kruger NP, dominant dogs were also found to have higher basal corticosterone levels than subordinates (Creel *et al.* 1996a). Rank in African Painted Dog society is dictated by aggressive interactions (i.e. increased glucocorticoid levels) with the cost of social stress offset by gaining dominance and greater access to mates and food (Creel *et al.* 1996a; Sands & Creel 2004). In females, endocrine and behavioural patterns were correlated with alpha females being significantly more aggressive than subordinates only during the mating season. While alpha and beta males had testosterone levels comparable to each other while being higher than lower-ranking males. During a breeding season the rate of aggression between males decreased but was accompanied by a marked increase in severity when aggressive encounters did occur (Creel *et al.* 1997a). As glucocorticoid levels were elevated for higher ranked individuals it was also suggested that a non-glucocorticoid-mediated mechanism could be responsible for reproductive suppression in African Painted Dogs (Creel *et al.* 1997a). Van der Weyde (2013) found evidence to support this view with hormone concentrations in animals of this study being similar between age and social status; and that pseudo pregnancies in nearly all single sex female groups were observed. Further characterisation of reproduction biology in this species by Van der Weyde (2013) has highlighted similarities and differences with other canids providing greater support to both *in situ* and *ex situ* breeding programs.

Table 5.1. Studies investigating glucocorticoid levels in African Painted Dogs

Glucocorticoid metabolite	Study purpose	Biological material	Sample size	Concentration (ng/mL or g)	Reference
Corticosterone	Social stressor and dominance	Faecal	22 females: 34 males; n=216 samples	Males: ~90-150 Females: ~100-255	Creel *et al.* (1996a)
Corticosterone	Rank and reproduction	Faecal	Dataset used in Creel *et al.* (1996a) extra data from 18males; n=503 samples	Males: 120-90 Females: 225-100 skewed dom>sub	Creel *et al.* (1997a)
Corticosterone	Radio collaring animals	Faecal	Same dataset as Creel *et al.* (1996a)	Collared: ~100 Uncollared: ~120	Creel *et al.* (1997b)
Corticosterone	Adrenal function	Faecal	3 females; 2 males; sample no. not stated	Males: ACTH: Initial = ~100 Post = ~2,269 Females: ACTH: Initial = ~376 Post = ~3,275	Monfort *et al.* (1998)
Corticosterone	Laboratory technique	Faecal	4 captive bred males; n=23 (7yr)	Comparative mean values ranged from 101.1 to 160.3 ng/g of faeces	Santymire and Armstrong (2010)
Corticosterone	Oder cues, enrichment	Faecal	2 males (12yr)	Gazelle faeces elicited the greatest response with a 10.6% increase in activity ACTH: Initial = 56 (±1.7) Post = 174.3 (±4.6)	Rafacz and Santymire (2014)
Cortisol	Laboratory technique and adrenocortical activity	Faecal	2 females; 2 males; n=143 (4-8yr)	Response to ACTH challenge, a 125-325% increase in cortisol. Ranged from 103-657ng/ml	Ven (2009)
Cortisol	Female reproduction	Faecal	16 captive and wild females, n=62	Various reproductive stages ~200-550 ng/g of faeces	Van der Weyde (2013)
Cortisol	Female reproduction	Plasma	4 females; n=34 samples	Skewed dom>sub ~45.3-86.9	van Heerden and Kuhn (1985)
Cortisol	Immobilisation stressor	Plasma	12 females: 13 males; n=17	Both sexes: 52(±27)	De Villiers *et al.* (1995)
Cortisol	Immobilisation stressor and social dynamics	Plasma	9 females; 10 males; n=64	Both sexes: 65(±15) ACTH: Initial = 41(±17) Post = 122(±18)	De Villiers *et al.* (1997)

Whilst investigating social stressors and dominance, Creel *et al.* (1996a; 1997b) also investigated the effect that handling (i.e. radio collaring) had on survival showing that there were no significant differences in faecal corticosteroid levels between collared and uncollared animals. They observed that an increase in stress hormones was related more to sex and rank than to the actual field handling and wearing of a collar. De Villiers (1995; 1997) examined the effect of an immobilisation stressor on captive and free-ranging dogs focusing on the alternative cortisol metabolite from blood samples. Plasma cortisol concentrations were found to differ significantly from initial levels after a period of 10-20 minutes from immobilisation and again after approximately 70 minutes (De Villiers *et al.* 1995). Interestingly the concentration of cortisol decreased significantly after 30-40 and 40-50 minutes.

This observation is reflective of a secretory pulse and a subsequent attempt to return the system to homeostasis (Kolevská *et al.* 2003). In general there was an a 2.19 fold (or 119%) increase from initial cortisol levels for all animals after darting (De Villiers *et al.* 1995). Later De Villiers *et al.* (1997) assessed the effect of immobilisation in relation to age and rank for captive animals while also undertaking a adrenocorticotropic (ACTH) challenge. This test resulted in a 3.06 fold increase in cortisol concentrations compared to only a 1.51 fold increase in those that did not have any ACTH administered.

A number of studies have assessed the differing laboratory techniques used to monitor targeted hormones relative to reproduction, adrenal function and stress in the African Painted Dog. Monfort *et al.* (1998) tested the usefulness in monitoring faecal corticosterone levels in captive African Painted Dogs confirming its validity as a non-invasive approach for evaluating adrenal function and hence the measurement of stressors. This study administered a long acting ACTH preparation (400 IU) with entire scats collected for a period of 72 hours prior to and 144 hours post ACTH administration. Faecal excretory profiles were developed for both sexes with female dogs experiencing a 10 fold increase in mean faecal corticosterone concentrations compared to pre-ACTH levels. Corticosterone concentrations in the females were highest in the first 24 hours and later returned to initial levels by 48 hours. Samples gathered from the two male dogs had a much lower starting level with corticosterone concentrations increasing 25 and 13 fold respectively after the administration of ACTH. Like the females corticosterone concentrations in the males returned to basal levels by 48 hours. In general, measured changes for an acute stimulus were apparent within a 24 hour period being consistent with that also found in Grey Wolves (Sands & Creel 2004).

Identification of the most appropriate enzyme immunoassay (EIA) based on faecal glucocorticoid measurements was undertaken by Ven (2009). Results suggested that the cortisol EIA was the most suitable for measuring changes in faecal glucocorticoid metabolite levels in African Painted Dogs. Santymire and Armstrong (2010) compared a range of extraction methods and storage approaches with the view of developing a 'field friendly' technique for hormone analysis. They demonstrated that homogenization of samples was more efficient than handshaking with hormone stability not compromised when stored in plastic tubes for a period of 6 months at room temperature.

There is significant value in monitoring endocrine hormones as they can assist not only in developing a hormone profile for a species but they can also investigate what relationship a stressor has on the well-being of an individual and hence a population (Monfort *et al.* 1998). Within zoo populations enriching an animal's environment is central to improving an animal's quality of life and well-being. This can be achieved through increasing physical activity, stimulating natural behaviours and preventing or reducing stereotypical behaviour. One form of stimulus is to use odour cues with Rafacz and Santymire (2014) testing the response that African Painted Dogs had relative to activity and faecal corticosterone levels, when dogs were exposed to known and unknown competitors/prey. The greatest behavioural response was reported in a familiar prey species (i.e. Gazelle) with the more subordinate individual having the greatest response. This could suggest that in a social species with defined hierarchies, lower ranked individuals might benefit the most from enrichment activities.

5.3 Oxidative stress

The element of oxygen is necessary for life in all mammals but it can also be highly toxic or lethal in aerobic environments, particularly when found in excess. There is no formal definition for the term oxidative stress (OS) but in essence it is a serious imbalance between the production of free radicals or reactive species (RS, oxygen or nitrogen) and antioxidant defences (Costantini *et al.* 2010; Halliwell 2007). Sarma (2010) describes free radicals as molecules with an unpaired electron. In this state they are very unstable and react rapidly with other compounds to try and capture the needed electron to gain stability, consequently promoting oxidative stress. Young-Joon (2005) describe members of the reactive oxygen species (ROS) family as the superoxide radical anion ($O_2^{\cdot -}$), hydroperoxyl radical ($HO_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$).

Free radicals that are produced from the oxidation process cause structural and functional alterations in both biological membranes and lipoproteins (Armstrong 2011). Harmful effects can lead to DNA mutation, oxidation of polyunsaturated fatty acids in lipids, oxidation of amino acids in proteins or an inactivation of specific enzymes, ultimately resulting in the senescence of a cell (Sarma 2010). The deleterious impact of oxidative stress can not only be observed in cells (e.g. disrupted metabolism and apoptosis) but also in most biological systems (Mandelker 2011). In the course of an innate immune response, for example, phagocytic cells produce reactive species to assist in the destruction of a pathogen (Costantini *et al.* 2010). This induced oxidative stress causes a physiological cost to an individual with side effects including an inflammatory response or can lead to a chronic elevation of a baseline glucocorticoid metabolite, hence being a double edged sword (Hõrak & Cohen 2010). Reactive oxygen species are known to be responsible for normal regulatory function such as the induction of host defence genes and mobilisation of ion transport systems (Sarma 2010). Other benefits of ROS are noted in wound repair and blood homeostasis release where additional platelets can be recruited to an injury site (Winrow *et al.* 1993).

Research relating to oxidative stress in the field of ecology, has focused on a broad range of areas that can affect organismal behaviour, physiology, distribution and health/fitness (Brearley *et al.* 2012; Costantini 2008; Costantini *et al.* 2010). A number of studies have examined the relationship between antioxidants and immunity though these have concentrated mainly on avian species with other species groups being poorly represented in the literature (see Blount 2003; Costantini & Dell'Omo 2006; Hõrak *et al.* 2007). In the management of endangered species few studies have directly incorporated oxidative stress parameters. Schultz *et al.* (2011), for example, investigated the health of captive bred Brush-tailed Rock-wallabies (*Petrogale penicillata*) after being released into the wild, while Valdivia *et al.* (2007) derived baseline oxidative stress indicators for the Pacific Green Turtle (*Chelonia mydas agasizii*) to assist with a wildlife health assessment that could detect early exposure to environmental pollutants or emerging diseases.

A range of infectious diseases both in humans and animals have in some way been linked to oxidative stress (Pohanka 2013). In diseases involving neurodegeneration such as Alzheimer's or Huntington's, or in age-related cancers, there has been a direct link to elevated OS levels (Nunomura 2006). Epidemiologists noted biochemical differences in a mouse model of the rabies infection, finding that individuals which experienced higher levels of OS were more likely to have greater functional abnormality (Jackson 2010).

Jackson (2010) stated that oxidative stress played a central role in the degeneration of neuronal processes. Similar effects were observed in a case study involving the death of two captive lions. These individuals succumbed to lentivirus with it believed that oxidative stress played a part in enhancing the neurological damage which resulted in severe encephalopathy (Brennan *et al.* 2006). In domestic dogs, Panda *et al.* (2009) found oxidative stress indices were more pronounced for individuals suffering viral gastroenteritis compared to those that tested negative. For African Painted Dogs, little is known about the effect that oxidative stress has during disease pathogenesis or invasive research approaches. Examining oxidative stress under a range of management scenarios could introduce an alternative perspective to the handling debate and the potential cost of OS on the individual fitness of *L. pictus.*

5.3.1 Antioxidants

In circumstances where there are excessive amounts of RS, mammals have evolved antioxidant defences to limit the damage sustained by free radicals. Young-Joon and Lester (2005) describe antioxidants as a broad range of substances that act to help prevent cell and tissue damage. These compounds typically donate an electron to prevent or delay oxidation of an oxidisable substrate. Antioxidant enzymes are either water or lipid soluble being synthesized within the body or are obtained from an animal's diet. Vitamins C, E and beta-carotene are considered primary antioxidants (Clarkson & Thompson 2000), while other redox active substances include polyphenols and bioflavonoids are considered antioxidant sinks (Young-Joon & Lester 2005). Costantini and Dell'Omo (2006) suggest that carotenoids also play an important role in modulating immune function with higher circulating carotenoid levels produce larger T-cell mediated and humoral immune responses. Additional contributors to antioxidant defences include the lipoic acid, coenzyme Q_{10}, and various micronutrient metals (e.g. selenium, copper, zinc, manganese, iron) which are essential for continued activity in antioxidant enzymes (Young-Joon & Lester 2005). There are a number of key enzymes that assist with this process of neutralising or preventing free radicals, namely superoxide dismutase, catalases, glutathione and thioredoxin peroxidasis (Clarkson & Thompson 2000; Young-Joon & Lester 2005). Costantini (2008) summarises the function of antioxidants as being to, (i) quench the singlet oxygen, (ii) bind metal ions to avoid the production of RS and decomposition of peroxides to radicals, (iii) reduce the concentration (scavenging) of RS, (iv) chain breaking to prevent continued hydrogen abstraction from biomolecules, and (v) catalase reactions involving H_2O_2.

As interest in the role of antioxidants has increased so have the techniques in which to measure antioxidant damage and/or an individual's capacity to deal with oxidative stress (Abuja & Albertini 2001). Costantini *et al.* (2010) indicate that ecologists typically measure oxidative stress at a molecular level, thus having a more generalised view on anti-oxidative capacity. In a clinical laboratory there is no set group of biochemical assays that represent a routine analysis when testing for oxidative stress. Palmieri and Sblendorio (2007) indicate that measuring one or two oxidative parameters can produce misleading results as there can be many possible interactions between antioxidants. Using such measurements can consequently under represent the overall antioxidant status of an individual. Conversely, a small set of parameters may have some merit for evaluating a particular antioxidant therapy. Caution should also be used when selecting the appropriate marker as direct or indirect (inhibition vs. redox capacity) analytical approaches are based on different principles requiring users to have an understanding on the biological function they are targeting (Hõrak & Cohen 2010). Ultimately, each assay is a proxy for a given biochemical process which should provide a greater understanding to how a system works. This independent view should however, be interpreted with some care as these biochemical processes are part of a highly interdependent and complex system (Hõrak & Cohen 2010).

5.4 Summary

There is no doubt that stressful events can have significant consequences on the health of an individual. In the management of the African Painted Dog there is a perception that stress caused through research activities has contributed to the decline of some populations. Despite the differing opinions on this subject, stress-related research in this species remains limited with scant information available on how acute physiological changes can lead to a chronically stressed state. An individual's inability to return to a normal physiological function, or a maladaptive response to stress, is central to Burrows (1992) handling-stress hypothesis. In Chapter 6, the physiological response of the African Painted Dog to a repeated immobilisation stressor is assessed. In this experimental approach the primary stress hormone in mammals, cortisol, will be targeted both through invasive and non-invasive approaches.

6.0 Introduction

The African Painted Dog has experienced significant population declines over past decades requiring intensive management to both monitor populations and better understand the causal factors that limit abundance (Woodroffe & Sillero-Zubiri 2012). Research on this species has involved both invasive (e.g. deploying radio collars, vaccination, treatment of disease or injury, translocation) and non-invasive approaches (e.g. observation, faecal collection), with there being some conjecture on the effect that invasive approaches have had on the survival of individuals and local populations (Burrows 1992; 2011c). Understanding stress physiology is important for species management as high levels of stress can reduce individual fitness and limit reproduction (Van der Weyde *et al.* 2016). Despite capture and handling being a routine conservation activity, limited information is available on how dogs respond. This chapter addresses the sixth study objective by investigating the stress response of the African Painted Dog when immobilised and the subsequent relationship between invasive vs. non-invasive sampling approaches.

6.1 Capture stress

Although there is no formal definition for the term stress, it is an expression commonly used to describe an adaptive change to a physiological, psychogenic or environmental stimulus (Moberg 2000). This adaptive response is aimed at protecting an individual from a perceived threat and can involve changes in behaviour, and perturbations in the neuroendocrine and autonomic nervous systems (Mendoza *et al.* 2000; Selye 1946). A fundamental component in dealing with a stress response involves the activation of the hypothalamic-pituitary-adrenocortical (HPA) axis, which typically leads to increased secretion of glucocorticoids (Palme *et al.* 2005). While glucocorticoids are able to be adaptive in the short term, prolonged secretion due to chronic stress can be disruptive and can result in a physiological state known as 'distress' (Moberg 2000). Chronic stress or repeated activation of a stress response can have serious effects on an individual which can lead to lower reproductive rates, immunosuppression, muscle and bone atrophy, hypertension or the apoptosis of nerve cells (Romero 2004; Sapolsky 1990). The most important and biologically pertinent glucocorticoid metabolites are cortisol and corticosterone (Palme *et al.* 2005). Monitoring such hormones can provide useful information on the level of stress experienced by individuals and has increasingly been used

in conservation related studies to assess an animals' well-being or alternative management approaches (Busch & Hayward 2009).

The level of capture stress experienced by an individual can be related to the degree of invasiveness of that capture approach (e.g. manual handling, chemical immobilisation), the number of times captured and the coping mechanism of the individual. Such physiological responses may change over time with individuals becoming either more habituated or sensitive to such a procedure (Hämäläinen *et al.* 2014; Walker *et al.* 2006). Existing approaches used to measure a physiological stress response are determined by the amount of circulating glucocorticoid levels, with monitoring occurring invasively through repeated blood sampling or non-invasively by testing alternative biological materials (e.g. faeces). There are however notable differences between each approach. Glucocorticoid levels obtained from blood samples do not typically represent a baseline level as there is often a delay between capturing an animal and collecting the sample (>3 minutes), coupled with a rapid increase in circulating hormones resulting from capture (Wilkening *et al.* 2013).

Monitoring faecal glucocorticoid concentrations (FGC) is one of the most widely accepted and utilised non-invasive approaches but again there is a lag effect in FGC that coincides with an individual's gut transit time (Harper & Austad 2000). FGCs are also more reflective of biologically active unbound glucocorticoid concentrations than total glucocorticoid concentrations as seen in blood (Breuner *et al.* 2013; Sheriff *et al.* 2011a; Touma & Palme 2005). Measuring FGC provides an assessment to an individual's overall stress load as opposed to circulating levels that can represent a single point in time. This non-invasive approach is not affected by short term fluctuations in glucocorticoid levels as they are metabolised by the liver and secreted via the gut (Palme 2005). Additional factors that can further influence a meaningful result relate to an individual's diet, composition of gut flora, metabolic rate and the storage and analysis of samples (Goymann 2005; 2012; Millspaugh & Washburn 2004; Sheriff *et al.* 2011a).

There have been a limited number of studies that have investigated capture physiology in the African Painted Dog (Creel *et al.* 1996a; Creel *et al.* 1997b; De Villiers *et al.* 1995; De Villiers *et al.* 1997). Respectively these have focused on glucocorticoid concentrations in faecal material or separately in blood plasma with both targeting different metabolites. To date however, there has been no research in this species that has examined the same metabolite in two different biological materials. Similarly, there is limited information on how individuals respond to single or recurrent capture approaches and alternative handling

methods. This chapter aims to (i) measure the glucocorticoid response to an immobilisation stressor, during single, as well as, repeated capture events involving captive bred African Painted Dogs, (ii) assess changes in metabolite concentrations after an adrenocorticotrophic hormone stimulation test (ACTH challenge) and a low dose dexamethasone test to suppress adrenocortical activity, (iii) validate an enzyme immunoassay (EIA) to quantify a glucocorticoid metabolite in African Painted Dogs, (iv) make comparisons between invasive and non-invasive approaches to capture.

6.2 Materials and methods

6.2.1 Experimental animals

Thirty two captive born African Painted Dogs (19 males and 13 females) ranging in age from 3 to 12 years old were included in this study. All male dogs were housed at Monarto Zoo, South Australia and were either immobilised repeatedly as part of a concurrent experiment (see Chapter 4) or samples were collected opportunistically when there was a need to manage animals individually. Dogs participating in the simultaneous experiment were also randomly assigned to one of three groups that reflected the approach in which they were immobilised. At MZ all dogs are run through a crush facility that contains a squeeze cage. This apparatus was utilised as a primary method for immobilising dogs with 7 individuals mechanically restrained to facilitate blood collection. Another 6 male dogs were given an oral sedative to decrease sensory capabilities and locomotion prior to being mechanically restrained, while the final group of 6 animals were restrained and chemically immobilised yielding a complete general anaesthesia (GA).

Samples from female dogs were pooled together from four zoological institutions within Australia (Adelaide Zoo, AZ; Taronga Western Plains Zoo, TWPZ; Werribee Open Range Zoo, WORZ; and Monarto Zoo). In contrast to females held at MZ that were chemically immobilised after being mechanically restrained, all other females were chemically immobilised through the use of a remotely delivered dart. Two females (B09056, B09058) were transferred from MZ to TWPZ as part of routine breeding recommendations with the receiving institution performing their own health examinations. Samples obtained from the endpoint of this transfer where included to provide some insight into the cumulative effects of a transportation stressor, changed holding conditions and repeated handling of individuals typical to this management activity.

Dogs were immobilised using a range of drug regimens reflecting either partial or complete anaesthesia. Male dogs that were given the oral sedative were given a small piece of meat

injected with Medetomidine (2mg TD per animal, Pfizer Animal Health) prior to being immobilised with Atipamezole (1.2mls, Troy Laboratories P/L) administered as the reversal agent once sample collection had concluded. Complete anaesthesia in approximately half the animals was achieved using Medetomidine (1mg TD, Troy Laboratories P/L) and Ketamine (100mg TD, Troy Laboratories P/L) with Atipamezole (5mg TD) again used as the reversal agent for the alpha-2 agonistic. General anaesthesia was also induced by using Fentanyl (2.5mg TD, Kyron Laboratories) and Xylazine (25mg TD, Illium Veterinary Products) with Naltrexone (50mg TD, Greens Dispensary) and either Yohimbine (10mg/ml, Bomac Animal Health) or Rx (0.3mg TD, Sigma Life Sciences) used as the reversal agents.

6.2.2 Cortisol enzyme immunoassay

All immobilised animals had approximately 1.5ml of whole blood collected by venepuncture from either the cephalic or saphenous vein and placed into a sterile serum tube. For conscious animals only one blood sample was obtained, while for those individuals that were completely anaesthetised most had two blood samples collected. The first was collected after it was deemed safe to directly handle the animals, which was approximately 10 minutes after the administration of the anaesthetic agents, and the second twenty minutes later. During one sampling event 4 male dogs had blood collected every 5 minutes describing an immediate cortisol response post capture. Collected samples were centrifuged for 5 minutes at 13,600g on the day of collection and the serum placed into a screw top container and stored at -80˚C until required for laboratory analysis. Serum samples from other zoos were collated at AZ with samples appropriately packaged to maintain their integrity.

Cortisol concentrations were measured using a solid phase enzyme-linked immunosorbent assay (ELISA) kit (Demeditec Diagnostics DE1887) developed for use in humans. Antibody specificity and cross-reactivity to other steroids were stated as: corticosterone 45%, progesteron <9%, deoxycortisol <2%, dexamethazone <2%, other steroids <0.01%. Assays were performed in duplicate by adding 20µl of sample (or standard) to each well in the microplate followed by 200µl of an enzyme conjugate (horseradish peroxidase) and thoroughly mixed for 10 seconds. The microplate was left for 1 hour to incubate at room temperature where it was then repeatedly washed and blotted dry by sharply hitting the plate onto paper towel to remove any residual droplets. In each well 100µl of tetramethylbenzidine (TMB) was added and allowed to incubate for a further 15 minutes.

Optical density (OD) of cortisol was read at a 450nm wavelength using a Cobas Bio (Roche) analyser after the reaction was terminated by adding 100µl of 0.5M sulphuric acid solution.

Cortisol concentrations were calculated according to a standard curve with intra and inter-assay coefficients of variation being 2.24% and 10.03% respectively. The minimum level of detection was 0.43ng/ml and sensitivity in the 0ng/ml standard was found to be 1.3 ng/ml (n=16). A pooled plasma sample was prepared utilising 100 µl from all participating males. Recovery was estimated by adding a 10µl of the 800ng/ml ELISA standard to 90µl of the pooled serum sample and subsequently assayed. The mean recovery from plasma was calculated as 104% (Standard deviation, SD $\pm$ 6%).

6.2.3 ACTH challenge and dexamethasone suppression test

To substantiate the observed stress response a subset of adult male and female dogs underwent two complementary tests that reflect the differing feedback mechanisms utilised within the HPA. The function of ACTH is to regulate levels of glucocorticoids that are released from the adrenal gland. An ACTH stimulation test was used to pharmacologically validate the stress response by making comparisons between the maximum values obtained here to that of collected samples. Six animals participating in this research component (3M:3F, 6yo) were anaesthetised completely using Fentanyl and Xylazine and maintained on approximately 2% Isoflurane (Henry Schein) for the duration of the procedure with sexes processed separately and outside of breeding season.

Following De Villiers *et al.* (1997), blood collection occurred at 10 minute intervals for 70 minutes to develop an initial cortisol profile, after which one single dose of exogenous Synacthen (Tetracosactrin, Novartis Pharmaceuticals Australia P/L) was administered intramuscularly to 4 randomly selected individuals. Blood sampling continued to occur at 20 minute intervals for another 2 hours. The two remaining dogs (1M:1F) did not receive any Synacthen and were therefore utilised as a control with blood sampling occurring at the same time intervals as their conspecifics. Each animal was given Atipamezole and Natrexone to reverse anaesthesia and recovered in an appropriate crate where they were either assimilated back into the larger pack or in the case of the females transferred to another zoo.

A low dose dexamethasone suppression test was performed to measure the negative feedback response in the HPA. Administration of dexamethasone suppresses pituitary ACTH secretion causing a protracted decline in circulating cortisol. Plasma cortisol

concentrations in canids are significantly reduced within 3 hours and remain suppressed for 8 hours (Feldman & Nelson 2004). As animals in this study needed to be anaesthetised for longer than 8 hours, only individuals that were independently recommended for euthanasia were considered for this test. This group was comprised of 3 post reproductive animals (2M:1F, 12yo) and a female (6yo) that had had notable health issues over an extended period of time. Induction agents varied between the sexes with males anaesthetised using a regime of Medetomidine and Ketamine while Fentanyl and Xylazine were used for the females. Dexamethasone (Dexone-5, Virbac Australia P/L) was administered intravenously at 0.01 mg/kg (0.28mg TD) immediately after an initial baseline blood sample was collected, repeated sampling for all animals occurred at 1, 2, 4, 6 and 8 hours. Changes in cortisol concentrations were assayed using the previously described ELISA kit.

6.2.4 High-performance liquid chromatography

To confirm the glucocorticoid metabolite, cortisol, was being measured in the ELISA kit a comparison was made between the hormone properties observed in a series of pooled serum samples to that of two referenced standards (hydrocortisone, corticosterone). Stock solutions of hydrocortisone (1.45mg/ml, Sigma-Aldrich) and corticosterone (1.05mg/ml, Sigma-Aldrich) were prepared with high performance liquid chromatography (HPLC) grade methyl alcohol. Four standard concentrations (8, 80, 400, 800µl) were made and stored at -4˚C till analysis. A calibration curve for each reference standard was calculated from the peak height of each concentration with the resulting function used to estimate values in test samples.

Three pooled samples were prepared for each sex incorporating serum collected during either one of the scheduled capture events (n= 5M, 5F), the ACTH stimulation test (n= 3M, 2F) or low-dose dexamethasone suppression test (n= 2M, 2F). Known sample volumes were initially extracted by adding 200µl of ethyl acetate and 200µl absolute alcohol to an Eppendorf tube, then vortexed and centrifuged at 13,500g for 5 minutes. The supernate was removed and combined with 200µl of demineralised water in a fresh tube. The precipitate of the original solution was washed with 500µl of hexane and added to the aqueous solution in the new tube. Again these were vortexed and centrifuged with the organic layer taken off and added to another tube containing a small amount of sodium sulphate crystals (~0.3gm). The aqueous solution was washed a second time with hexane and added to that which was previously collected. All samples were vortexed and centrifuged for 2 minutes with the hexane solution removed and placed into a glass tube.

The sodium sulphate crystals were washed with hexane and combined with the latter and allowed to evaporate to dryness.

Detection of the glucocorticoids in the serum of *L. pictus* was performed by reverse phased HPLC with analysis conducted on a Waters (600) pump system equipped with a 2489 wavelength detector set at λ240nm and an Alltech Apollo C18 column. The mobile phase was isocratic consisting of water:methyl alcohol (30:70% v/v) at a flow rate of 1.3mL/min. Samples were reconstituted with 70µl of mobile phase with the injection volume being 35µl.

6.2.5 Non-invasive faecal monitoring

African Painted Dogs at MZ were observed weekly for approximately 3 months to ascertain their use of their 4.3 hectare exhibit. Parallel transects were placed in high activity areas at 10 metre intervals. The total transect length equated to 3.84 kilometres distributed across 25 transects with lengths ranging from 40 to 245 metres. A systematic sampling approach was employed during each scheduled handling period with transects walked on consecutive days by either one or two people for a 7 day period. A baseline glucocorticoid level was established from faecal samples collected in the first 3 days. Animals were handled on day 4 with samples collected on days 5-7 used to assess the stress response in each treatment group. The total sampling effort across the study period was 215 kilometres.

To identify individual faecal pellets from animals held at MZ, each dog was prescribed a specific marker comprised of either two natural seed types (i.e. canary, corn, safflower, sorghum, white millet, sunflower), coloured glitter (red, blue, green) or a combination of both. Appendix 3 displays the individual markers given to the male dogs held at MZ. The collective weight of the two different seed types ranged from 6 to 10 grams depending on seed type (e.g. markers containing corn generally weighed more) with approximately 1gm (~½ teaspoon) of glitter for specified individuals. Peanut butter was initially used to bind the seeds and/or glitter together which was then inserted into a self-made pocket in a small piece of meat. This binding agent was later modified to minced meat as a progressively larger proportion of dogs started to shake out the peanut butter, thereby reducing the actual amount of marker consumed. A preliminary investigation showed that gut transit time was between 16 and 30 hours, indicating that in the majority of cases a faecal pellet would be voided the next day. Hence, markers were opportunistically dispensed to all individuals the day before faecal collection was scheduled to begin at approximately the same time each day (~8.30am). Smaller pieces of meat were used to distract dominant individuals whilst markers were being dispensed to targeted individuals. Collection of entire fresh faecal

pellets commenced immediately after the markers had been dispensed and all dogs had been moved into off-limit enclosures. Gathered samples were weighed and frozen at -20˚C on the day of collection with all samples from that week later stored at -80˚C until required for further processing. All faecal samples were dried at 55˚C in a fan forced convention oven for 24 hours after which a dry weight was obtained. As the composition of faecal matter varied between samples a pilot study was conducted to assess which of the biological materials the cortisol metabolite bound more readily too. Individual samples were subsequently homogenised targeting this element with a known amount of faecal matter placed into an Eppendorf tube with 1ml of methanol and heated at 60˚C for 20 minutes. All samples were vortexed and centrifuged at 13,500g for 5 minutes where the resulting supernate was removed and placed into a glass tube. The sample was washed and centrifuged twice more with the new supernate solutions combined in a glass tube where collectively it was allowed to evaporate to dryness. The glass tube was rinsed with 1ml of methanol, vortexed for 1 minute with the solution transferred into an appropriately labelled screw top tube.

Samples were assayed using the same ELISA kit as previously described. A proportion of samples exceeded the maximum standard value requiring them to be diluted 1:10. A recovery was performed in a subset of samples by adding 20µl of the 100 ng/ml ELISA standard to a dry faecal sample prior to extraction. The mean recovery from faecal samples was calculated as 104% (SD $\pm$ 6%).

6.2.6 Statistical methods

Cortisol enzyme immunoassay

All analyses were performed using the computer software program GraphPad Prism (2013). Cortisol values that were not normally distribution were subsequently log transformed for statistical analysis. Using blood samples that were initially collected (i.e. time = 0) it was assumed that there would be no differences in mean cortisol levels relative to how animals were restrained and immobilised. A one-way ANOVA with a Dunn's multiple comparison test was employed to test respective differences in cortisol levels within each sex, as well as, between males and females. Comparative differences in cortisol levels were examined between darted female dogs at AZ and TWPZ, in addition to male and female animals that only underwent a general anaesthetic. To address age related factors associated with cortisol production, male dogs at MZ were targeted with it assumed that there would be no differences in cortisol levels between each cohort.

Repeated measures two-way ANOVA with a Tukey post hoc test was utilised to see if there were any dissimilarities between cortisol levels across each of the capture approaches for male dogs that were consecutively bled over the 14 month period. While individual differences in cortisol concentrations over time were assessed using a one-way ANOVA. A subset of the individuals from the hand injected (GA) group had blood taken every 5 minutes to assess their immediate response to capture with this information analysed using a repeat measures one-way ANOVA. A paired *t*-test was employed to similarly determine if there were significant differences between initial blood samples to those that were collected 20 minutes later for a subset group of dogs. To assess possible cumulative effects of immobilising and transporting dogs from one zoological institution to another an ordinary two-way ANOVA with a Sidaks multiple comparison test was employed on the samples gathered from the two female animals transferred from MZ to TWPZ.

Cortisol response to ACTH and Dexamethasone

For the ACTH stimulation test initial cortisol concentrations (INIT) were measured and utilised as an approximate basal concentration. A peak value (PEAK) was also recorded within the first 70 minutes of the procedure representing the maximum cortisol concentration obtained within this time frame. After the administration of ACTH a maximum cortisol concentration (A-MAX) was documented in the 2 hours following for each animal. This response is reflective of the dose given and the relative size of the adrenal gland. Basic statistical measures were calculated for each category identifying the mean, range and the proportional change.

A Receiver Operator Characteristic (ROC) curve is a popular and gold standard method for displaying the discriminatory accuracy of a diagnostic test and has been broadly used in a range of scientific areas (Fluss *et al.* 2005; Shultz 1995). A contingency table was constructed incorporating data gathered from the ACTH stimulation test (control vs. treated) with sensitivity and specificity computed. This assisted in determining whether the episodic cortisol release that occurred within the first 70 minutes was distinguishable from the ACTH induced response. A ROC curve plots the sensitivity versus 1-specificity for all results and is capable of illustrating the concession between the true positive rate and the false positive rate for a given scenario (Kumar & Indrayan 2011). The standard summary variable, Area Under the Curve (AUC), was calculated to evaluate the overall discriminative power or accuracy of the test. To determine the point where there is a clear difference between the true positive and false positive value a Youden's (1950) index *J* was derived (Equation 1). This index is frequently used as a summary measure that can assist

in establishing an optimal threshold value. Here, this would define acutely stressed and non-stressed individuals with the division of states reflected on a 0 to 1 scale. This also corresponds to a particular cortisol concentration. Graphically the index is shown as the maximum vertical difference between the diagonal chance line and the ROC curve.

$$J = \text{maximum } \{Sensitivity(c) + Specificity(c) - 1\} \qquad \text{Equation 1}$$

A dexamethasone suppression test assesses whether the HPA (i.e. the brain) is able to make the necessary negative feedback adjustments by reducing ACTH and cortisol production (Sheriff *et al.* 2011b). For recurrent samples a Friedman's test was applied to assess whether cortisol concentrations were equal across time. Changes in cortisol levels were examined individually with the lowest concentration (TROUGH) identified. Basic statistical measures (e.g. mean and proportional change) were also calculated for each time period.

Non-invasive faecal monitoring

An initial pilot study was conducted on a number of faecal samples (n=7) to ascertain which biological material cortisol was more readily bound too. Pellets were separated into specific material types including hair, bone, vegetative matter (e.g. grass), wood and indeterminate particulates otherwise referred to here as 'dust'. An independent *t*-test was used to compare mean faecal cortisol concentrations in sample groups comprising of different faecal components. It was hypothesized that no difference in cortisol concentrations would be found between each of the faecal materials.

Despite the usefulness and practicality of non-invasive monitoring approaches no study to date has focused on correlating cortisol plasma levels to non-invasive FGC levels in the African Painted Dog. A Pearson's bivariate correlation coefficient was computed and a scatterplot using both faecal and plasma cortisol concentrations was constructed. This provides opportunity to assess the relationship between plasma cortisol levels on the day of capture to that found in faecal samples collected in the days following immobilisation. An unpaired *t*-test was utilised to determine if there were any significant differences between samples that were identified to an individual versus those that were not, to determine whether all faecal samples could be pooled for further analysis.

Finally, faecal cortisol concentrations before and after immobilisation were investigated not just for the different immobilisation approaches (manual restraint, sedation, hand injected and unknown) but also across time (capture events 1 to 8). As there were multiple variables

involved in each of the explanatory variables and an uneven sample size an unweighted means analysis was employed. To investigate whether immobilisation does cause an acute stress response, as seen by a non-invasive approach, it was hypothesised that there would be no differences in faecal cortisol concentrations before and after immobilisation. This was coupled with an assessment on whether capture method had an impact on faecal cortisol concentrations. In a separate analysis, it was further hypothesised that there would be no differences in faecal cortisol concentrations across time. Table 6.1 provides an overview of the statistical tests conducted using plasma and faecal samples in this chapter.

Table 6.1. Statistical tests used to assess a stress response

Sample type	Statistical test	Purpose
Plasma	Friedman's test	Evaluated the differences in medians among the associated time periods for the low dose dexamethasone suppression test.
	Independent *t*-test	Compare cortisol concentrations between male and female animals that only underwent a general anaesthetic. An immediate response to capture was assessed in a subset of male dogs (GA group) from blood taken every 5 minutes (for 20 minutes).
	One-way ANOVA	Compare initial cortisol concentrations (time 0) between the sexes. Assess age related factors associated with cortisol production. Compare cortisol concentration of darted female dogs housed at different zoos. Compare individual differences in cortisol concentrations over time. Examine initial response to capture from all animals (time 0).
	Paired *t*-test	Assess acute stress response in both sexes, using cortisol samples collected at time 0 and then 20 minutes later.
	ROC curve Youden's index	Employed to statistically discriminate between plasma cortisol concentrations gathered during the first 70 minutes (i.e. control period) to those collected after the administration of ACTH. Derived to determine acutely stressed and non-stressed individuals, with the division of states reflected on a 0 to 1 scale.
	Two-way ANOVA	Compare cortisol concentration of female dogs transferred between zoological institutions (MZ and TWPZ). Evaluate capture approach on faecal cortisol concentrations over the total study duration.
Faecal	Independent *t*-test	To ascertain which biological material cortisol binds more readily too.
	Two-way ANOVA	Assess differences in faecal cortisol concentrations before and after immobilisation; and separately different immobilisation approaches across time.
	Unpaired *t*-test	Make comparisons in faecal cortisol concentrations from identified and unidentified individuals.
Plasma/faeces	Linear regression	Used to predict the concentration of FGC based on the plasma cortisol levels.
	Pearson's correlation	Calculated to assess the relationship between plasma cortisol levels on the day of capture (day 4) and the faecal glucocorticoid concentrations in the 3 days following the procedure.

6.3 Results

6.3.1 Plasma cortisol enzyme immunoassay

Cortisol values, determined from initial blood samples, were pooled for both sexes (n=164) and analysed using a nonparametric one-way ANOVA (i.e. Kruskal-Wallis test). Results showed that there was a statistical difference in cortisol concentration between capture approaches, $\mathcal{X}^2(3) = 20.62$, $p = 0.0001$, with a mean rank score of 72.7 for dogs manually restrained, 64.55 for sedated animals, 100.6 for dogs anaesthetised via hand injection and 113.8 for darted individuals (Figure 6.1a). Reanalysing the data without the inclusion of female dogs showed the same trend in the male dogs captured by manual restraint, sedated or chemically immobilised by hand injected ($\mathcal{X}^2(2) = 13.48$, $p = 0.0012$). Male dogs that were given a sedative prior to being restrained had the lowest cortisol concentrations (44.57 ng/ml, $CI_{95\%}$ 34.28 to 57.91) compared to those that were manually restrained (53.95 ng/ml, $CI_{95\%}$ 45.19 to 64.42) or anaesthetised by hand injection (76.03 ng/ml, $CI_{95\%}$ 65.15 to 88.51).

Based on an approximate significance value, the Kruskal-Wallis test identified that there were differences in cortisol levels between age cohorts (Figure 6.1b), $\mathcal{X}^2(3) = 12.14$, $p = 0.0069$. Male dogs aged 6 had the greatest mean cortisol levels at 71.45 ng/ml ($CI_{95\%}$ 60.81 to 83.75) being closely followed by the 5 year olds (57.68 ng/ml, $CI_{95\%}$ 46.99 to 70.79). Older animals aged 7 and 11 years had the lowest mean cortisol levels at 43.45 ($CI_{95\%}$ 32.96 to 57.41) and 41.11 ($CI_{95\%}$ 24.66 to 68.55) respectively.

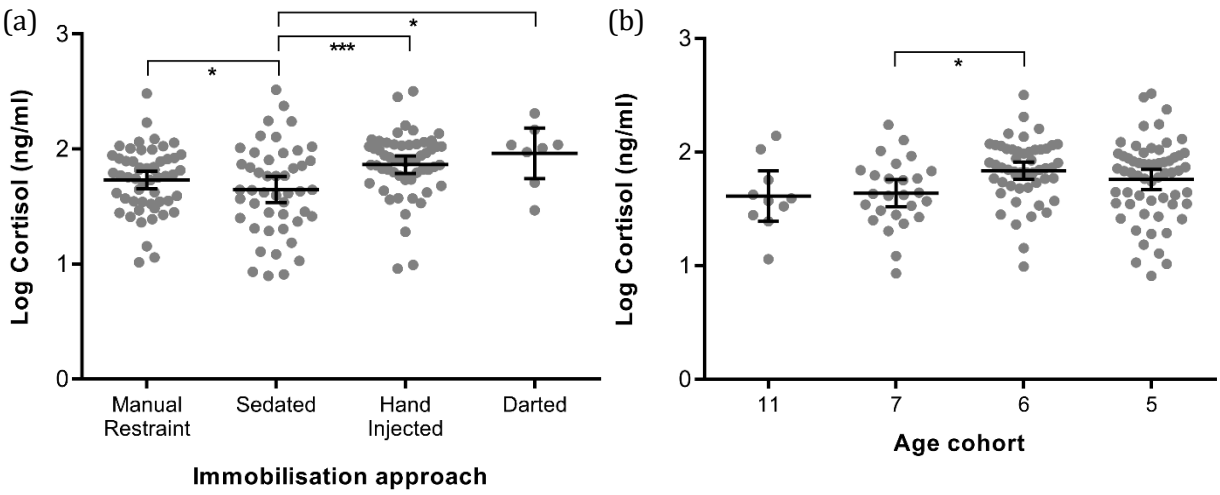


Figure 6.1. Initial cortisol response relative to (a) capture approach in both sexes; (b) males of varying age

An independent *t*-test was used to compare the cortisol concentrations between males and females. The Mann-Whitney *U* test showed no significant differences between the sexes overall ($U = 719$, $p = 0.0221$) or for those dogs that were completely anaesthetised (darted vs. hand injected), $U = 295$, $p = 0.3523$. To examine whether cortisol concentrations differed between female dogs housed at AZ, MZ and TWPZ (excluding transferred dogs) a Kruskal-Wallis one-way ANOVA was utilised. This showed no notable differences in concentration levels between each respective institution, $\mathcal{X}^2(3) = 0.6038$, $p = 0.7622$. It also permitted samples collected from female dogs to be pooled.

Figure 6.2 illustrates the mean cortisol response for male dogs captured over successive periods. Significant differences in the concentration of cortisol were noted across time ($F_{r(7)} = 9.37$, $p = <0.0001$) with a pairwise comparison identifying differences in plasma cortisol levels only within the sedated and GA'ed groups. Across the total capture period, the fifth capture event had the highest mean cortisol level recorded overall ($M = 108.1$ng/ml, $SD = 77.08$) as opposed to the lowest being in the second capture event ($M = 31.71$ng/ml, $SD = 27.25$). Reviewing the mean cortisol levels between successive time points identified the third and fifth capture events as having the greatest increase in cortisol compared to previous capture sessions, respectively 97% and 70%. Comparing against initial levels, however, it was only the fifth capture event that exceeded initial levels (16.5% increase).

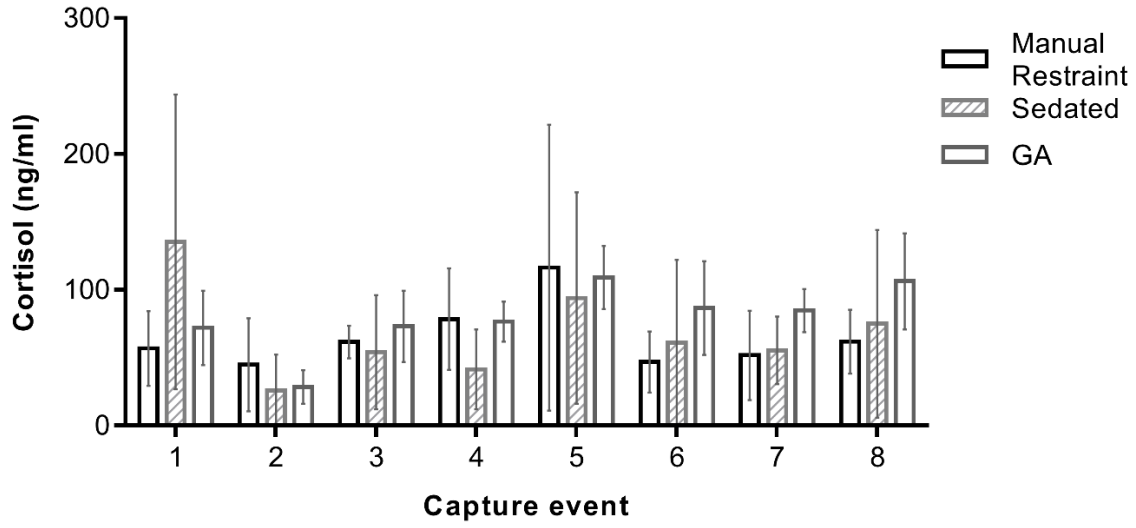


Figure 6.2. A mean comparison of plasma cortisol levels with 95% confidence intervals after repeated capture events

Significant differences in the concentration of plasma cortisol were noted between individual male dogs ($F_{r(15)} = 5.497$, $p = <0.0001$). A Z-score was computed using the mean

cortisol concentration for each dog (across all capture events) and subsequently compared to the initial (*M* = 88.06ng/ml, *SD* = 71.59), as well as, peak value (*M* = 138.54ng/ml, *SD* = 72.84) obtained across all capture events. Using an independent *t*-test showed no significant differences between the initial cortisol concentrations of each animal compared to their individual mean. But observable differences were however noted between the mean values and the individuals' peak concentrations (t =2.0687, p = 0.0013). Only two dogs appeared to have relatively consistent cortisol concentrations throughout the experimental period (<1 *SD*), with one (*A59173*) being from the manual restraint group and other being (*A69120*) from the sedated group. Peak cortisol concentrations in most dogs exceeded the mean value by approximately 1.9 *SD*, with two other dogs (*A69113* and *A69115*) both having 4 *SD* from their mean value. Dog *A69113* from the sedated group had the highest recorded cortisol concentration for all individuals during the first capture session at 327.6ng/ml. This animal was also observed to have the maximum cortisol level for individuals in four out of eight capture events.

A subset of male dogs from the GA group had their blood taken every 5 minutes with there being no statistical difference in cortisol levels across respective time intervals (Figure 6.3). Concentrations of cortisol for these individuals varied by 30.74% in the first sample with two notable responses observed over the 20 minute period. Cortisol levels increased by 230% and 123% after 5 minutes in *A59175* and *A59171* respectively, while it decreased by 14% and 23% in the other two individuals. Dogs that had the highest levels compared to the baseline reading also had the highest cortisol values at the 20 minute sample. For *A50172* and *A50174* however, the concentration of cortisol had either returned to or had dropped below their baseline level. The variability in cortisol concentrations for all individuals did narrow across the three intermediate sampling periods, but by 20 minutes variability was shown to be only marginally lower (i.e. 28.81%) than the baseline level.

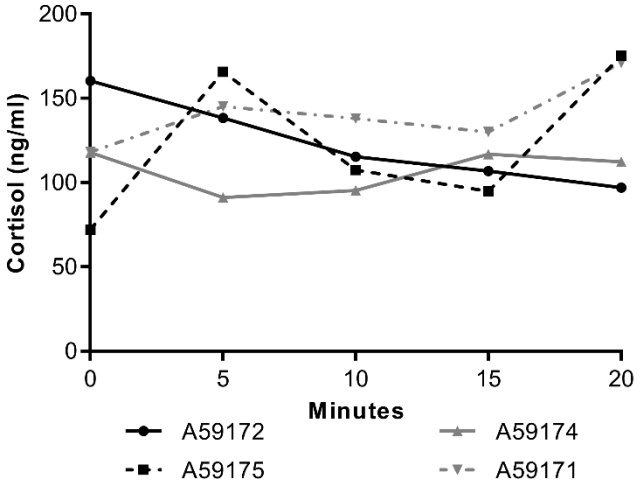


Figure 6.3. Cortisol response within the first 20 minutes of being immobilised for a subset of male dogs

To examine whether plasma cortisol concentrations taken when an animal was initially immobilised (M = 76.91, SD =1.86) were within a similar range to samples taken 20 minutes later (M = 63.10, SD =2.00), a paired t-test was used involving 61 paired samples from animals of both sexes. Prior to conducting the analysis, the assumption of normally distributed difference scores was examined. This assumption was satisfied, as the skew and kurtosis levels were estimated at -0.460 and 1.779 respectively, which is less than the maximum allowable values for a t-test (Posten 1984). It should also be noted that the correlation between the two blood samples was estimated at r = 0.647, p = <0.0001, suggesting that the dependant samples t-test was appropriate in this case. The null hypothesis that the mean cortisol concentrations were equal was therefore rejected, t (60) = 2.760, p = 0.0077. Thus, the cortisol concentration taken in the initial sample was statistically higher than that taken 20 minutes later suggesting that an acute stress response was elicited as a response to capture.

Transportation of animals has been attributed to higher plasma cortisol levels in a range of species. A two-way analysis of variance tested the plasma cortisol concentrations of two female dogs before and after they were transported from one zoological institution (MZ) to another (TWPZ) with blood samples taken 7 days apart. Both individuals that were transferred showed significant differences in cortisol levels compared to each other with *B09056* having repeatedly higher cortisol concentrations (M = 288.3, SD = 90.03) than *B09058* (M = 45.36, SD = 22.34), $F_{(4)}$ = 21.15, p = 0.01. However, when comparing samples taken at each respective zoo there were no differences in cortisol levels $F_{(4)}$ = 0.5308, p =

0.5066 suggesting that transporting and holding these animals in unfamiliar enclosures did not significantly contribute to increased cortisol levels.

6.3.2 Cortisol response to ACTH and dexamethasone

In the ACTH stimulation test the mean cortisol concentration for the initial sample was 123.5ng/ml, with females having a higher concentration than males (137.1 and 109.9ng/ml respectively). In all female dogs their peak was during the second blood sample at 10 minutes while the male dogs' peak fluctuated between 20-40 minutes (Table 6.2). This variation in sexes was further reflected in the pooled results where slight increases of only 2% and 5% were observed at respective time periods when compared to previous blood draws. With the exception of *A69113* there was an average plasma cortisol increase of 62% in peak concentrations (within 70 minutes) compared to initial levels. The highest percentage change recorded was for *B09057* who subsequently died under anaesthetic 30 minutes into the procedure suggesting that there were additional physiological factors at play. In contrast, the concentration of plasma cortisol in the control dog showed an approximate increase of 43% 10 minutes after the initial sample was collected. For this dog a negligible difference in cortisol concentration was recorded between its PEAK and A-MAX values. Cortisol concentrations for each individual animal are presented in Appendix 4.

Table 6.2. Initial and peak responses to the ACTH stimulation test

Animal ID	Sex	Wgt (kgs)	Synacthen Dose (IU)	INIT	PEAK		A-MAX		Percentage Change	
				Conc*	Conc	Time (mins)	Conc	Time (mins)	INIT vs. PEAK	INIT vs. A-MAX
A69113	M	29.0	16.5	209.61	90.03	30	162.63	130	-57.05	-22.42
A69117	M	25.4	14.5	57.52	92.48	40	105.72	110	60.80	83.81
B09056	F	30.4	40	318.79	390.53	10	372.28	150	22.50	16.78
B09058	F	29.2	40	60.45	99.49	10	141.05	170	64.58	133.33
A69119	M	30.0	Control	62.58	89.97	20	63.49	150	43.76	1.45
B09057	F	29.4	Control	32.04	70.63	10	-	-	120.41	-

* Cortisol concentrations represented as ng/ml

Following the administration of Synacthen all except one dog (*A69113*) had discernible increases in plasma cortisol concentrations compared to initial values. The greatest increase was noted in a beta female (*B09058*) with a 133% increase. A maximum cortisol concentration was not achieved in two individuals (*A69113* and *B09056*) when compared to naturally derived levels. *B09056* had a peak cortisol value of 390.55ng/ml 10 minutes

after the initial sample was collected within the ACTH trial, and *A69113* was recorded as having a concentration of 327.6ng/ml in the first capture event. Both of these dogs had baseline levels approximately 3.5 times greater than the other individuals that were administered ACTH (i.e. *A69117* and *B09058*). Figure 6.4 shows graphically the initial response to capture and after the administration of ACTH. Mean (±SD) values are shown with the range depicted as minimum and maximum values for the pooled group. Cortisol concentration values for male and female dogs have been separated to reflect potential sex related differences. The percentage change in the concentration of cortisol is shown across time with the raw data displayed in Appendix 4.

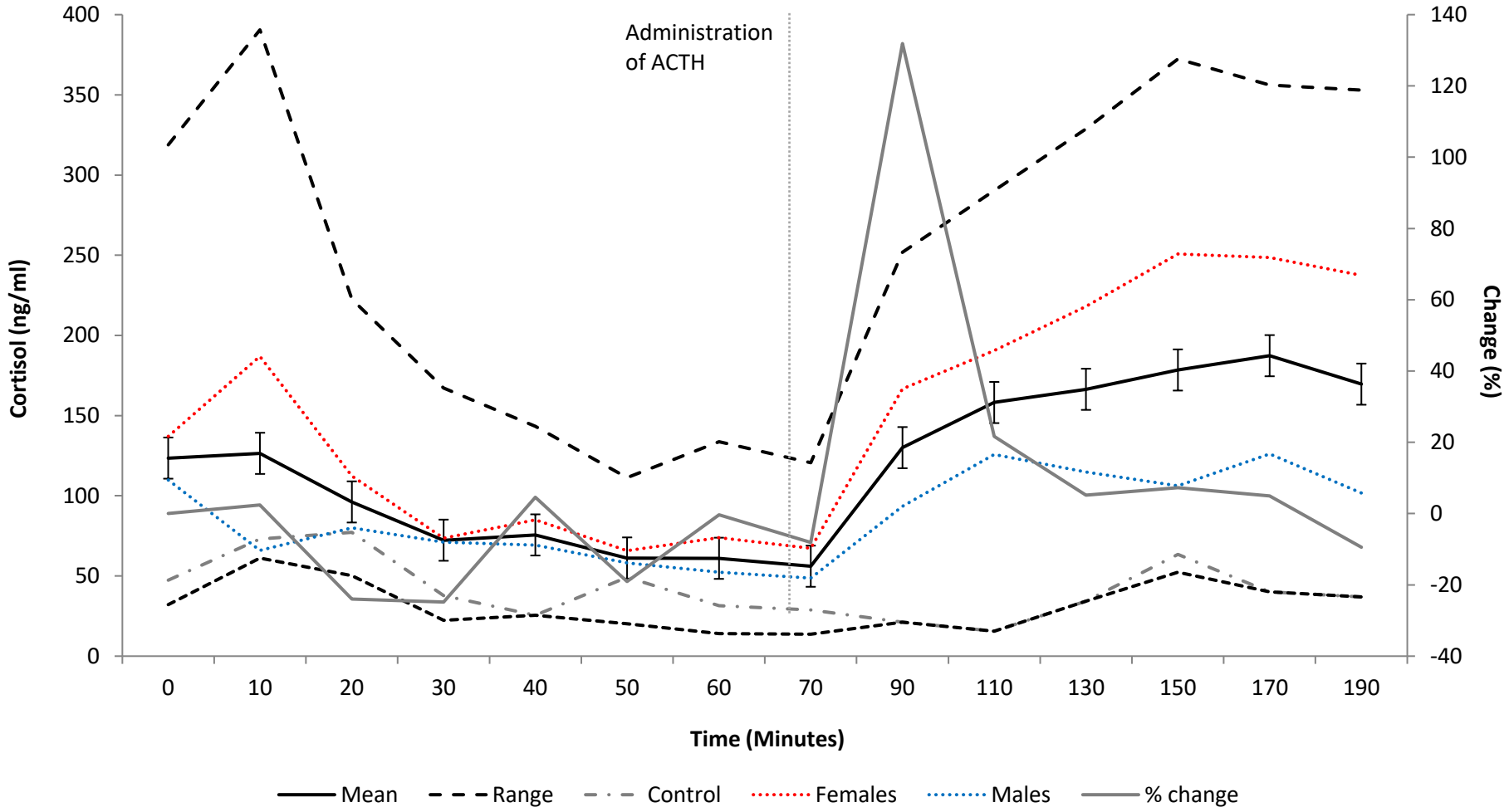


Figure 6.4. Cortisol response to capture and administration to ACTH in blood plasma (±SE)

As a maximum adrenal response was not attained, due to naturally occurring concentrations exceeding the stimulated response, the capacity to develop a profile representing an upper limit was reassessed. Given the small sample size and variability amongst participating individuals was diverse, a ROC curve was employed to statistically discriminate between plasma cortisol concentrations gathered during the first 70 minutes (i.e. control period) as opposed to those values collected after the administration of ACTH. With an AUC score of 0.8097 (SE 0.05234, $CI_{95\%}$0.7070 to 0.9123, p <0.0001) accuracy of the test was considered good suggesting that an acute stress response was elicited and that it was better than chance alone.

Graphpad Prism further produced tabular data reflecting incremental sensitivity and specificity values and a corresponding positive likelihood ratio. The former were used to apply Youden's index to determine the cut-off criterion. Figure 6.5 shows graphically the ROC curve and the maximum vertical difference indicative of Youden's index. This index was found to be 0.56 with sensitivity and specificity deemed to be 87.5% and 68.18% respectively. The optimal threshold value between a non-stressed vs. a stressed state was determined by a plasma cortisol concentration of greater than 80.72ng/ml, with a positive likelihood ratio of 2.75. In a group of animals, it could therefore be assumed that 12.5% of individuals could have a predisposition to be acutely stressed. Moreover, individuals above the threshold value of 80.72ng/ml are 2.75 times more likely to be experiencing a degree of stress. In developing a contingency table, positive and negative predictive values were able to be calculated. This indicated that 28.2% of animals with a positive test result were actually acutely stressed, while in converse 97.45% of animals that tested negatively were considered to not be stressed.

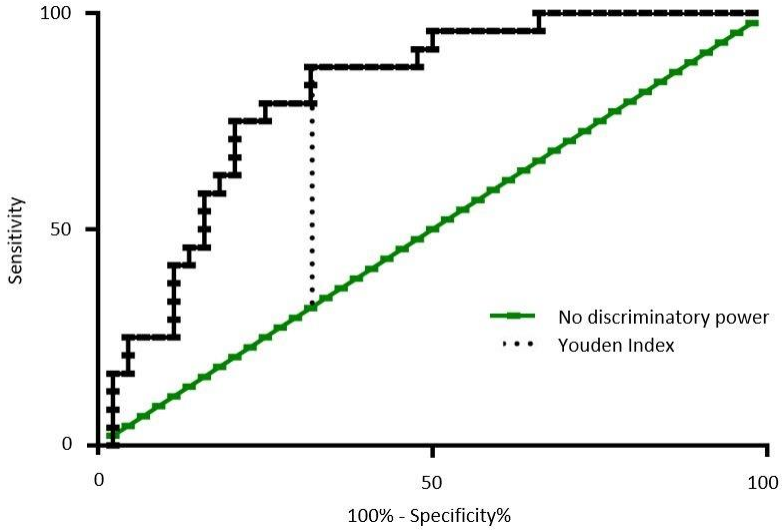


Figure 6.5. ROC curve differentiating between control and ACTH administered animals

As the sensitivity and specificity values (i.e. 87.5% and 68.18%) are consistent regardless of population size there is scope to apply these statistics to the data obtained in the immobilisation trial for male dogs at MZ. Plasma cortisol concentrations for each animal across all capture events were reviewed and samples above the threshold value, 80.72ng/ml, identified. A proportion in terms of the number of times an animal was above this value compared to the number of times the individual was immobilised was calculated (Appendix 5). Three dogs were identified as having plasma cortisol levels greater than the threshold value in more than half of the capture events, these being *A59172*, *A59171* and *A69113*. The first two individuals had increases in cortisol from the fourth capture event with *A59172* having concentrations marginally higher than the threshold value in two cases, while *A69113* had consistently high levels. The minimum number of animals seen to have elevated concentrations fits within the general parameters of the predicted values displayed in Table 6.3.

Table 6.3. Extrapolated predictive values in relation to the immobilisation trial at Monarto Zoo

	Stressed	**Not stressed**	**Total**
Positive test	2.0	5.0	7.0
Negative test	0.3	10.7	11.0
Total	2.3	15.7	18.0

The alternate negative feedback mechanism in the HPA was assessed by administrating a single dose of dexamethasone, which under normal conditions would elicit a decrease in plasma cortisol concentrations over time. Administration of dexamethasone occurred immediately after the animal was stable (from the perspective of the anesthetic) and the first blood sample had been obtained. A non-parametric Friedman's test evaluated the differences in medians among the associated time periods finding that there were significant differences, $\mathcal{X}^2(6) = 11.19$, $p = 0.0175$. Mean plasma cortisol concentrations are displayed in Table 6.3 along with the percentage change for each period, with individual responses presented in Appendix 6. The maximum suppression of cortisol for this group of animals was found to be 120 minutes after the commencement of the procedure with there being an average reduction of 65.05% compared to initial values. There was a clear suppressing trend in 75% of animals with *A29038* deviating from this response. Plasma cortisol concentrations in the majority of animals at this time were 12.31ng/ml ($\pm$0.95) with *A29040* recording the lowest concentration of 8.99ng/ml an hour later at the 240 minute blood draw.

Initial cortisol concentrations for *A29038* increased by approximately 18% after the first hour with a reduction of only 22% compared to baseline levels seen after the second hour. This individual was in very poor body condition and died under anesthetic. The lowest concentration level for *A29038* was approximately 54% higher than the mean level of all other individuals at TROUGH. The initial rise in cortisol observed in this animal demonstrates a dysregulation in the feedback mechanism and is indicative that this individual was suffering from a chronic physiological stressor.

Table 6.4. Percentage change compared to baseline levels and the mean cortisol response at individual time intervals for the low dose dexamethasone suppression test

Animal ID	Sex	Wgt	Dose	INIT Conc	Percentage Change* (mins)					TROUGH	
					60	120	240	360	480	Conc	Time
A29040	F	26.1	0.28mgTD	65.40	-57.32	-81.82	-86.25	-77.35	-46.46	8.99	240
B09059	F	28.1	0.28mgTD	65.91	-71.88	-82.70	-31.46	-16.51	-12.31	11.40	120
A29037	M	20.8	0.05ml	39.39	-73.36	-65.40	-56.59	15.15	53.53	10.49	60
A29038	M	16.0	0.04ml	74.33	18.38	-22.26	-69.72	-	-	22.51	240
Mean percentage change					-46.05	-65.05	-61.00	-26.24	-1.75		
Mean cortisol conc			Overall	61.25	36.23	23.67	23.44	38.40	51.09		
			Females	65.65	23.22	11.65	27.08	34.92	46.40		
			Males	56.86	49.24	35.70	19.80	45.35	60.47		

* Compared to INIT levels

After 240 minutes a steady increase in plasma cortisol production was noted with concentrations only being 16.5% lower than initial levels at the experiments conclusion (i.e. 480 minutes). Mean plasma cortisol concentrations with standard deviation values are shown in Figure 6.6 with the range depicted as minimum and maximum values for the pooled group. Separate values reflecting each sex along with the percentage change are also illustrated.

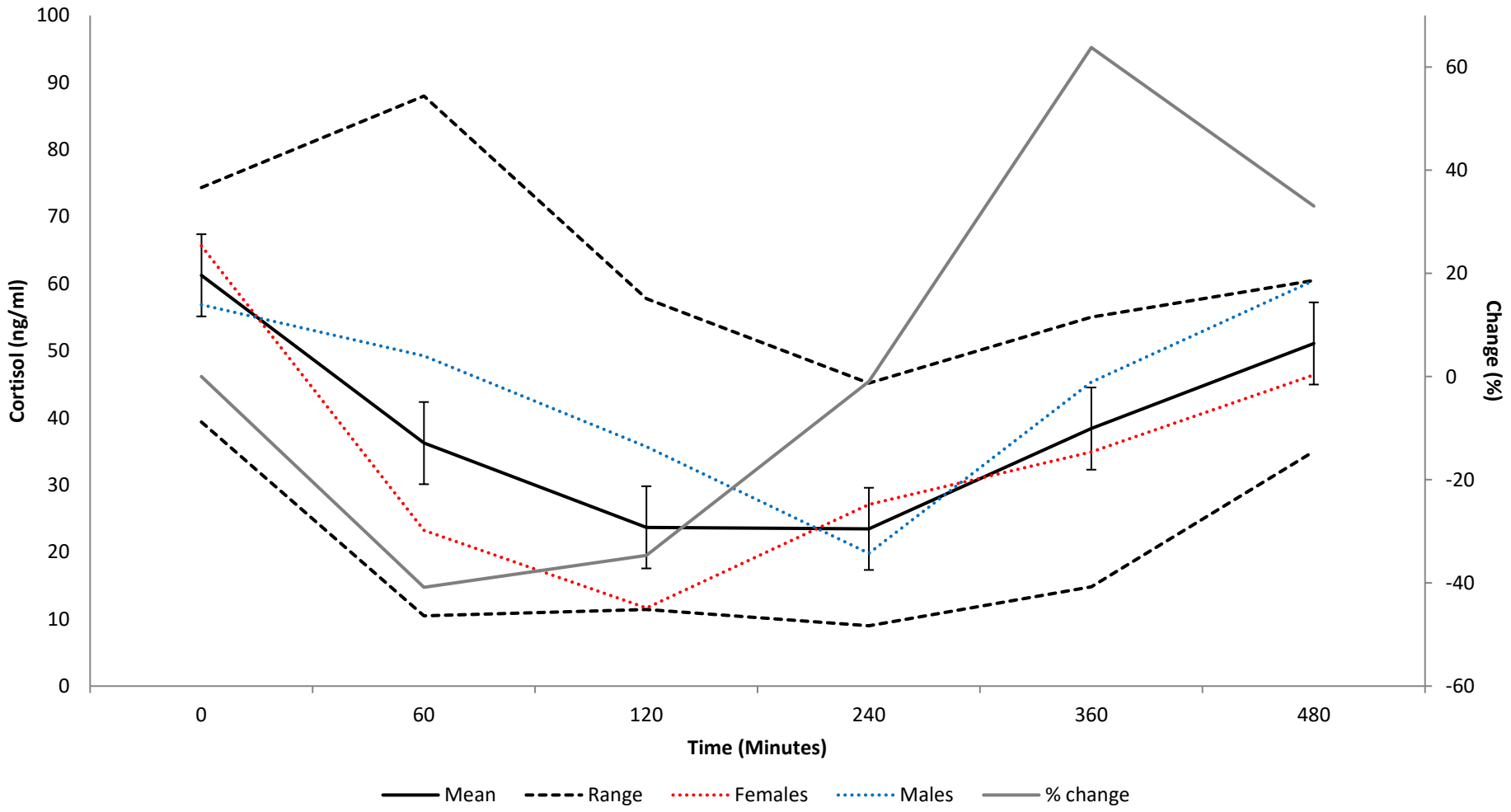


Figure 6.6. Cortisol response to dexamethasone (±SE) for both sexes in blood plasma

Note: dexamethasone was administered directly after the first blood sample

6.3.3 High-performance liquid chromatography

The specificity of EIA can vary depending on the commercially available kit used resulting from a cross-reactivity between some steroids and the antisera present. To identify whether the cortisol ELISA kit used in this study appropriately identified the correct metabolite in the samples, the hormone property in a sample was compared through HPLC to that of a referenced standard.

Qualitative detection of glucocorticoids in the serum of male and female African Painted Dogs was performed by reserve phase HPLC. The HPLC chromatogram (Figure 6.7) shows the separation of each of the glucocorticoid metabolites. The retention times of cortisol and corticosterone standards were 4.25±0.25min and 5.89±0.001min respectively. The concentration of the standards was calculated using a regression equation derived from the area and height of serially diluted solutions relative to each metabolite. The retention time of the extracted serum samples was 3.97±0.10min, which demonstrates that the metabolite targeted by the kit was more likely to be cortisol rather than corticosterone.

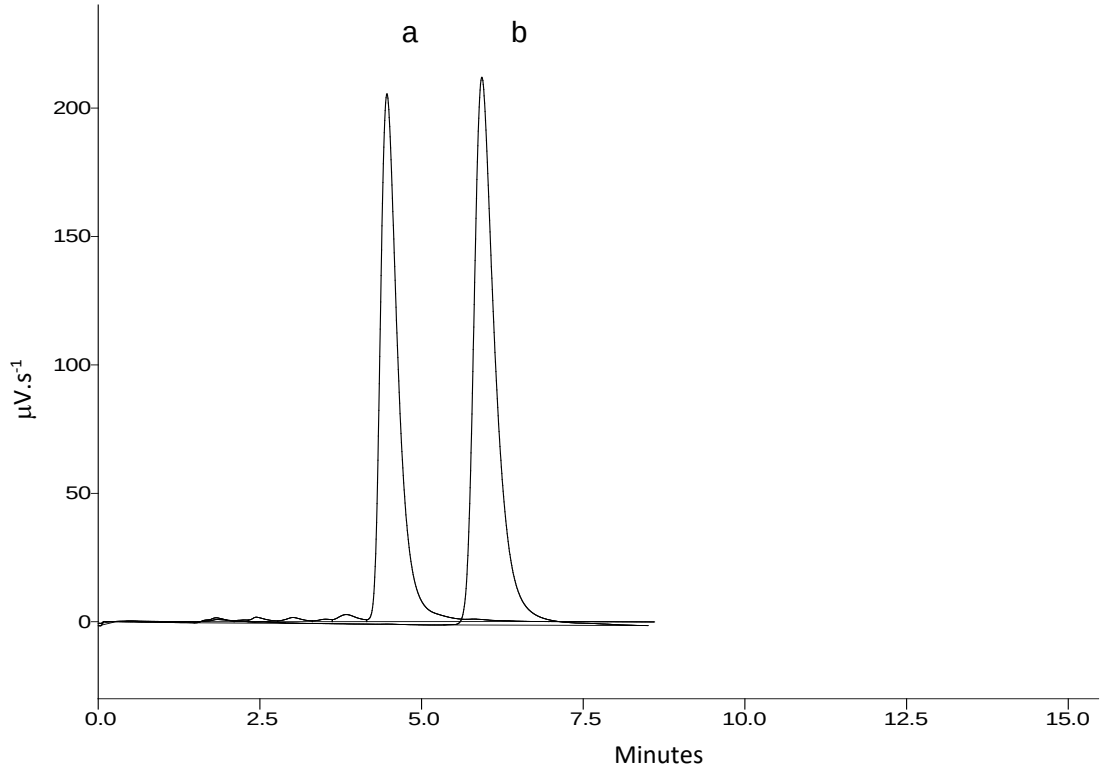


Figure 6.7. HPLC separation of (a) cortisol and (b) corticosterone

6.3.4 Non-invasive faecal monitoring

Cortisol concentration in partitioned samples

A small number of faecal samples (n=7) were partitioned into the individual material types with each component weighed and the cortisol extracted. Broadly these categories included hair, indeterminate particulates (or dust), bone, vegetative matter and woody material. As to be expected all samples contained dust with 85% of them having either some hair and/or bone present. A surprising finding was that approximately 71% of the partitioned samples contained an obvious amount of grass with one sample containing evidence of wood (~14%). Collectively examining the extracted cortisol concentrations from the various components showed significant differences in mean values particularly between: bone (M = 130.7ng/g, SD = 35.48) and dust (M; 173.8ng/g, SD = 62.55) $t(31) = 2.189$, $p = 0.0363$; dust and veg (M = 120.7ng/g, SD 62.53) $t(32) = 2.406$, $p = 0.0221$; bone and wood (M = 199.9ng/g, SD = 5.162) $t(13) = 3.279$, $p = 0.0060$; hair (M = 162.7ng/g, SD = 51.07) and veg $t(23) = 2.076$, $p = 0.0492$; wood and veg $t(14) = 2.142$, $p = 0.0403$. Consequently, the null hypothesis was rejected. Figure 6.8 displays each material type and the disparity in glucocorticoid concentrations between each of the collected samples.

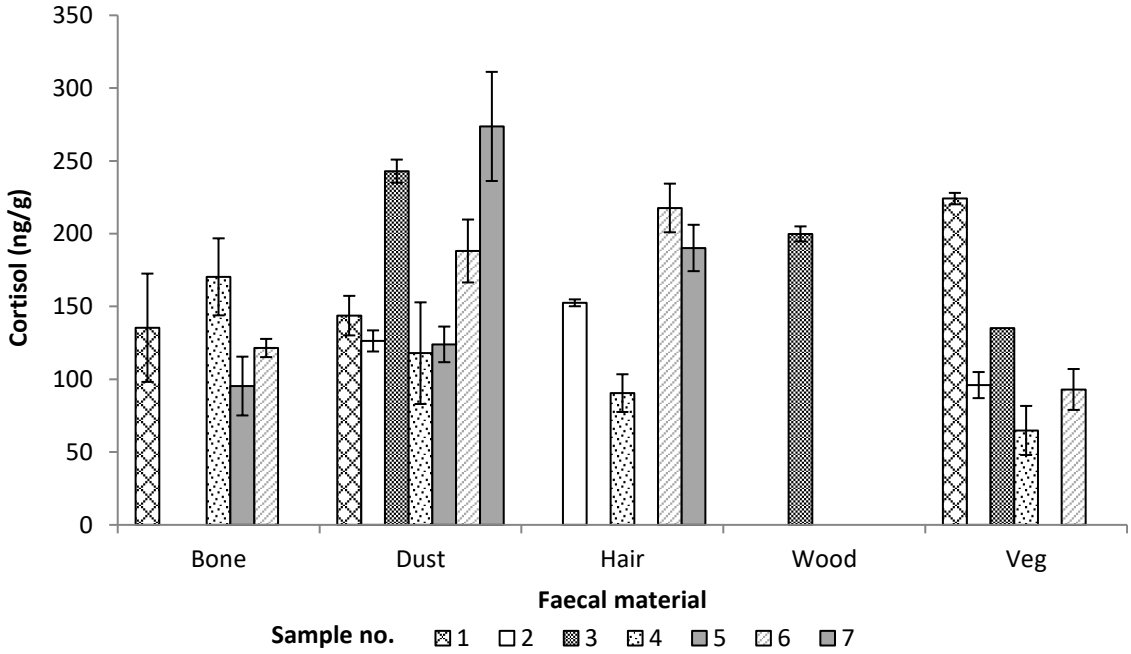


Figure 6.8. Comparative differences in cortisol concentrations (±SE) found across all partitioned samples

The concentrations observed within each sample were variable depending on the different types of faecal material present. Samples that were partitioned into two material types identified dust as having higher glucocorticoid concentrations when compared to the hair

(p = 0.0237) and separately the bone (p = 0.1037). Where there were three or more different components in a sample, however, there was no clear trend in determining which material the cortisol bound more readily too. Cortisol concentrations of the indeterminate particulates were marginally lower but not significantly different to both the hair and bone components in these samples. Given the presence of dust was consistent between all samples and that a marginal difference in cortisol concentrations was noted between other material types (e.g. bone and hair), a decision to target the dust in the extraction process was made. Focusing on this component also potentially limits the contribution of cortisol that may be found in the consumed prey species.

Correlation between faecal and circulating blood cortisol

A Pearson's bivariate correlation coefficient was calculated to assess the relationship between plasma cortisol levels on the day of capture (day 4) and the faecal glucocorticoid concentrations in the 3 days following the procedure. FGC from days 4 to 7 across seven capture events were used to generate the correlation coefficient. No faecal samples were able to be collected during the second capture event as social dynamics and instability had caused the group to split with animals unable to be moved into the off-limit area. The only positive correlation found (Table 6.5) between blood and faecal cortisol was on day 6 (r =0.4851, p = 0.0006), while for all other days (i.e. 4, 5 and 7) a non-significant result was recorded.

Table 6.5. Pearson's correlation coefficient for assessing the relationship between plasma and faecal cortisol (day 4 is day of capture).

Day	r	N	Sig (2-tailed)	R^2
4	0.0063	71	0.9581	0.00004
5	-0.0957	27	0.0634	0.00917
6	0.4851	46	0.0006*	0.2353
7	-0.0033	40	0.9838	0.00001

Given the significance of the correlation coefficient for day 6, a simple linear regression was then used to predict the concentration of FGC based on the plasma cortisol levels. A significant association was found between plasma and faecal cortisol ($F(1,44) = 13.54$, p = 0.0006) with 23% of the variation explained by the concentration observed in the plasma sample. A sample's predicted FGC is equal to 1.480 + 202.6 (plasma cortisol) when plasma is measured in ng/ml. FGC increased 202.6 for each ng/ml of plasma cortisol. A scatterplot as shown in Figure 6.9 summarizes the relationship. The mean cortisol concentration for

plasma on the day of capture was 70.81ng/ml (SD = 51.09) whereas the mean concentration found in faecal samples on day 6 was 291.87ng/g (SD = 167.3). This equates to approximately a 4-fold difference between each of the biological materials collected.

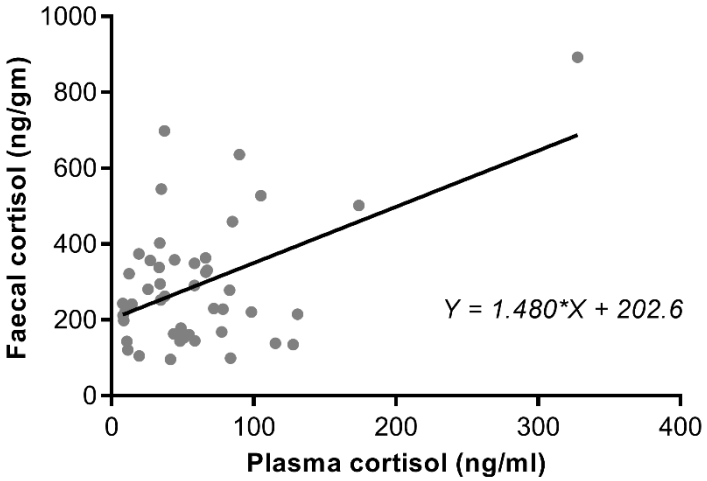


Figure 6.9. Scatterplot identifying the relationship between plasma and faecal cortisol concentrations

Effects of handling as seen by FGC

Delivery success of faecal markers over each capture event was variable (refer to Appendix 7) resulting from individual dominance, cohort age and group dynamics. The oldest animals (*A29037, A29038*) were found to contribute the least to off target uptake, which is in converse to the youngest cohort (*A69113, A69114, A69115, A69116, A69117, A69118, A68119, A69120*) who were highly competitive and frequently took baits assigned to other animals. Individuals from this cohort were typically ranked higher than other individuals of this social group. Across the eight capture events the final session was the most successful with 90% of baits reaching their intended target.

Over all capture periods (1-8) a total of 542 faecal samples (both before and where possible after) were collected (Figure 6.10) and analysed with approximately 33% originating from an unidentified individual. Samples from known individuals were competently identified using the stated faecal markers (Appendix 3) in conjunction with recorded delivery uptake. An unpaired *t*-test was used to compare faecal cortisol concentrations in samples from known and unknown individuals. There was no significant difference in concentrations for identified samples (M = 222.6ng/g, SD = 138.6) and unknown samples (M = 212.9ng/g, SD = 91.42; $t(540) = 0.9932$, $p = 0.3211$). This indicates that concentrations of unknown samples can be utilised and where possible pooled for subsequent analyses.

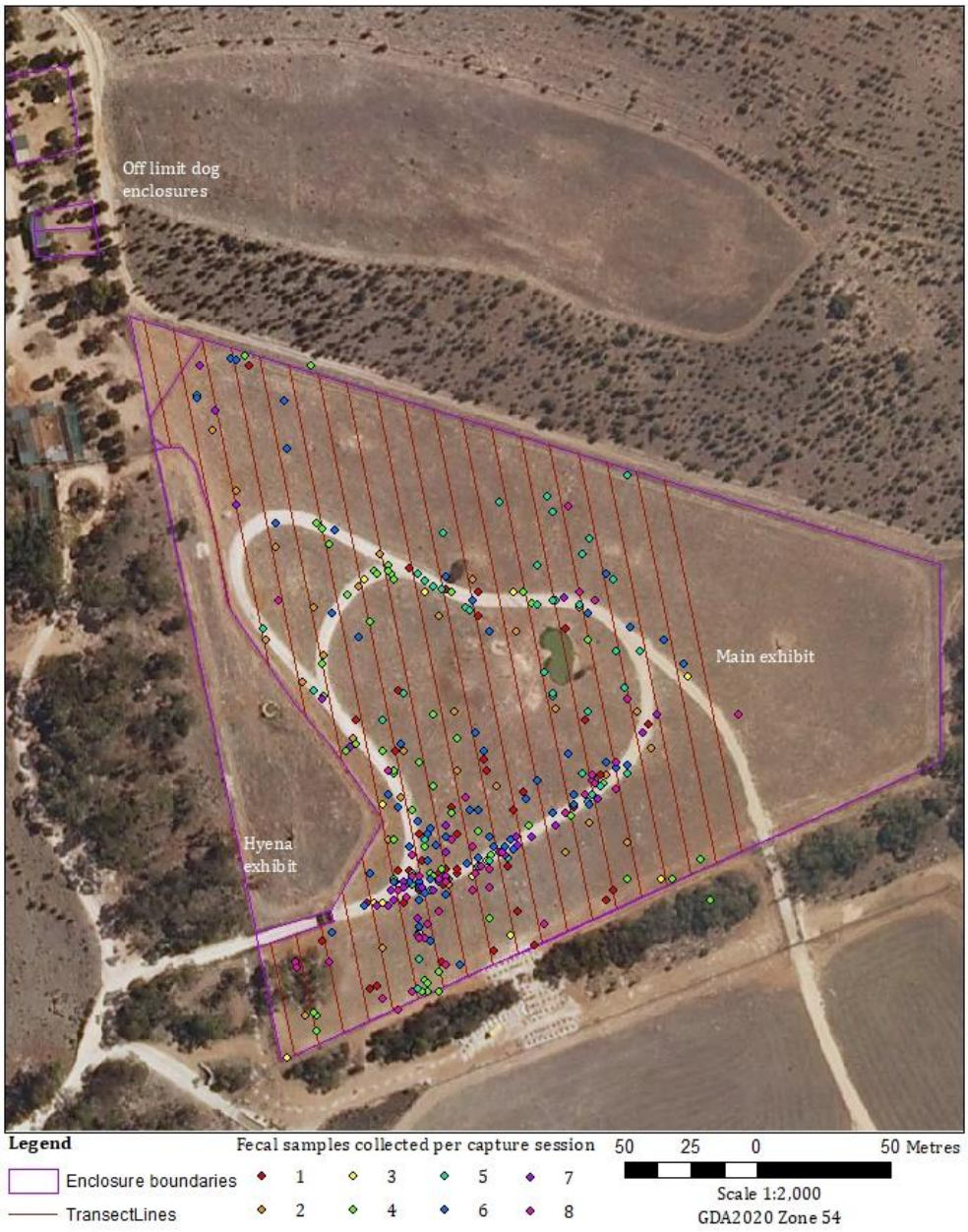


Figure 6.10. Transect lines within the African Painted Dog exhibit, Monarto Zoo

An unweighted means analysis was conducted to assess differences in faecal cortisol concentrations before and after immobilisation, as well as, across the different immobilisation approaches (including unknown samples). All effects were significant at the 0.05 significance level. The main effect relating to time (i.e. before/after) yielded an *F* ratio of $F(1,534) = 78.83$, $p = <0.0001$, indicating that the mean change in score was significantly greater for samples collected after animals have been immobilised. Capture approach returned an *F* ratio of $F(3,534) = 8.71$, $p = <0.0001$, signifying that the mean change score was significant between manually restrained animals (M = 203.62ng/g, SD = 108.34) compared to sedated animals (M = 145.5ng/g, SD = 97.11), and separately sedated animals

to those that were hand injected (M = 220.86ng/g, SD = 109.78). Faecal samples collected from unknown individuals followed a similar trend over time (see Figure 6.11). The interactive effect between immobilisation approach and capture event was significant $F(3, 534) = 4.24$, $p = 0.0056$. This suggests that the observed concentrations of cortisol before and after capture was just as important as the method in which animals were immobilised.

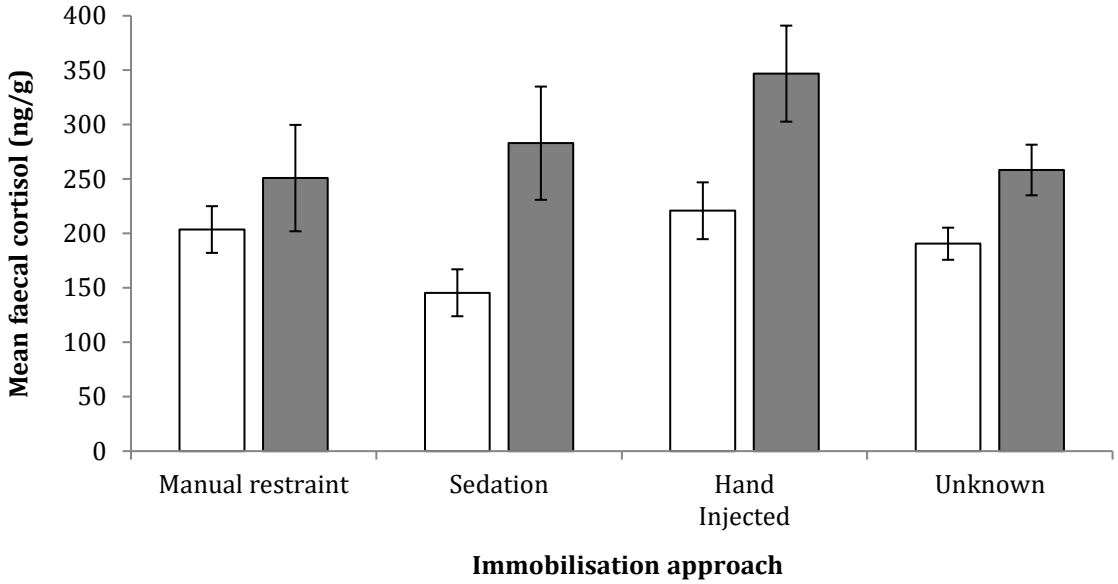


Figure 6.10. Mean faecal cortisol concentrations (95% CI) before (white bars) and after (grey bars) capture based on immobilisation approach

A similar statistical approach was employed using an 8 x 2 ANOVA to evaluate the pooled effect of capture on faecal cortisol concentrations over the total study duration (Figure 6.12Figure 6.11). The main effect relating to time (or capture number) accounted for the greatest total variance found (27.11%) across samples with significant differences noted between capture events $F(7, 573) = 32.42$, $p = <0.0001$. Faecal cortisol concentrations after immobilisation (M = 279.42ng/g, SD = 144.88) were generally shown to be statistically different from before values (M = 190ng/g, SD = 101.62); $F(1, 573) = 11.20$, $p = 0.0009$. With the exception of capture events 2 and 4 (Figure 6.12), the trend was for faecal concentrations to be higher. Follow up analysis incorporating pairwise comparisons to this main effect identified capture events 6 and 8 as being significantly different, respectively $p = <0.0001$ and $p = 0.0227$. The interactive effect between capture event and faecal cortisol concentrations before and after immobilising was statistically significant $F(7, 573) = 3.738$, $p = 0.0006$, suggesting that only under certain circumstances where additive effects (e.g. social stress) are known could immobilising dogs induce a statistically significant acute stress response. Overlaying the mean plasma cortisol levels on those collected non-

invasively shows a less exaggerated but similar trend thereby supporting the view that alternative biological materials are able to represent similar expressive profiles.

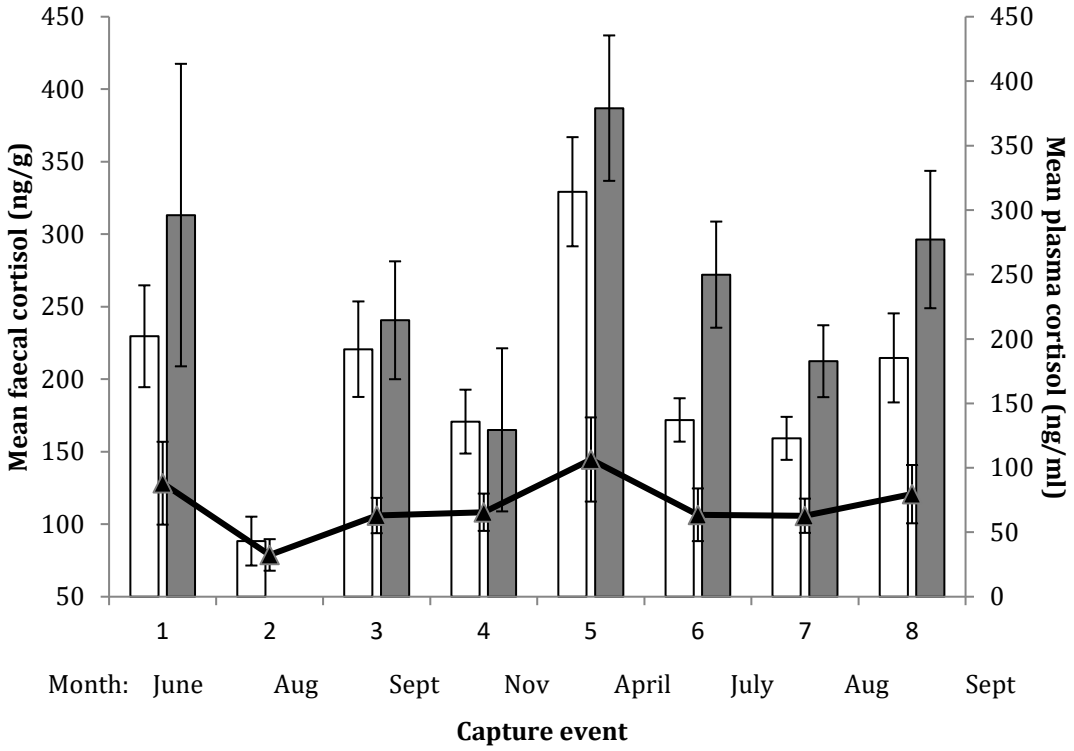


Figure 6.11. Mean faecal cortisol concentrations (95% CI) before (white bars) and after (grey bars) capture overlaid with plasma (black line) cortisol concentrations for each capture event.

6.4 Discussion

Capture physiology has increasingly emerged as a sub disciple of conservation biology focusing on the cause and consequence of physiological stress on individuals (Dantzer *et al.* 2014). Here, the process of capture and handling was used experimentally to assess how the African Painted Dog responded to a frequently employed management tool. The main glucocorticoid metabolite for mammals, cortisol, was targeted with the same EIA kit used to determine the concentration in both plasma and faecal samples. The cortisol metabolite targeted in the EIA was verified by using HPLC as there was a large cross-reactivity with corticosterone. Plasma cortisol concentrations presented in this study are equivalent to that of other immobilised carnivores (see De Villiers et al. 1997).

Animals given a sedative prior to being restrained had significantly lower plasma cortisol concentrations than those that were manually restrained or those that were completely immobilised (e.g. darted and GA). This would suggest that the tranquilizing action of medetomidine encouraged a relaxed demeanour which lessened the effect of an acute stress

response imposed by handling these animals. Despite significant differences not found between individuals that were manually restrained and those that underwent a general anaesthetic, the trend observed between immobilisation approaches is consistent with similar findings in De Villiers *et al.* (1997). Where safety concerns and logistical requirements for working with dangerous animals are met it is recommended to use a sedative during conscious restraint in this species.

Assessing the response to capture across the study's duration identified that there were some significant temporal differences in both blood plasma and the faecal metabolite. As male dogs had not been routinely immobilised at MZ this process was novel to them. During the first immobilisation event dogs would generally run with some speed in the raceway, and being unfamiliar with the use of multiple slide gates, would frequently collide into them whilst being drafted. Within the actual crush they would often growl, expel excreta, bite the surrounding mesh and struggle vigorously until the squeeze mechanism had been activated and secured. Animals that were bled whilst conscious were coaxed into a recumbent position with metal forks positioned horizontally where the majority of individuals would remain motionless until handling had concluded. For a small proportion of individuals significant physical exertion occurred requiring multiple attempts to squeeze or reposition the forks. Such behaviours within the raceway did become less intense as time progressed reflecting an individual dog's cognitive reappraisal of the situation, thus highlighting both the physical and psychological dimensions of an immobilisation stressor.

From an endocrinological perspective, the initial capture event did elicit an exaggerated response in the blood plasma when compared to the other periods of immobilisation, suggesting that these naïve animals had a heterotypic stress response. Subsequent immobilisation of dogs showed a lower and more consistent level of plasma cortisol being more commonly associated to a homotypic stress response. This indicates that the sympathoadrenal system of *L. pictus* was able to adapt to a homotypical stressor as a result of changes in a higher regulatory centre. Dronjak *et al.* (2004) observed a similar response in rats after they were repeatedly exposed to an immobilisation stressor. It is less likely that these dogs became habituated to this stressor as repeated activation of the HPA did not reveal a declining trend in plasma cortisol levels over progressive capture events (Grissom & Bhatnagar 2009). The rise in plasma cortisol observed during the fifth capture event could be attributed to hormonal changes and increases in aggression between conspecifics relevant to the breeding season. Immobilisation during this reproductive phase constituted a rise in mean plasma cortisol of 42% as opposed to a 30% increase at the time of first

capture. By monitoring overall changes in plasma cortisol in this small group of male dogs it does not generally appear that they were chronically stressed as a result of repeated immobilisation.

The elevation of plasma cortisol was reflected in the faecal pellets collected after immobilisation. This biologically validates that a stress response to capture did occur in this study. Partitioning faecal samples into different materials identified higher concentrations of bound cortisol in an assortment of indeterminate particulates (i.e. dust). The gastrointestinal tract causes a lag effect in observing circulating unbound cortisol associated with plasma to its appearance as bound cortisol in excreted faeces. This delay is often correlated to gut transit time with times being species specific (Palme 2005; Schwarzenberger *et al.* 1997). Marked increases in faecal cortisol concentrations were observed mainly in the second faecal sample collected after animals had been handled (i.e. day 6). The delay between immobilisation and the appearance of peak concentrations was 24-48 hours which is in keeping with previous research on this species by Ven (2009) and Monfort *et al.* (1998), respectively being 12-38 hours and 24-30 hours. This delay is also similar to other studies involving domesticated dogs and cats (24-48 hours, Graham & Brown 1996; 25 hours, Schatz & Palme 2001), Cheetah (24-72 hours, Terio *et al.* 1999) and the Spotted Hyena (16-50 hours, Goymann *et al.* 1999). The peak in faecal cortisol was significantly greater than baseline values in all but those that were manually handled. For sedated animals and dogs that underwent a GA, faecal cortisol increased by approximately 48% and 32% respectively, while manually restrained animals had higher basal levels and an increase of less than 20%.

Over time faecal cortisol levels were shown to be greater after immobilisation than before. In situations where this did not occur, such as the second capture event, samples were not able to be collected due to increased intraspecific aggression. This resulted in animals separating into their respective cohorts and an inability for them to be moved into an off-limit area. Increases in aggression were directly associated to handling and their social hierarchy being disrupted. In the fourth capture event however, a high degree of variability was found predominantly in samples collected after immobilisation. Greatest differences between pre and post immobilisation values were observed in the sixth and eight capture events. After the breeding season (capture event 5) dogs were separated into two groups stemming from a high degree of social stress and a fatality due to increased aggression. Sample collection from animals in the off-limit area was more meticulous due to the survey area being considerably smaller and a minimum amount of vegetation being present in the

yards comparative to the main exhibit. Having a reduced enclosure area improved the accuracy in both dispensing markers and the subsequent identification of pellets to specific individuals. Overall, this study was able to test and confirm the utility of faecal cortisol as a means to measure the stress of immobilisation in conjunction with plasma cortisol. It was shown that short term fluctuations in plasma cortisol levels were discernible in samples excreted over a longer period of time.

An ACTH stimulation test pharmacologically induced a stress response in a subset of animals, which was further used to determine a threshold value between a stressed and unstressed state (i.e. ROC curve and Youden's Index). No other study appears to have conducted this type of statistical analysis for African Painted Dogs or carnivores generally. While the statistical approach used did subsequently identify individuals that had a higher than expected plasma cortisol value, the sample size from which this threshold was derived was very small. Greiner *et al.* (1995) suggest that the distribution of data through parametric and non-parametric methods in addition to sample size is an important consideration in determining a cut-off value. This study represents a novel application of a statistical approach commonly used in epidemiology to stress related studies. To increase the utility of this statistical approach and to refine the cut-off value presented here for African Painted Dogs, a greater number of animals experiencing different types of stressors should be collectively analysed.

Throughout this study, the use of the calculated threshold value showed a small proportion of individuals with elevated levels of plasma cortisol. While at an individual level this could be suggestive of a chronic stress response, it is in contrast to population level effects where the relative influence of immobilisation and handling was not overtly significant. The most notable individual response was dog *A69113* from the sedated group. This dog appeared to experience ongoing social stress, which became more evident after this individual and a full brother were transferred to another institution where intraspecific aggression between the two resulted in *A69113* being fatality mauled. The two other dogs that had values slightly above the cut-off threshold were one of the lowest and highest ranked dogs from a separate cohort. This cohort of 6 year olds had over the years been in conflict with animals aged 5 indicating that social stress had an additive effect to elevating the concentration of cortisol.

From the ACTH stimulation test itself, individuals *A69113* and *B09056* had the highest plasma cortisol levels during the initial phase (<70minutes). These animals were either part of a dominant cohort or were asserting themselves as a dominant individual within a single

sex group. Peak values for these dogs occurred at 40 and 20 minutes respectively for the male and female, which was consistent with that also observed by De Villiers *et al.* (1997). This supports the view that rank does play an influential role in both initial and peak cortisol concentrations especially within the first hour after immobilisation. Being of higher rank could potentially lower ones' individual fitness as their coping mechanism could be maladaptive due to having a lower capacity to return to an unstressed state. As shown here high ranked individuals (or those trying to achieve a high rank) are more likely to suffer from consistently elevated glucocorticoid levels which are akin to a chronic stress response. This has direct implications in managing small groups with regard to appropriately targeting specific individuals for invasive handling purposes (e.g. deploying radio collars).

In contrast, a low dose dexamethasone suppression test can reflect the functional sensitivity of glucocorticoid receptors at the hypothalamic-pituitary level. This synthetic glucocorticoid suppresses the secretion of ACTH through negative feedback inhibition resulting in the suppression of secreted cortisol. A review of the literature has not identified any publication that has performed a low-dose dexamethasone suppression test on the African Painted Dog. Administering dexamethasone did suppress adrenal activity resulting in plasma cortisol levels falling by approximately 62% when compared to initial concentrations. The cortisol response in one dog, however, was noted to rise within the first two hours before declining. While caution should be used in interpreting such results these findings may be suggestive of mild hyperadrenocorticism in this individual. This syndrome is most common in domestic dogs that are middle aged or older, > 9 years (Lane & Rizzi 2010). Moreover, the excessive production of cortisol and this animals weight being <20kg could be reflective of a pituitary dependent hyperadrenocorticism being commonly associated with a pituitary adenoma (Peterson 2007). The response observed in the other animals receiving dexamethasone showed a rapid onset of complete suppression but a transient effect, which is in keeping with the normal HPA function of domestic dogs (Kirk *et al.* 1990). It is believed that the results obtained from both the suppression and stimulation tests present a greater understanding of adrenal function and pattern of cortisol secretion in this canid species.

No significant sex related differences were noted in the concentration of cortisol when immobilising dogs using a complete anaesthetic regime. In spite of this, disparities were observed during the ACTH stimulation test with female dogs consistently having a higher concentration than male individuals. A similar trend between the sexes was detected by Creel *et al.* (1996b) and De Villiers *et al.* (1997) with female dogs having elevated

concentration levels compared to their male counterparts. During the stimulation test the greatest difference was seen during the second blood sample being approximately 20 minutes after the anaesthetic was administered with females on average having a concentration of 186ng/ml, while samples collected from male dogs had a mean value of 66ng/ml. Keller-Wood *et al.* (1983) demonstrated that the canine adrenal cortex responds in a dose-dependent manner. This pharmacokinetic study also established that a response was proportional to the total dose administered and the means in which it was delivered e.g. intravenous vs. intramuscular. As the administered dose to the males was relative to their weight, this may have contributed towards some of the differences in cortisol concentration between each of the sexes in the ACTH trial.

Over the course of the study only a minimal seasonal shift in regulating the HPA was observed with maximal cortisol values seen during the breeding season (i.e. April). Changes in glucocorticoids have also been tied to photoperiod which is the primary proximate factor used by many species to adjust time of reproduction (Pereira *et al.* 2010). In this temperate zone April is generally when the photoperiod decreases below 13 hours (Gupta 2007), which could be considered a cue for oestrus in some females and greater competition and/or aggression in male dogs.

In both domestic and African Painted Dogs there is limited information on the physiological variability of the cortisol metabolite relating to age associated changes (Mongillo *et al.* 2014). In some studies on domesticated canines plasma cortisol has been reported to increase with age (e.g. Goy-Thollot *et al.* 2007), while others have stated it does not (e.g. Hennessy *et al.* 1997). Research on adrenal activity in *L. pictus* has shown that glucocorticoid levels were highest in male yearlings (Van der Weyde 2013). In this study, age related differences in the concentration of plasma cortisol were noted to occur with older animals having lower mean values compared to other age groups (Figure 6.1b). Reul *et al.* (1991) indicate that aging is associated to a progressive dysfunctioning of the HPA which can lead to a diminished ability to maintain equilibrium. This inactivation can overexpose the individual to mediators of neural, endocrine and immune stress with different pathophysiological consequences (Ferrari *et al.* 2001). During the dexamethasone suppression test one dog (*A29038*) was observed to hypersecrete cortisol basally with some degree of dexamethasone resistance in the first hour when compared to other participating individuals. While this could be indicative of a borderline hypercortisolism condition, this elevated concentration in cortisol may also reflect a chronically stressed state resulting from a deteriorating physiological condition. Further investigation into the biology of aging

in this species could help determine what constitutes normal function, which has benefits in assessing and comparing different disease states.

This study has a number of limitations. For completely anaesthetised dogs initial blood samples were taken approximately 10-12 minutes following the administration of the anaesthetic. This delay is not reflective of a basal concentration, which in an ideal situation should have been collected within the first couple of minutes of handling. Differences in cortisol concentrations for dogs that underwent a GA compared to other approaches could in part denote the differing amounts of time that elapsed between being immobilised and blood collection. Furthermore, the use of anaesthetics can in itself illicit just as intense or stronger cortisol response than that related to physical restraint (Jessup 1999).

Small sample size can also lead to less dependable findings due to a lack of independence between subjects, low statistical power and a violation of underlying assumptions. Such challenges of small *N* in zoo research is commonplace with Plowman (2008) providing a general guideline to statistical analysis in which to limit potential errors and extract the maximum amount of information from a dataset. Sample size was further compromised by individual dogs not coming into the raceway to be processed and intraspecific aggression disallowing the collection of faecal pellets post immobilisation. Equally, animals that were included in the low dose dexamethasone test had existing health issues which potentially introduced some bias to the results. Given the length of the procedure however, there was ethical considerations that needed to be weighed up between using healthy animals or those that were already identified through management processes to be euthanised.

Further to these limitations is the use of the ELISA kit, which was designed for human rather than canine subjects. While derived cortisol concentrations were in a similar range as other studies (see De Villiers *et al.* 1995; 1997) differences in the antigenic properties of each species should not be discounted. Values presented in this study cannot be considered a direct measurement of cortisol but rather a cortisol equivalent.

6.5 Conclusion

Stressful experiences are an inherently natural part of daily life resulting from a broad range of pressures (Creel 2001; Van Meter *et al.* 2009). For species that are actively managed, whether that is a free-ranging or captive population, they may have additional stressors which relate to capture and handling. This study reports the stress response of the African Painted Dog to a series of immobilisation events by monitoring the concentration of cortisol

in both faecal material and blood plasma. While some differences in the concentration of cortisol were noted between immobilisation approaches it cannot be said that repeated capture of this group of animals generally elicited a chronic stress response. Cortisol concentrations derived from pharmacologically inducing a stress response were able to determine a cut of value for stress for animals in this study. This statistical investigation provided an initial foundation in which subsequent studies can expand on and further refine such a value. In utilising this approach a small number of animals were observed to have an elevated cortisol response, inferring that they had a limited ability to effectively manage or cope with immobilisation on top of normal social stressors. Comparing cortisol values gathered by invasive and non-invasive means showed a similar temporal trend and sensitivity to being immobilised with concentrations being significantly correlated after 24 hours. This highlights the usefulness in monitoring known stressors through non-invasive approaches. In the following and final chapter of this thesis an overall discussion of the research undertaken in this study will be made.

CHAPTER 7
CONCLUSION

7.0 Introduction

A strategic approach to conservation planning is essential for the recovery of endangered species. Conservation strategies that directly address significant threats also guide the development of effective conservation action (McGowan *et al.* 2017). Integration of *in situ* and *ex situ* management along a continuum of conservation actions broadens the suite of skills and resources available to a species under threat (Byers *et al.* 2013). For the African Painted Dog, control and prevention of all significant disease threats has been highlighted as a priority conservation issue (ARAZPA 2008; Mills *et al.* 1998; Woodroffe *et al.* 2005; Woodroffe *et al.* 1997). To increase the contribution that *ex situ* populations have to *in situ* conservation, this study examined both vaccine immunogenicity in one infectious disease (i.e. CPV) and explored how individuals responded to routine management procedures (i.e. immobilisation). This study showed that whilst the vaccine was safe to use, a modification to the inoculation schedule is required. It was also shown through invasive and non-invasive sampling that the African Painted Dog was generally able to cope with being repeatedly handled. This chapter aims to not only bring together the findings made in previous chapters but also examines implications for management for both *in situ* and *ex situ* populations. Limitations of this study and directions for future research are also discussed.

7.1 Review of research

7.1.1 Infectious disease and vaccination

Threats relating to infectious disease in wildlife populations are difficult to evaluate and manage as there is only a limited understanding of the ecology and dynamics of diseases (Tompkins *et al.* 2011). Mitigating disease risk through preventative medicine approaches is considered important for any wildlife restoration program involving threatened species. Vaccination provides a critical step in avoiding catastrophic population declines during epidemics (De Castro & Bolker 2005). Chapter 3 reveals that disease related research conducted on the African Painted Dog has focused predominantly on rabies and CDV. For CPV, a comparatively less pathogenic but still significant disease, there is little information not only on the prevalence of CPV in free-ranging populations but also on the serological response that African Painted Dogs have to vaccination. This research collated published studies across all major canine pathogens combined with research undertaken by Popelin (2010), identified 29 vaccines that have been utilised in the African Painted Dog with mixed

outcomes (Appendix 1). Vaccinating this canid species has seen some attenuated vaccines commercially produced for domestic dogs reverting to virulence in the African Painted Dog, e.g. Durchfeld *et al.* (1990); McCormick (1983); van Heerden *et al.* (1989).

Clinical investigations provide a rational and systematic way to examine disease dynamics and preventative treatments. As previously described, only three studies have documented the serological response in the African Painted Dog after being inoculated against CPV. To expand upon this basic knowledge, Chapter 4 assessed the immunity in a group of captive bred dogs (n=18) after being vaccinated with an inactivated monovalent CPV vaccine (Parvac™). This research is the first to assess this vaccine's DOI in a non-domesticated carnivore. Parvac™ is deemed safe to use in the African Painted Dog with an adequate humoral response inferring protection. However, DOI was not consistent with that described by the manufacturer (Pfizer) for domestic dogs (i.e. protective immunity for 12 months) despite adherence to the prescribed schedule of inoculations. Paralleling earlier findings by Spencer and Burroughs (1992), the results presented in this study also highlight species specific differences in immunity within Canidae.

This study demonstrated that protective immunity after the first two inoculations lasted for approximately 90 days, while after the annual booster all dogs had protected immunity for a minimum period of 77 days. Retesting a subset of the study group (n=6) one year after the annual booster showed half (n=3) still had a seroprotection rate above the threshold value of 1:80. Evaluating mode of delivery showed no statistical differences in immunity between administration routes (i.e. hand injection, remote delivery). Despite this, there was a trend in the remote delivery group for lower titre values when compared to animals that were vaccinated by hand injection. This gives some support to the argument made by Goddard *et al.* (1990) in that administration route can influence long term protection against CPV.

7.1.2 Immobilisation and stress

Increasing the understanding that capture physiology has on species can lead to better animal welfare and research practices. Studies that have investigated the two glucocorticoid metabolites, cortisol and costicortisone, in the African Painted Dog are limited (see Table 5.1). Prior to Burrows' (1992) article claiming that there was a link between handling dogs, lower survival and localised extinction, there was only one publication that had investigated the production of cortisol in this species (i.e. van Heerden & Kuhn 1985). Not only was Burrows' claim refuted by a number of conservation biologists

but it also initiated a series of scientific investigations into stress physiology, social dynamics and adrenal function in the African Painted Dog. In spite of this, there remains a limited amount of information available on the acute stress response in this species with no publishable information on how individuals respond to repeated capture events and chronic stress.

This research targeted one of the primary stress mediators, cortisol, and monitored the physiological stress response of capture in captive bred dogs undergoing routine management procedures and those involved in the vaccination trial (Chapter 4). Validation of this metabolite was achieved through HPLC. This study demonstrated through invasive and non-invasive approaches that an acute stress response did occur because of immobilisation. This was reflected in significant rises in both in plasma cortisol concentrations during the handling procedure and the elevated FGC collected in the days following each capture event.

Comparing three immobilisation approaches (e.g. manual restraint, sedation, GA) showed that animals given an oral sedative had the lowest plasma cortisol concentrations at the time of handling. Mean plasma cortisol levels in this study were marginally higher but still comparable to those recorded in De Villiers *et al.* (1995) for this species. Marked temporal fluctuations in plasma cortisol levels were observed for male dogs at MZ that were repeatedly immobilised. Higher mean cortisol concentrations were primarily seen in the first capture event in June highlighting the naivety of these dogs to being immobilised, and later in April of the following year. These male dogs were housed initially as a bachelor group with the elevated result noted in April coinciding with increased aggression, reflective of this species general breeding season. Assessing plasma cortisol concentrations across cohorts identified some age related differences with there being a trend for older males to have a lower plasma cortisol level. Similar findings relative to age were also made by De Villiers *et al.* (1997) and Van der Weyde (2013) with younger dogs having higher cortisol concentrations than older animals. Plasma cortisol levels were also shown to be marginally higher for dogs that were in the cohort that were directly subordinate to the dominant cohort.

This study addresses one knowledge gap by examining the relationship between faecal and plasma cortisol levels in the African Painted Dog. A positive correlation between the two different biological materials was found approximately two days after the capture event with similar responses found to other carnivore species. In the present study, there was a

trend for FGC to increase significantly after capture and handling. Empirical data collected here support faecal glucocorticoid metabolite analysis as a robust non-invasive approach in detecting a stress response in this species. FGCs were relatively consistent across time indicating that dogs were generally able to cope with being immobilised. The exception was when increased aggression between conspecifics occurred, which led to fractures of the all-male group based on age and cohort dominance. The effects of immobilisation in the faecal metabolite were more pronounced both immediately before the breeding season and after the changed housing arrangements of the MZ study group (i.e. capture events 6-8). This suggests that immobilising dogs while existing stressors are at play (e.g. social stress) has an additive effect on the overall stress response, which may challenge an individual's coping mechanism and adaptability.

This study also examined both the positive and negative feedback mechanisms of the HPA. No previous study has documented the negative feedback mechanism in this species through a low dose dexamethasone test. Despite having both a small sample group and aged and health compromised animals, a protracted decline in mean plasma cortisol was still observed, which is in keeping with normal HPA function for domestic dogs. The ACTH stimulation test has been utilised several times in this species to assess adrenal function and to pharmacologically validate a stress response, however no previous study has derived a cut-off value to indicate animals being in a stressed state. The novel application of Youden's index identified two dogs which had higher mean cortisol levels. One dog in particular, *A69113,* who had a subordinate rank in the dominant cohort not only had consistently high plasma cortisol levels throughout the study's duration but also had a poor response to Parvac™ in the vaccination trial.

In the handling-stress hypothesis, it is suggested by Burrows (2011b) that handling may adversely affect the normal function of the immune system and inhibit the body's natural response to viral infections. The concurrent results for *A69113* suggest that this animal was experiencing chronic stress during the vaccination trial that could have contributed to a reduced immunological response. While not discounting the handling-stress hypothesis as a plausible explanation for these findings, consideration also needs to be given to the social dynamics of the study group and the lack of information on the immunogenicity of the vaccine prior to administration. It would be difficult to attribute immobilisation and handling as being the primary reason for the elevated cortisol levels and lower seroprotection rate in this particular animal.

The long term effect of social stress cannot be overlooked especially as intraspecific aggression increased between cohorts and individuals. The social environment of the African Painted Dog is highly complex as individuals have to maintain diverse relationships with multiple animals, with social instability and the formation of new social groups being major stressors. It is likely that repeated social stress acted cumulatively with the acute immobilisation stressor to maintain high cortisol levels in *A69113.* The interval time between acute stressors is perhaps of greater importance in characterising a chronic stress response, rather than making a distinction between the stressors origin.

By monitoring glucocorticoid levels it was also shown that the majority of dogs were able to cope with and adapt to a range of routine management activities, i.e. immobilisation, vaccination and transportation. With the exception of *A29038,* all dogs involved in the concurrent vaccination and stress related study survived for a minimum of two years from the final inoculation.

7.2 Implications for management

For the management of any species, an adaptive scientific approach that revisits and assesses current strategies in a progressive and rigorous manner is needed to develop future management options (Ray *et al.* 2005a). Captive bred animals have an important role in supporting research initiatives and collaborating with *in situ* conservation efforts. The safe and effective use of vaccines remains a principal consideration in reducing the incidence of disease and the immediate threat to individuals. Monitoring vaccine efficacy is therefore critical to optimise the availability of limited resources and can increase the success of a preventative health program. Demonstrating the impact that a vaccine has on health outcomes (e.g. protection rate, herd immunity) help to justify the logistical and financial costs in implementing a program.

For *ex situ* populations, financial costs are considerably less as facilities and husbandry techniques are often already well established. Costs for *in situ* conservation are significantly greater as an investment of time and resources are required to locate animals before vaccination can even occur. Similarly, organisations holding *ex situ* populations can also make calculated decisions in terms of the need to mitigate the risk of a particular pathogen through a cost-benefit type analysis, e.g. high density of susceptible species in city zoos; low possibility of exposure in an open range zoo. While for *in situ* populations often there are major deficiencies in health monitoring data which can compound the significance in disease risk and therefore the need to vaccinate. Assessing vaccine efficacy can also identify

possible inadequacies in current management protocols and stimulate the development of improved vaccines.

As captive bred animals live in relatively controlled environments, there is greater capacity to investigate the effect that different vaccines and inoculation schedules have on immunogenicity. Such practical information can be difficult or impossible to obtain from free-ranging animals. The research undertaken in this thesis contributes to on-going conservation objectives, relating to drug and vaccine testing, by increasing the understanding in the immunogenicity of a monovalent inactivated vaccine when administered to the African Painted Dog. From this investigation, it is recommended that adult dogs that are seronegative to CPV be inoculated with three initial standard doses of Parvac™ four weeks apart, with biannual vaccinations occurring thereafter. The use of Parvac™ is better suited to captive environments where animals can be repeatedly inoculated to maintain protective immunity. Under field conditions the implementation of this vaccination schedule would be less practical, given the ranging behaviour of this species and the logistics in locating and darting animals to maintain an adequate level of protection. This vaccine could however serve some role during an episodic disease event for animals that were contained within a rehabilitation facility or boma where handling or darting could occur. Vaccinating dogs during opportunistic or planned immobilisation events could stimulate an initial immune response as a reactive measure but as shown in this study protective immunity would not be achieved without a minimum of 2 doses.

To fulfil a range of conservation objectives, whether it is for *in situ* or *ex situ* conservation, dogs are required at times to be immobilised and handled. The methods used to capture and restrain wildlife species have been refined over time with greater focus now given to the welfare of animals and the effects that these types of procedures have in inducing stress or potential changes in fitness. The choice of approach is however dependent on a wide range of factors including the reason for immobilising (e.g. deploying radio collars, translocation), environmental conditions (e.g. climate, location), species and number, temperament, facilities, the experience and number of attending personnel, the safety and welfare of staff and animals, as well as, the total cost and cost per animal (Gibert 1993; Tribe & Spielman 2009). The alternative approaches used to immobilise dogs in this study are commonly applied to captive environments, while chemical restraint via a remotely delivered dart is the primary means of immobilising dogs *in situ.* As to be expected an acute stress response to handling was elicited in dogs included in this study. In the majority of situations the FGC after capture was lower than the before levels in the following capture

event, inferring that these animals were generally able to cope with being repeatedly immobilised and vaccinated. It is therefore postulated that the intermittent capture of free ranging dogs does not in itself result in a chronic stress response. A caveat to this is that there is a high degree of individual variability along with a general lack of understanding on the cumulative effect that other stressors have on individuals. While the inability to cope with stressful events has been associated with the aetiology and pathophysiology of many health conditions, meeting the demands of a stressor can also promote resilience in the health of an individual and/or population.

The animals identified as undergoing chronic stress in this study were either ranked as a subordinate animal from the dominant cohort of 5 year olds (*A69113*) or a higher ranked individual from the cohort that was directly subordinate to the dominant group of dogs (6 year olds, *A59172*). This could suggest that animals that are having more aggressive interactions with those conspecifics that are ranked both higher and lower than themselves may have a greater propensity for a lower immune response due to elevated cortisol levels. As the effect of subclinical stress and the additive effect of successive or simultaneous stressors cannot always be appreciated there may be a need to give greater consideration to the health care demographics of subordinate or beta ranked individuals. For *in situ* conservation, a strategy that could be applied to address this shortfall may include extending the primary treatment (i.e. number of inoculations) for these animals to increase the rate of herd immunity. Conversely, for *ex situ* populations dietary supplements such as vitamin E could be added to increase antioxidant levels and therefore the capacity to deal with stress better. Furthermore, it is suggested that handling dogs for routine purposes (e.g. vaccination, deploying radio collars, etc.) be conducted outside of the main breeding season or during times when it is known that packs are slightly fractured to further minimise intra-specific aggression and the opportunity for pack dissolution.

7.3 Limitations

It is important to recognise and acknowledge the limitations of any research. Despite having a small study group (i.e. <30), data obtained from each experimental approach was able to provide meaningful results. Sample size was constricted by the number of animals held in captivity with the majority of individuals being from one specific zoo. As described by Plowman (2008) small sample size is a common issue for zoo research and small free-ranging populations. To compensate for this, non-parametric tests were used to limit violating any statistical assumptions during analysis.

The results of the vaccination trial relate specifically to adult male dogs. Despite this sex bias a generalisation is made here that adult females have a comparative immune response. The availability of veterinary vaccines in different countries is also subject to national legislation and the current disease status for specific pathogens in those countries. For developing countries vaccines can be relatively expensive to acquire with disease control and management being driven by risk-based surveillance and risk management principles (Lubroth *et al.* 2007). The administration of Parvac™ within the range states of *in situ* populations or outside of Australia more generally appears to be limited despite this research showing protective immunity was elicited and is safe to use.

As with any study, analysis processes become more refined as a project progresses. Retrospectively it would have been more appropriate to dry and homogenise all faecal samples, then select a subset number across all capture events to pool, analyse and derive a standard curve, instead of performing this process on batched samples. Even though the inter-assay coefficient of variation was within an acceptable range, changing the workflow in analysing samples could minimise the introduction of variability in results. Furthermore, due to resource constraints the cortisol test kit used in this study was designed for humans rather than canines. Differences in antigens and the capacity of proteins to bind at such sites are common between species and species hybrids (McGibbon 1944), with there likely being some discrepancy in the measured cortisol concentration to the actual level found in the sample.

All male animals that were involved in the vaccination trial and the assessment of capture approaches were randomly assigned to a treatment group through the use of a random number generator. However, looking more closely at each group during analysis it was noted that there was an uneven distribution of animals from different cohorts across treatment groups, which could have introduced some age or dominance related bias when monitoring cortisol concentrations.

Behavioural changes were also observed immediately after dogs were immobilised and processed with some animals chasing members of other cohort groups (mainly between the 5 and 6 year olds). Despite this, there were very few definite acts of active aggression (e.g. being held down, biting) observed between conspecifics. A range of alternative approaches were utilised to try and limit this behaviour including carcass feeding, double feed the daily amount of meat offered in pieces, release small mixed cohort groups to the main exhibit, hold all dogs in the off-limit areas and release to the main exhibit as one group. With the

exception of the first capture event where dogs were cohesive the day following immobilisation, the next four successive capture events found dogs in two main groups with each utilising alternative areas of the exhibit, often without visual contact. The two oldest animals generally moved between each group while the 7 year olds (3 dogs) aligned themselves predominantly with the eight 5 year olds who were the dominant cohort. It was usually the six 6 year old animals that isolated themselves during these times with these animals being hesitant to participate in normal management activities, e.g. moving into off-limit areas. In the lead up to the group being permanently separated, segregation between cohorts continued with some chasing observed by keepers. In the days immediately prior to the dominant cohort being overthrown, these behaviours appeared to increase with vocalisations also noted. Audible communication between individuals/cohorts (e.g. high-pitched twitter and hoo-calling) were previously absent or occurred infrequently during times of group instability. The subsequent take back by the deposed dominant cohort resulted in the death of *A69114* and injury to a number of other 6 year old animals. While social stress was not quantified in this study it is expected that the dynamics between dogs (and cohorts) would have contributed to elevated cortisol levels.

The trend in the FGC was more distinct in the latter capture events (i.e. 6-8). Given the changed housing arrangements, it is necessary to highlight two aspects that could have influenced results. These include, (i) faecal samples were easily identified within the off-limit enclosures due to the widespread presence of bare soil, compared to the main exhibit where more extensive vegetation made the detection of scats challenging; and (ii) the social dynamics within each group (animals housed in off-limits vs. those on exhibit) changed with greater focus on the relationship and alliances between individual animals rather than against specific cohorts.

It should also be stated that the stressors experienced by different populations whether *in situ* and *ex situ* are often different. Similarly, there is a high degree of variation within individuals as their coping mechanisms evolve as a result of social-environmental conditions and learned experience. This makes comparisons across an individual's lifetime and also between populations more challenging. Having a species or taxon specific set of basic guidelines outlining which glucocorticoid metabolite to focus on, laboratory method (e.g. ELISA and kits used), and validation approaches would help make population level comparisons more meaningful.

7.4 Directions for future research

There is still much research needed in mitigating disease risk with both *in situ* and *ex situ* populations each having a complementary role in this process. Canine parvovirus continues to be an important pathogen for all canine species being responsible for serious occurrences of morbidity and mortality. In Australia this canine pathogen is the greatest viral threat to dogs. Conducting molecular epidemiological studies at an appropriate geographic scale for *in situ* populations, and for conspecific canid species within the same regional areas that *ex situ* populations reside can provide an in depth understanding of which CPV-2 strains (and other pathogens) are circulating in local populations. Combining disease surveillance data across susceptible species with relevant demographic information can also show a greater appreciation of the real disease threat and herd immunity, particularly as mass vaccination is the main tool for controlling and preventing disease (Belsare & Gompper 2013). Such studies would also provide a better insight into the regulatory role that CPV has in African Painted Dog populations and the risks to different age classes (e.g. < 1 year).

CPV has over the years mutated from the original CPV-2 to CPV2a, 2b and more recently 2c with CPV-2 no longer considered to exist in the field (Bajehson 2010). The Parvac ™ vaccine contains CPV-2, with efficacy studies on domestic dogs generally demonstrating that there is only partial protection for 2a and 2b when an original CPV-2 virus vaccine is used (Martella *et al.* 2005; Truyen 2006). The current generation of vaccines targeting CPV is yet to include the more recently discovered CPV-2c variant, thus requiring additional research into the cross variant protection offered by existing vaccines.

Equally, greater research should be conducted into the efficacy of modified live vaccines as there are some advantages to their use compared with inactivated vaccines (e.g. longer protective immunity, fewer inoculations). Scientific investigations should examine the combined use of inactivated vaccines as a primer to a modified live vaccine, as this may decrease the incidence of clinical cases if there is a likelihood that a live vaccine will revert to virulence (e.g. CDV). Determining which multivalent vaccines are safe for the African Painted Dog and threatened canid species more generally, is essential for conservation actions to have the greatest impact. Investigations into vaccine delivery methods, particularly those of oral preparation are particularly important for free-ranging animals. Information on bait uptake rates and efficacy are vitally important for broader scale vaccination efforts.

To expand on the vaccination trial described in Chapter 4, antibody titres for vaccinated females and their pups should be monitored to assess the influence that maternal antibodies have on immunogenicity and the scheduling of inoculations. Similarly, inoculating dogs with a double dose, instead of one standard dose, and assessing the serological response may reduce the need to administer the third booster (as per recommendation) and increase DOI. Collectively, this would be an interesting and complimentary avenue of scientific enquiry. In Australia the Parvac ™ vaccine is also frequently administered to other threatened canid species (e.g. Maned Wolf, Fennec Fox) that are held in captivity. Conducting trials of immunogenicity in these species may not only better define any species specific differences in immunity for this taxon group, but could assist in the development of a targeted vaccination schedule for these species and non-domesticated canids more broadly.

The concept of allostatic load, or chronic wear and tear on the body, has provided a useful framework for organising research on stress-related diseases. Approximately 16 different biochemical (e.g. glucose, various vitamins) and oxidative stress parameters along with a complete blood count were collected for most dogs in this study. Analysis of this data in conjunction with the cortisol results within an allostatic load model will provide an alternative insight into the interactive effect these mediators have during stressful events and disease susceptibility. The frequency and interval time between stressors should also be given greater consideration when trying to account for individual responses and chronic stress.

Glucocorticoid investigations should wherever possible be coupled with a behavioural study as there is a continuing need to better understand how social dynamics affect coping mechanisms and fitness consequences of individuals and populations. Affable behavioural patterns during initial social interactions are believed to be a prerequisite for the formation of a stable social unit (McCreery 2000). Little information is however known on how glucocorticoids change during pack fission and fusion, as well as, for dispersal events. Pack formation and group augmentation under intensive management conditions (e.g. in captivity or bomas) can be challenging due to the potential for conspecific aggression. Examining the co-operative relationships between and within the sexes may better understand kin selection, the development of social bonds and the formation of social groups. This would have direct benefit to both *in situ* and *ex situ* conservation.

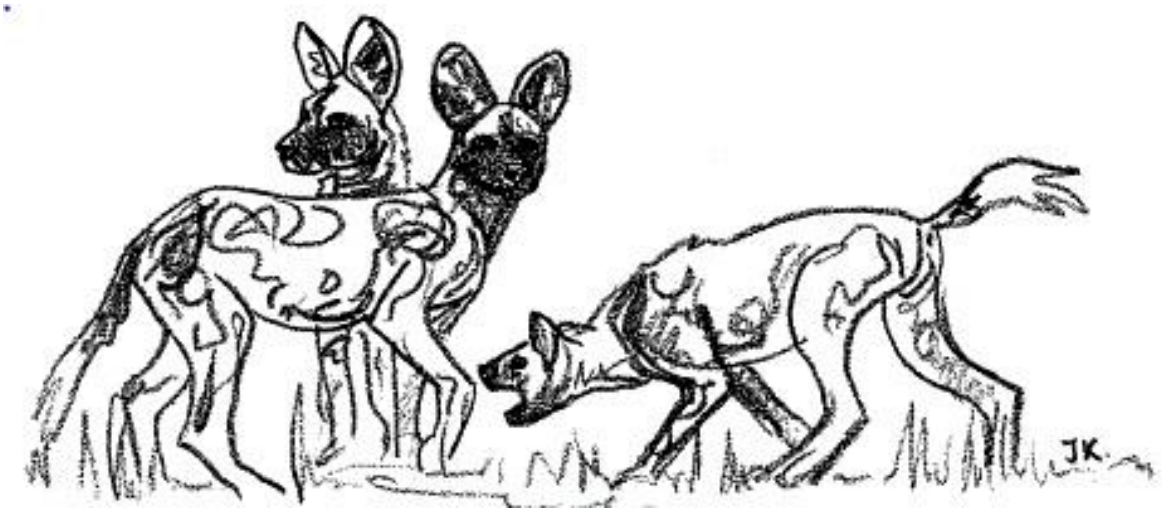
7.5 Conclusion

Ex situ conservation programs have contributed significantly to the conservation of many threatened species through breeding programs and targeted research efforts. To mitigate the risk of disease for endangered species it is critical to improve the understanding and effectiveness of preventative programs. This research has demonstrated that the inactivated Parvac™ vaccine used to prevent canine parvovirus was safe to use in the African Painted Dog. Modifying the inoculation schedule used in domestic dogs to include additional boosters will increase this vaccines duration of immunity in the African Painted Dog. Only slight differences in immunogenicity were noted between the delivery methods. Consolidating information about CPV and the African Painted Dog highlighted a significant gap in knowledge, especially regarding this pathogens prevalence in *in situ* populations, its regulatory role and the possible effects it has on recruitment rates.

This study was also able to provide a better understanding in the physical response that the African Painted Dog has to capture. It was clear that captive bred animals in this study had an acute stress response as seen by the elevated plasma and faecal cortisol concentrations respectively during capture, as well as in the days following. The simultaneous collection and analysis of these two biological materials showed a direct correlation, thereby confirming the utility of this non-invasive approach as a means to monitor stress in this species. Monitoring the stress response across repeated capture events identified two animals that experienced a degree of chronic stress. This highlights the need for additional research into the cumulative effects of stressors (particularly social stress), the frequency in which dogs experience stressful events and possible impacts this has for management (e.g. increasing number of boosters during vaccination). Generally, however, dogs were able to adequately cope with a range of conservation activities undertaken in this project (i.e. immobilisation, vaccination, transportation between zoos). *In situ* populations are likely to experience different stressors than the animals in this study but it is fair to assume that free-ranging dogs would have a similar coping mechanism to being immobilised and handled.

As the abundance of the African Painted Dog has continued to a decrease over time there has been significant effort made to develop more structured conservation plans at both national and regional scales to limit further losses of *in situ* populations. There has also been a major emphasis on *ex situ* populations to investigate different vaccination protocols to address disease risk and guide better management decisions. There is still a continuing need however, for additional engagement between *in situ* and *ex situ* conservation to

broaden the scope of research areas available to *ex situ* populations. Identifying ways to develop and improve collaborative efforts along the conservation continuum of wild and captive management, will increase the effectiveness that conservation actions have in protecting the African Painted Dog against extinction.



Appendix 1. Vaccine use and serological response in African Painted Dogs for all major canine pathogens

Target Pathogen	Vaccine	Type and Delivery	Study Purpose	Description	Reference
Adenovirus (Type 1)	Vanguard DA2P + CPV (Smith Kline Animal Health)	Modified live, Delivery not discussed	Monitoring antibody response after annual vaccination (n=9)	Antibody response to CAV-1 measured in dogs held at De Wildt Research Centre. Retained antibodies were found in the pre vaccination bleed. Second blood sample taken 1 month after revaccination. Anamnestic responses were detected in 6 of the 9 dogs tested with there being only one dog showing a significant response. The remaining dogs showed no or a slight increase in antibody titres. It was suggestive that the vaccine was safe and effective.	Spencer (1991)
Distemper	Enduracel d-h (Smith Kline Animal Health)	Modified live, Delivery not discussed	Vaccine trial (n=16)	A cohort of puppies held at National Zoological Gardens Pretoria were divided into 3 groups and vaccinated at different times and frequencies. Group A (n=3) was first bled at 9 weeks and then at 6 months with vaccination occurring 3 times at monthly intervals. Group B (n=4, approx. 6 months old) were vaccinated once and bled 2 months later. The last group were vaccinated at 3 months and bled 2 weeks later. This serological investigation showed an increase in antibody levels but results were deemed by the authors to be inconclusive.	van Heerden *et al.* (1980)
Distemper	Quantum 4, Quantum 6, (Pitman Moore)	Live attenuated Delivery not discussed	Captive management (n=13)	Distemper like clinical signs were observed in two unassociated groups of pups (8 and 11 weeks old) at Hartebeestpoortdam Snake Park and the National Zoological Gardens, RSA respectively. In both groups clinical signs were evident 13 days after vaccination with a number of animals dying within 36hrs after onset. Laboratory analysis confirmed distemper and circumstantially links it to the live vaccine used during inoculation which were designed for domestic dogs only.	van Heerden *et al.* (1989)
Distemper	Vanguard 5 (Smith Kline Animal Health)	Modified live Delivery not discussed	Vaccine trial (n=7)	Antibody response measured 1 month after vaccination from animals held at De Wildt Research Centre. Two-fold increase in antibody titres noted for distemper. Recommended that vaccine was effective and safe to use.	Spencer and Burroughs (1992)
Distemper	Oil Emulsion CDV vaccine	Inactivated, IM[a] into thigh Hand inject and blow pipe	Vaccine trial (n=9)	Captive held animals housed in the BioPark of Rome had not previously been vaccinated against CDV and returned a seronegative result at first blood sampling in vaccine trial. Vaccination regime consisted of an initial dose with revaccination occurring 30 days later for only 5 animals. A second blood sample occurred at day 50. No overt reaction except a mild transient limp in three animals. Protective antibody titres were detected in all but one dog with the highest titre values detected in animals that were vaccinated twice. It was suggested that oil adjuvants may be another good way to enhance immune responses in inactivated vaccines but would still require further investigation.	Cirone *et al.* (2004)

Target Pathogen	Vaccine	Type and Delivery	Study Purpose	Description	Reference
Distemper	PureVax™ Ferret (Merial)	Recombinant Live Delivered orally and hand inject 1ml IM	Vaccine trial (n=21)	Captive held dogs from WCS Bronx Zoo received 3 inoculations with the recombinant canary pox vectored vaccine designed for use in ferrets. Initial vaccinations occurred at 2 months of age with revaccination occurring 2 and 4 weeks later. For oral administration animals (n=8) were encouraged to bite a wooden block with vaccine spray over the gingival mucosa. Blood collection ceased for this group at 6.5 months because of a poor immune response. All pups seroconverted after 3 doses with no detectable antibodies by 20 months.	Connolly *et al.* (2013)
Parvovirus	Vanguard DA2P + CPV (Smith Kline Animal Health)	Modified live Delivery not discussed	Monitoring antibody response after annual vaccination (n=7)	Antibody response measured 1 month after vaccination from animals held at De Wildt Research Centre. Five dogs showed a positive seroconversion at 1 month with the 2 other animals showing an increase in antibody levels after being bled again two weeks later. No obvious effects of immunosuppression shown in study group.	Spencer and Burroughs (1990)
Rabies	Imrab (Merial)	Inactivated, Delivery not discussed	Disease outbreak (n=23)	Reports the first confirmed case of rabies in African Painted Dogs from the Masai Mara, Kenya. Prior to the disease outbreak blood was collected from 2 individuals with a further 3 vaccinated at a later date. Antibodies were found for a variety of pathogens including CPV, CAV, coronavirus, herpesvirus. In total 21 dogs died with the retrieval of 4 carcasses confirming the rabies virus. Of the two surviving animals one was vaccinated. Genetic analysis of the viral variant was consistent with that found in the regional domestic dog population. It was suggested that a carefully developed and maintained vaccination program for domestic dogs would assist conservation efforts in sympatric wild canid populations.	Kat *et al.* (1996)
Rabies	Madivak (Hoechest, Hanover)	Inactivated, Hand inject and by dart 1ml IM	Preventative medicine and reactive response to disease outbreak (n=34)	Adults dogs (n=29) and pups (n=5) from two packs in the Serengeti-Mara ecosystem were vaccinated in an attempt to minimise the effects of a disease outbreak. Serum neutralising antibodies were found for rabies in 3 of 12 dogs blood sampled prior to vaccination. Serum collected from two of the inoculated animals showed increased antibody levels post vaccination. After four months some individuals had disappeared with signs of lethargy observed in other pack members. Rabies was confirmed in one carcass found in the Mara area. This vaccination program failed to protect the population with all study animals subsequently disappearing. This disease outbreak initiated the localised extinction event of dogs in the Serengeti-Mara region.	Gascoyne *et al.* (1993a)

Target Pathogen	Vaccine	Type and Delivery	Study Purpose	Description	Reference
Rabies	Madivak (Hoechest, Hanover)	Inactivated, Hand inject and by dart 1ml IM	Vaccine trial and field experiment (n=4)	Management decision by the Tanzanian NP to implement a vaccination program in the Serengeti ecosystem. An initial trial was conducted at Frankfurt Zoo with no adverse effects being recorded. Serum samples were collected 5 weeks from inoculation with all dogs seroconverting. A field trial was performed on adults and pups greater than 12 weeks old with two recaptured animals having increased antibody titres.	Gascoyne *et al.* (1993b)
Rabies	Rabisin, (Rhône-Poulenc, South Africa)	Inactivated, 1ml IM, Delivery not discussed	Preventative medicine and reactive response to disease outbreak (n=24)	Mixed group of 3 males (wild) and 3 females (captive) were translocated to Madikwe GR, RSA forming a coherent group and having two successful litters. Translocated males were vaccinated during the process. Unusual behaviours were observed and 20 animals died in the following weeks from disease or unexplained aggression. The remaining animals were captured, vaccinated and placed in a boma where only three animals survived. Serological monitoring showed the presence of antibody titres soon after the primary vaccination but declined despite booster vaccinations.	Hofmeyr *et al.* (2000)
Rabies	SAG-2 (Virbac Laboratories, France)	Modified live Oral bait	Vaccine trial (n=14)	Demonstrated the use of chicken head baits as a delivery method for the live SAG-2 vaccine in captive bred adults and pups. 11 of the 14 captive bred dogs picked up the vaccine baits with all 11 animals seroconverting. Significant differences were found in pre and post vaccination titre levels.	Knobel *et al.* (2003)
Rabies	Rabisin (Merial)	Inactivated, 1ml IM, Hand inject and by dart	Preventative medicine of free ranging animals (n=5)	Multiple doses of the vaccine given to all adults, with pack consisting of a further 12 pups. A rabies outbreak occurred where only pups died. The remaining animals were transferred to a boma and monitored. Post exposure vaccination occurred to all captured dogs to reduce the potential for shedding. Six pups and all adults survived the episode. It was suggested that multiple vaccinations against rabies were effective in increasing immune response and reducing the pathogens effect on the Painted Dog population.	Hofmeyr *et al.* (2004)
Rabies	Imrab3, (Merial)	Inactivated, 1ml IM, manual restraint	Preventative medicine of captive dogs Vaccination trial (n=35)	Pups were administered Purevax Ferret (Merial) prior to being inoculated against rabies. Naïve animals(3-5mth old) were assigned to two groups, receiving either a single or double dose of Imrab3. No adverse effects noted suggesting vaccine safe to use.	Connolly *et al.* (2015)
Distemper, Adenovirus, Parainfluenza **Parvovirus** Rabies	Paramune 5 and Parvocine (Dellen Laboritories) Trimune (Rolynn Laboratories)	Modified live Inactivated, Delivery not discussed	Captive population that suffered a CDV outbreak (n=7)	Transfer of a group of seven captive bred animals from Namibia to New York. All dogs were approximately 5 months old weighing approximately 14-16kg each. General condition of the individuals on arrival in New York appeared to thin with skeletal deformities suggestive of rickets. All animals were vaccinated upon arrival. Clinical signs developed 10 days later with laboratory analysis confirming CDV. This infection was fatal in all but one animal who gradually recovered over a 3 month period. Canine parvovirus or any other pathogen were not considered.	McCormick (1983)

Target Pathogen	Vaccine	Type and Delivery	Study Purpose	Description	Reference
Distemper, **Parvovirus**, Adenovirus, Leptospirosis, Canicola	Candur® (SHLP)	Modified live and inactivated	Captive population that suffered a CDV outbreak (n=4)	Four captive bred animals were vaccinated at 57 days. Clinical signs of disease first developed 18 days after vaccination with all animals reported dead 6 days later. Timing of clinical symptoms followed vaccination with pathological results confirming CDV. Authors recommended the use of killed vaccines to avoid vaccine induced CDV infections in exotic carnivores. No serological results were shown for any of the other pathogens.	Durchfeld *et al.* (1990)
Rabies Distemper	Rabisin, (Rhône-Poulenc, South Africa) Not stated	Inactivated, Delivery not discussed	Reintroduction attempt (n=13)	Captive bred animals were released into Etosha NP, Namibia. Animals ranged in age from 1-4 years and were vaccinated annually for rabies and at 12 weeks for CDV. After persecution from humans and significant predation from lions (n=6) the release group was reduced to 4 individuals. These animals began to scavenge rather than hunt and were seen by tourists killing a black backed Jackal. All dogs were recaptured with clinical signs of rabies developing two weeks later with a fatal outcome for all. Diagnosis was confirmed by laboratory analysis. Monitoring antibody titre levels for rabies or CDV was not performed.	Scheepers and Venzke (1995)
Distemper Rabies **Parvovirus**, Leptospirosis	CDV-ISCOM Rabdmum (Schering-PloughAnimal Health) Dohyvac I-LP, (Solvay Duphar) Vanguard Lepto-CI (Pfizer)	Inactivated S/C[b]; blow pipe Inactivated IM Inactivated S/C; blow pipe	Captive management (n=25)	Captive group was created at Mkomazi GR, Tanzania from a founder group of 25 wild born pups. Inactivated vaccines were used to minimise risk of unwanted side effects and to prevent the spread of viruses in the field. The vaccine used to protect against CDV was developed for use in harbor seals and dogs for protection against the phocine distemper virus. All dogs were initially blood sampled and vaccinated (12-15 weeks old) for all pathogens with revaccination for CDV occurring 3 weeks later. After a further 5 weeks revaccination for CPV, CDV and Leptospirosis occurred with a repeat blood sampling occurring 2 weeks after that. Antibody testing showed that all but three dogs had an adequate level of neutralising antibodies for CDV; All dogs were well protected for CPV with two animals had a positive Ig M suggesting that they were at the point of having a parvo-infection. After 2½ months there was no response in immunity to the rabies vaccine with only 13 of the 25 dogs showing some result. This prompted a review into the vaccination policy for the rabies virus. The vaccine for parvovirus and leptospirosis was changed due to an inability to source Dohyvac I-LP. No anti-body testing occurred when Vanguard Lepto-CI (Pfizer) was administered.	George Adamson Trust (1996); Visee (2001)
Distemper Rabies **Parvovirus**, Leptospirosis	CDV-ISCOM Rabdmum (Schering-PloughAnimal Health) Dohyvac I-LP, (Solvay Duphar)	Inactivated, Inactivated Inactivated Delivery not discussed	Captive population that suffered a CDV outbreak. (n=52)	Captive group established at Mkomazi GR, Tanzania. Vaccination schedule consisted of three consecutive vaccinations at 2-4 week intervals and annual revaccination. Blood samples were routinely collected to monitor immune response. Approximately one month after an annual revaccination infection became apparent with 49 of the 52 animals dying within 8 weeks (Dec 2000-Feb 2001). CDV was identified as the causal agent with a discussion on transmission. Vaccination failure was seen as being responsible with possible reasons to be investigated by authors. Antibody titre levels for rabies and CPV and Leptospirosis were not discussed.	van de Bildt *et al.* (2002)

Target Pathogen	Vaccine	Type and Delivery	Study Purpose	Description	Reference
Distemper	Akzo Nobel (Intervet South Africa)	Inactivated	Vaccine trial (n=10)	Animals were held at Sasol and the De Wildt Research Centres. Control animals (n=2) just received the inactivated canine vaccine. Four animals received the live attenuated vaccine for distemper (Synder Hill strain), parvovirus and rabies intramuscularly. The remaining four dogs received the live attenuated distemper and parvovirus vaccine and the oral rabies vaccine. Prior to the live CDV vaccine all dogs received the inactivated distemper vaccine (Rockborn strain). Dogs just receiving the inactivated distemper vaccine did not seroconvert but a cellular response was presumed to occur, whereas all dogs inoculated with the live attenuated vaccine did seroconvert. Antibody titres for CPV were present prior to the administration of the vaccine. A temporary increase in titre levels was noted in most dogs after vaccination. Antibodies were present for 451 days for both pathogens. For animals vaccinated against rabies seroconversion was achieved in 7 of 8 dogs with titres dropping to very low concentrations within 100 days. The animal deemed to be the exception received the parenteral vaccine. Administered boosters increased titre levels in all dogs. Recommended that the vaccine was safe and that revaccination should occur between 3-6 months after first inoculation. Serology for CAV and CPI was not reported. There was no indication of vaccine induced disease in this study caused by the live distemper, parvovirus or rabies vaccines. The distemper vaccine could not however be recommended as safe for use in Painted Dogs. One control animal died during the experimental period from bacteria enteritis possibly a result of capture stress. van Heerden *et al.* (2002) reports unpublished observations by R.E. Burrows that 3 and a half month old pups exhibited vaccine induced distemper after being vaccinated with Vanguard Puppy 5).	van Heerden *et al.* (2002)
Distemper, **Parvovirus,** Adenovirus, Parainfluenza	Vanguard Puppy 5 (Pfizer Animal Health)	Live attenuated, S/C in neck			
Rabies	Defensor® (Pfizer Animal Health)	Inactivated IM			
	SAG-2 (Virbac Laboratories, France)	Live oral vaccine			
Major canine pathogens	Depending on level of threat	Remote delivery favoured	Captive management in European zoos and wildlife parks	Additional vaccines not previously described in the above studies. Nobivac (Intervet); Duramine (Fort Dodge); Hexidog, Eurican P, Eurican L, Leptodog (Merial); Vanguard CPV Lepto, Canigen (virbac). Contrary to what was stated in this study, Parvac (Pfizer) was not actually given to collection animals, only one pup died assumed to result from food poisoning with adults dogs too becoming ill (*pers comm.* Maria Krakowiak, Curator of Carnivores, Warsaw Zoo, 28th October 2014). Refer to study for additional information.	Popelin (2010)

[a] Intramuscular, IM;
[b] Subcutaneously, S/C;
Note: Some information regarding vaccine types were obtained from (Woodroffe *et al.* 2004a)

Appendix 2. Vaccines available in Australia that inoculate against CPV

Company	APVMA No.	Vaccine	Type	Pathogens	Other Constituents
Boehringer Ingelheim	59730	Duramune Adult C3	Live attenuated	CAV-2, CDV, CPV-2b	Nil
	59731	Duramune Adult C4	Live attenuated	CAV-2, CDV, CPV-2b, CPI-2	Nil
	51487	Protech C3	Live attenuated	CAV-2, CDV, CPV-2b	Nil
	51490	Protech C3+2i	Live attenuated	CAV-2, CDV, CPV-2b, inactivated Canine Coronavirus, Leptospira	Nil
	51486	Protech C4	Live attenuated	CAV-2, CDV, CPV-2b, CPI-2	Nil
	51489	Protech C4+2i	Live attenuated	CAV-2, CDV, CPV-2b, CPI-2, inactivated Canine Coronavirus, Leptospira	Nil
Fort Dodge	52383	C3	Live attenuated	CAV-2, CDV, CPV-2b	Nil
	52382	C4	Live attenuated	CAV-2, CDV, CPV-2b, CPI-2	Nil
	39547	Protech C3	Live attenuated	CDV (Onderstepoort), Measles (Edmonston), CPV-2	Nil
Intervet Australia	56445	Companion C3	Live attenuated	CAV-2, CDV, CPV-2	Nil
	56438	Companion C4	Live attenuated	CAV-2, CDV, CPV-2, CPI	Nil
	59043	Nobivac DHP	Live attenuated	CAV-2 strain Manhattan, CDV (Onderstepoort), CPV-2	Nil
	56912	Nobivac DHPPI	Live attenuated	CAV-2, CDV, CPV-2, CPI	Nil
Pfizer Animal Health (Zoetis)	52773	Canvac 3	Live attenuated	CAV-2, CDV, CPV-2	Nil
	52778	Canvac 4	Live attenuated	CAV-2, CDV, CPV-2, CPI	Nil
	61300	Canvac 4 + CCI	Live attenuated	CAV-2, CDV, CPV-2, CPI, Bordetella Bronchiseptica (inactivated)	Nil
	52643	Canvac 4 + BB	Live attenuated	CAV-2, CDV, CPV-2, CPI, Bordetella Bronchiseptica (inactivated)	Thiomersal
	61477	Canvac Puppy 3	Live attenuated	CAV-2, CDV, CPV-2	Nil
	61475	Canvac Puppy 4	Live attenuated	CAV-2, CDV, CPV-2, CPI	Nil
	51777	Parvac	Inactivated	CPV-2	Thiomersal
Virbac (Aust)	47373	Canigen C4 DHA2PPI	Live attenuated	CAV-2, CDV, CPV, CPI	Nil
	40758	Canigen DHA2P	Live attenuated	CAV-2, CDV, CPV	Nil

Appendix 3. Individual markers employed during the faecal collection in the male dogs at Monarto Zoo

Treatment	Animal ID	Marker	Glitter Colour
Manual restraint only	A29038	Sorgham + Safflower	Red
	A69117	Sorgham + Safflower + glitter	
	A69118	Sorgham + Sunflower	
	A69116	Sorgham + Sunflower + glitter	
	A59173	Sorgham + Corn	
	A69115	Sorgham + Corn + glitter	
Sedation	A69120	White Millet + Safflower	Blue
	A69114	White Millet + Safflower + glitter	
	A49135	White Millet + Sunflower	
	A49134	White Millet + Sunflower + glitter	
	A49160	White Millet + Corn	
	A69113	White Millet + Corn + glitter	
General anaesthetic	A59176	Canary seed + Safflower	Green
	A59171	Canary seed + Safflower + glitter	
	A59174	Canary seed + Sunflower	
	A69119	Canary seed + Sunflower + glitter	
	A59175	Canary seed + Corn	
	A59172	Canary seed + Corn + glitter	
	A29037*		Purple

*This individual was not part of the vaccination trial and was immobilised opportunistically. A faecal marker was dispensed to this dog to exclude collected samples from the analysis.

Appendix 4. Individual cortisol concentrations before and after the administration of ACTH

Animal ID	Sex	Synacthen dose (IU)	Time (minutes) 0	10	20	30	40	50	60	70	90	110	130	150	170	190
A69117	M	14.5	57.52	61.53	79.31	78.83	92.48	80.09	77.64	56.02	62.79	105.72	66.84	52.40	103.21	89.34
A69113	M	16.5	209.61	61.15	70.52	90.03	89.96	45.14	48.05	61.25	123.98	145.78	162.63	159.83	148.99	114.15
B09056	F	40	318.79	390.53	222.58	167.20	143.29	111.31	133.78	120.67	251.94	290.35	328.73	372.28	356.14	352.95
B09058	F	40	60.45	99.49	50.18	22.26	26.73	20.25	14.09	13.68	81.34	90.75	107.35	129.28	141.05	122.00
A69119	M	Control	62.58	75.33	89.97	44.19	25.50	49.19	31.50	28.72	21.21	15.57	34.24	63.49	40.15	36.92
B09057	F	Control	32.04	70.63	64.42	31.27	-	-	-	-	-	-	-	-	-	-
Percentage change				2.38	-23.95	-24.82	104.56	-19.04	-0.30	-8.10	131.88	21.64	5.21	7.25	4.99	-9.47
Mean		Overall	123.50	126.44	96.16	72.30	75.59	61.20	61.01	56.07	130.01	158.15	166.39	178.45	187.35	169.61
		Females	137.10	186.88	112.39	73.58	85.01	65.78	73.94	67.18	166.64	190.55	218.04	250.78	248.60	237.48
		Males	109.90	66.00	79.93	71.02	69.32	58.14	52.40	48.66	93.39	125.75	114.73	106.12	126.10	101.74
		Control	47.31	72.98	77.20	37.73	25.50	49.19	31.50	28.72	21.21	15.57	34.24	63.49	40.15	36.92

Appendix 5. Individual cortisol concentrations for MZ males participating in the immobilisation trial

Capture Approach	Animal ID	Capture events								Proportion (%) of events >80.72ng/ml
		1	2	3	4	5	6	7	8	
General anaesthetic	A59172	85.08	34.02	66.32	87.91	73.10	136.76	105.71	160.38	62.5
	A59175	115.19	37.21	48.09	62.94	105.61	62.39	70.34	72.18	25
	A59174	50.67	27.14	54.52	58.83	113.81	36.47	80.78	117.99	37.5
	A59171	77.53	19.84	67.27	97.24	145.90	104.77	100.97	118.26	62.5
	A59176	66.19	43.58	81.16	73.03	107.51	-	-	-	40
	A69119	37.42	19.16	121.48	80.30	108.54	92.62	66.30	62.58	37.5
Manual restraint and sedation	A49160	173.98	12.23	33.79	42.04	79.14	23.45	92.28	20.24	25
	A69113	327.60	44.24	130.82	97.46	237.65	176.56	80.36	209.61	75
	A49135	127.60	27.93	34.59	41.33	25.14	62.59	43.06	37.11	12.5
	A69120	19.49	10.71	18.17	12.83	26.09	20.51	28.66	39.76	0
	A49134	58.55	18.58	58.43	28.10	102.42	68.24	44.13	69.41	12.5
	A69114	105.03	71.85	58.37	27.11	93.52	15.33	44.38	73.99	25
Manual restraint only	A69118	98.32	25.72	78.40	122.80	169.87	34.70	99.31	35.62	50
	A59173	48.88	14.37	58.35	23.07	28.27	29.44	69.88	53.99	0
	A29038	33.41	11.45	42.23	57.16	37.30	24.57	27.91	106.34	12.5
	A69116	83.74	83.08	69.04	65.50	78.51	66.13	10.36	62.22	25
	A69115	34.96	89.78	59.93	88.34	305.29	81.86	35.19	55.55	50
	A69117	41.48	-	-	113.83	78.77	44.62	68.31	57.52	16.6

Appendix 6. Cortisol response to the low dose dexamethasone suppression test

Animal ID	Sex	Wgt	Dose	Time						PEAK	TROUGH	
				0	60	120	240	360	480		Conc	Time
A29040	F	26.1	0.28mgTD	65.40	27.91	11.89	8.99	14.81	35.01	65.40	8.99	240
B09059	F	28.1	0.28mgTD	65.91	18.53	11.40	45.18	55.03	57.80	65.91	11.40	120
A29037	M	20.8	0.05ml	39.39	10.49	13.63	17.10	45.35	60.47	60.47	10.49	60
A29038	M	16.0	0.04ml	74.33	87.99	57.78	22.51	-	-	87.99	22.51	240
Mean cortisol concentration				61.25	36.23	23.67	23.44	38.40	51.09			

Appendix 7. Delivery success of markers and contribution of individuals to off target uptake

Animal ID	Capture event*								Percent
	1	2	3	4	5	6	7	8	
A29037	-	-	-	0.1	-	-	-	-	0.1%
A29038	-	-	-	-	-	-	-	-	0.0%
A49134	2	2	1	2	1.2	-	-	3	5.9%
A49135	1	1	-	1.9	1	1	-	-	3.1%
A49160	-	-	-	2	2.3	-	-	1	2.8%
A59171	-	-	-	-	1.8	1	-	0.1	1.5%
A59172	-	-	1	-	1	0.5	-	3	2.9%
A59173	-	-	-	1	1	1.5	-	2	2.9%
A59174	1	-	-	-	0.5	2	2	-	2.9%
A59175	-	-	-	-	-	6	4.1	-	5.3%
A59176	1	-	1	-	-	-	-	-	1.1%
A69113	-	1	3	1	2	1	1	-	4.7%
A69114	1	-	-	-	-	-	-	-	0.5%
A69115	7	1	7	-	5	2	1	-	12.1%
A69116	3	-	3	8	4.5	2	4	0.1	13.0%
A69117	2	-	4	3	-	4	-	1	7.4%
A69118	4	2	3.9	-	2	1	-	-	6.8%
A69119	7	1	3.5	3	11	-	-	-	13.5%
A69120	5	4	2	1	2.5	1	3	2	10.8%
Undetermined	-	-	2	-	-	2	1	-	2.6%
Dogs receiving off target baits	11	7	11	10	13	13	7	8	
Markers not dispensed (%)	0	29	13	15	0	17	0	0	
Delivery success (%)	73	58	58	64	73	59	87	90	

* Fraction refers to the proportion of bait that has been consumed, determined by subjective observation.
The duration of one capture event is 7 days with markers fed out daily.

REFERENCES

AAZV 2011, *Parvovirus*, Infectious Disease Manual, American Association of Zoo Veterinarians, Florida, USA, [Online], Available: http://www.aazv.org/associations/6442/files/idm_parvovirus.pdf [18th June 2012].

Abrantes, R. 2005, *The evolution of canine social behavior*, 2nd Edition, Wakan Tanka Publishers, Ann Arbor, Mi, USA.

Abuja, P. M. and Albertini, R. 2001, 'Methods for monitoring oxidative stress, lipid peroxidation and oxidation resistance of lipoproteins', *Clinica Chimica Acta*, vol. 306, no. 1–2, pp. 1-17.

Acevedo-Whitehouse, K. 2009, 'The importance of disease management programmes for wildlife conservation', *Animal Conservation*, vol. 12, pp. 185-186.

Aguirre, A. A., Ostfeld, R. S., Tabor, G. M., House, C. and Pearl, M. C. (ed), 2002, *Conservation medicine: Ecological health in practice*, Oxford University Press, Inc., Oxford, New York.

Alexander, K. A. and Appel, M. J. 1994, 'African Wild Dogs (*Lycaon pictus*) endangered by a canine distemper epizootic among domestic dogs near the Masai Mara National Reserve, Kenya', *Journal of Wildlife Diseases*, vol. 30, no. 4, pp. 481-5.

Alexander, K. A., Conrad, P. A., Gardner, I. A., Parish, C., Appel, M. J., Levy, M. G., Lerche, N. W. and Kat, P. W. 1993, 'Serologic survey for selected microbial pathogens in African Wild Dogs (Lycaon pictus) and sympatric domestic dogs (*Canis familiaris*) in Maasai Mara, Kenya', *Journal of Zoo and Wildlife Medicine*, vol. 24, no. 2, pp. 140-144.

Alexander, K. A., Kat, P. W., Munson, L., Kalake, A. and Appel, M. J. 1996, 'Canine distemper-related mortality among Wild Dogs (*Lycaon pictus*) in Chobe National Park, Botswana', *Journal of Zoo and Wildlife Medicine*, vol. 27, no. 3, pp. 426-427.

Alexander, K. A., McNutt, J. W., Briggs, M. B., Standers, P. E., Funston, P., Hemson, G., Keet, D. and van Vuuren, M. 2010, 'Multi-host pathogens and carnivore management in southern Africa', *Comparative Immunology, Microbiology and Infectious Diseases*, vol. 33, no. 3, pp. 249-265.

Ament, R., Clevenger, A. P., Yu, O. and Hardy, A. 2008, 'An assessment of road impacts on wildlife populations in U.S. national parks', *Environmental Management*, vol. 42, no. 3, pp. 480-96.

Andreka, G., Linn, I. J., Perrin, M. R. and Maddock, A. H. 1999, 'Range use by the Wild Dog in Hluhluwe-Umfolozi Park, South Africa', *South African Journal of Wildlife Research*, vol. 29, no. 1, pp. 1-9.

Apanius, V. 1998, 'Stress and immune defense', *Advances in the Study of Behavior*, vol. 27, pp. 133-153.

Appel, M. J. and Parrish, C. R. 1987, 'Canine Parvovirus Type 2', in *Virus Infections of Carnivores*, ed. M. J. Appel, Elsevier Science, Amsterdam, pp. 69-91.

APVMA 2012, *About the APVMA*, The Australian Pesticides and Veterinary Medicines Authority, Australian Government, [Online], Available: http://www.apvma.gov.au/ [2nd Decemeber 2012].

ARAZPA 2008, *Captive Management Plan for African Wild Dog (Lycaon pictus)*, Australiasian Regional Association of Zoological Parks and Aquaria, NSW, Australia.

ARAZPA 2009, *Wild Dog Annual Report*, Australiasian Regional Association of Zoological Parks and Aquaria, Mosman, NSW.

Armstrong, D. (ed), 2011, *Studies on veterinary medicine: Oxidative stress in applied research and clinical practice*, Springer Science, New York, USA.

Armstrong, D. P. and Seddon, P. J. 2008, 'Directions in Reintroduction Biology', *Trends in Ecology & Evolution*, vol. 23, no. 1, pp. 20-25.

AZA 2003, *African Wild Dog, Lycaon pictus, Species Survival Plan*, American Zoo and Aquarium Association, Population Management Centre, Chicago. pp. 1-29.

Bajehson, D. B. 2010, Molecular characterisation of canine parvovirus strains from domestic dogs in South Africa and Nigera, Master of Science (Veterinary Science), Faculty of Veterinary Science, University of Pretoria, South Africa, pp. 106.

Baldus, R. D. and Hahn, R. 2009, *The Selous-Niassa wildlife corridor in Tanzania: Biodiversity from the grassroots. practical experiences and lessons from intergrating local communities into transboundary natural resources management*, Joint Publication of FAO and CIC, Budapest, pp. 48.

Banyard, A. C., Gordon, C. H., Fooks, A. R., Sillero Zubiri, C. and Johnson, N. 2012, 'Conservation of canids: The impact of infectious viral disease', in Wolves: Biology, Behavior, and Conservation, ed. A. P. Maia and H. F. Crussi, Nova Science Publishers, pp. 49-78.

Barker, I. K. and Parrish, C. R. 2008, 'Parvovirus Infections', in *Infectious Diseases of Wild Mammals*, ed. Iowa State University Press, pp. 131-146.

Belsare, A. V. and Gompper, M. E. 2013, 'Assessing demographic and epidemiologic parameters of rural dog populations in India during mass vaccination campaigns', *Preventive Veterinary Medicine*, vol. 111, no. 1-2, pp. 139-146.

Berentsen, A. R., Dunbar, M. R., Becker, M. S., M'soka, J., Droge, E., Sakuya, N. M., Matandiko, W., McRobb, R. and Hanlon, C. A. 2013, 'Rabies, canine distemper, and canine parvovirus exposure in large carnivore communities from two Zambian ecosystems', *Vector borne and zoonotic diseases* vol. 16, pp. 1-7.

Berger, J. 1999, 'Anthropogenic extinction of top carnivores and interspecific animal Behaviour: Implication of the rapid decoupling of a web involving wolves, bears, moose and ravens', *Proceedings of the Royal Society London B*, vol. 266, no. pp. 2261-2267.

Binn, L. N., Lazar, E. C., Eddy, G. A. and Kajima, M. 1970, 'Recovery and characterization of a Minute Virus of Canines', *Infection and Immunity*, vol. 1, no. 5, pp. 503-508.

Blount, J. 2003, 'Carotenoid modulation of immune function and sexual attractiveness in zebra finches', *Science*, vol. 300, no. 5616, pp. 125.

Böhm, M., Thompson, H., Weir, A., Hasted, A. M., Maxwell, N. S., Herrtage, M. E., Coyne, M. J., Burr, J. H. H., Yule, T. D., Harding, M. J., Tresnan, D. B. and McGavin, D. 2004, 'Serum antibody titres to canine parvovirus, adenovirus and distemper virus in dogs in the UK which had not been vaccinated for at least three years', *Veterinary Record*, vol. 154, no. 15, pp. 457-63.

Boinski, S. and Garber, P. A. (ed), 2000, *On the move: How and why animals travel in groups*, The University of Chicago Press, Chicago, USA.

BOM 2012, *Climate Data Online*, Commonwealth Government of Australian, Bureau of Meteorology, [Online], Available: http://www.bom.gov.au/climate/data/?ref=ftr [10th April 2012; 12th December 2014].

Borer, E. T., Seabloom, E. W., Shurin, J. B., Anderson, K. E., Blanchette, C. A., Broitman, B., Cooper, S. D. and Halpern, B. S. 2005, 'What determines the strength of a trophic cascade?', *Ecology*, vol. 86, no. 2, pp. 528-537.

Brearley, G., Rhodes, J., Bradley, A., Baxter, G., Seabrook, L., Lunney, D., Liu, Y. and McAlpine, C. 2012, 'Wildlife disease prevalence in human-modified landscapes', *Biol Rev Camb Philos Soc*, vol. 88, no. 2, pp 427-42.

Breed, A. C., Plowright, R. K., Hayman, D. T. S., Knobel, D. L., Molenaar, F. M., Gardner–Roberts, D., Cleaveland, S., Haydon, D. T., Kock, R. A., Cunningham, A. A., Sainsbury, A. W. and Delahay, R. J. 2009, 'Disease management in endangered mammals', in *Management of Diseases in Wild Mammals*, ed. R. J. Delahay, G. C. Smith and M. R. Hutchings, Springer, Japan, pp. 215-239.

Brennan, G., Podell, M. D., Wack, R., Kraft, S., Troyer, J. L., Bielefeldt-Ohmann, H. and VandeWoude, S. 2006, 'Neurologic disease in captive lions (*Panthera leo*) with low-

titer lion lentivirus infection', *Journal of Clinical Microbiology*, vol. 44, no. 12, pp. 4345-4352.
Breuer, T. 2002, *Distribution, feeding ecology and conservation of the African Wild Dog (Lycaon pictus) in Northern Cameroon*, Yaounde, Cameroon.
Breuner, C. W., Delehanty, B. and Boonstra, R. 2013, 'Evaluating stress in natural populations of vertebrates: total CORT is not good enough', *Functional Ecology*, vol. 27, no. 1, pp. 24-36.
Brzeski, K. E., Harrison, R. B., Waddell, W. T., Wolf, K. N., Rabon, J. D. R. and Taylor, S. S. 2015, 'Infectious disease and red wolf conservation: assessment of disease occurrence and associated risks', *Journal of Mammalogy*, vol. 96, no. 4, pp. 751-761.
Buettner, U. K., Davies-Mostert, H. T., du Toit, J. T. and Mills, M. G. L. 2007, 'Factors affecting juvenile survival in African wild dogs (*Lycaon pictus*) in Kruger National Park, South Africa', *Journal of Zoology*, vol. 272, no. 1, pp. 10-19.
Burrows, R. 1992, 'Rabies in Wild Dogs', *Nature*, vol. 359, pp. 277.
Burrows, R. 1994, 'Rabies in African Wild Dogs of Tanzania', *Journal of Wildlife Diseases*, vol. 30, no. 2, pp. 297-299.
Burrows, R. 2003, *'Young wild dog male' is top dog*, The African Conservation Foundation, [Online], Available: http://www.africanwilddogwatch.org/library/documents/awdw0004.pdf [15th August 2012].
Burrows, R. 2011a, *A guide to the African Wild Dog*, George Adamson Wildlife Preservation Trust.
Burrows, R. 2011b, *The handling-stress hypothesis and its importance for the conservation and survival of the African Wild Dog*, [Online], Available: http://www.africanwilddogwatch.org/papers/ [29th October 2012].
Burrows, R. 2011c, *Invasive research, reduced longevity and mortality in an endangered species, The African Wild Dog (Lycaon pictus), with special reference to the extinction of the Serengeti-Mara study population 1985-91*, The African Conservation Foundation, [Online], Available: http://www.africanconservation.org/wilddogs/invasiveresearch.html [24th April 2010].
Burrows, R. 2011d, *Pack formation, protocols of social behaviour and conservation of the African Wild Dog (Lycaon pictus)*, African Wild Dog Watch, [Online], Available: http://www.africanwilddogwatch.org/library/documents/awdw0003.pdf [27th November 2012].
Burrows, R. n.d, *The case of the handled study population of Wild Dogs (Lycaon pictus) in Kruger National Park*, [Online], Available: http://www.africanwilddogwatch.org/papers/ [29th October 2012].
Burrows, R., Hofer, H. and East, M. L. 1994, 'Demography, extinction and intervention in a small population: The case for the Serengeti Wild Dogs', *Proceedings of the Royal Society London B*, vol. 256, no. 1347, pp. 281-292.
Burrows, R., Hofer, H. and East, M. L. 1995a, 'Population dynamics, intervention and survival in African Wild Dogs (*Lycaon pictus*)', *Proceedings of the Royal Society of London - B. Biological Sciences*, vol. 262, pp. 235-245.
Burrows, R., Hofer, H. and East, M. L. 1995b, 'Population dynamics, intervention and survival in African Wild Dogs (Lycaon pictus)', *Proc Biol Sci*, vol. 262, no. 1364, pp. 235-45.
Busch, D. S. and Hayward, L. S. 2009, 'Stress in a conservation context: a discussion of glucocorticoid actions and how levels change with conservation-relevant variables', *Biological Conservation*, vol. 142, no. 12, pp. 2844-2853.
Butler, J. R. A., du Toit, J. T. and Bingham, J. 2004, 'Free-ranging domestic dogs (Canis familiaris) as predators and prey in rural Zimbabwe: threats of competition and disease to large wild carnivores', *Biological Conservation*, vol. 115, no. 3, pp. 369-378.

Byers, O., Lees, C., Wilcken, J. and Schwitzer, C. 2013, 'The One Plan Approach: The philosophy and implementation of CBSG's approach to integrated species conservation planning', *WAZA*, vol. 14, pp. 3-4.

Carlson, A. A., Carlson, R. and Bercovitch, F. B. 2004, *African Wild Dog Conservation Project, Kafue National Park, Zambia 2004 Annual Report*, USA.

Carmichael, L. E., Schlafer, D. H. and Hashimoto, A. 1991, 'Pathogenicity of Minute Virus of Canines (MVC) for the canine fetus', *The Cornell Veterinarian*, vol. 81, no. 2, pp. 151-71.

Casal, J. I., Langeveld, J. P., Cortes, E., Schaaper, W. W., van Dijk, E., Vela, C., Kamstrup, S. and Meloen, R. H. 1995, 'Peptide vaccine against canine parvovirus: Identification of two neutralising subsites in the N Terminus of VP2 and optimisation of the amino acid sequence', *Journal of Virology*, vol. 69, no. 11, pp. 7274-7277.

Caughley, G. 1994, 'Directions in Conservation Biology', *Journal of Animal Ecology*, vol. 63, no. 2, pp. 215-44.

Cavalli, A., Martella, V., Desario, C., Camero, M., Bellacicco, A. L., De Palo, P., Decaro, N., Elia, G. and Buonavoglia, C. 2008, 'Evaluation of the antigenic relationships among canine parvovirus type 2 variants', *Clinical and Vaccine Immunology*, vol. 15, no. 3, pp. 534-539.

Ceballos, G., Ehrlich, P. R., Barnosky, A. D., García, A., Pringle, R. M. and Palmer, T. M. 2015, 'Accelerated modern human–induced species losses: Entering the sixth mass extinction', *Science Advances*, vol. 1, no. 5, e1400253.

Chalmers, W. S. 2006, 'Overview of new vaccines and technologies', *Veterinary Microbiology*, vol. 117, no. 1, pp. 25-31.

Chapin, S. F., Zavaleta, E. S., Eviner, V. T., Naylor, R. L., Vitousek, P. M., Reynolds, H. L., Hooper, D. U., Lavorel, S., SalaI, O. E., Hobbie, S. E., Mack, M. C. and Díaz, S. 2000, 'Consequences of changing biodiversity', *Nature*, vol. 405, no. 6783, pp. 234-242.

Childes, S. L. 1988, 'The past history, present status and distribution of the Hunting Dog *Lycaon pictus* in Zimbabwe', *Biological Conservation*, vol. 44, no. 4, pp. 301-316.

CITES 2007, 'PE 389.522', 14th Meeting of the Conference of the Parties to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), European Parliament resolution of 24 May 2007 on the EU strategic objectives, 3-15 June 2007, The Hague, pp. 5.

Clark, J. R. 1999, 'The ecosystem approach from a practical point of view', *Conservation Biology*, vol. 13, no. 3, pp. 679-681.

Clarkson, P. M. and Thompson, H. S. 2000, 'Antioxidants: what role do they play in physical activity and health?', *The American Journal of Clinical Nutrition*, vol. 72, no. 2, pp. 637s-646s.

Cleaveland, S. 2009, 'Viral threats and vaccination: Disease management of endangered species', *Animal Conservation*, vol. 12, no. 3, pp. 187-189.

Cleaveland, S., Appel, M. G. J., Chalmers, W. S. K., Chillingworth, C., Kaare, M. and Dye, C. 2000, 'Serological and Demographic Evidence for domestic dogs as a source of canine distemper virus infection for Serengeti wildlife ', *Veterinary Microbiology* vol. 72, no. 3/4, pp. 217-227.

Cleaveland, S., Kaare, M., Knobel, D. and Laurenson, M. K. 2006, 'Canine vaccination: Providing broader benefits for disease control', *Veterinary Microbiology*, vol. 117, no. 1, pp. 43-50.

CMS 2016, *African Wild Dog - Lycaon pictus*, Proposal for inclusion of species on the Appedices of the Convention on the Conservation of Migratory Species of Wild Animals, Proposal II/5, Ninth Meeting of the Conference of the Parties to CMS, Italy, pp. 12.

CMS 2017, *CMS and CITES join forces to protect Africa's carnivores*, The Convention on the Conservation of Migratory Species of Wild Animals, [Online], Available: http://www.cms.int/sites/default/files/PR_African%20carnivore%20initiative_0.pdf [8th November 2017].

Connolly, M., Thomas, P., Woodroffe, R. and Raphael, B. L. 2013, 'Comparison of oral and intramuscular recombinant canine distemper vaccination in African Wild Dogs (*Lycaon pictus*)', *Journal of Zoo and Wildlife Medicine*, vol. 44, no. 4, pp. 882-888.
Connolly, M., Thomas, P., Woodroffe, R. and Raphael, B. L. 2015, 'Single- versus double-dose rabies vaccination in captive African Wild Dogs (*Lycaon pictus*)', *Journal of Zoo and Wildlife Medicine*, vol. 46, no. 4, pp. 691-698.
Conway, W. 1999, 'The Changing role of zoos in the 21st Century', *Keynote for the Annual Confernce of the World Zoo Organisation*, 18 October, Pretoria, South Africa, pp. 1-8.
Costantini, D. 2008, 'Oxidative stress in ecology and evolution: lessons from avian studies', *Ecology Letters*, vol. 11, no. 11, pp. 1238-1251.
Costantini, D. and Dell'Omo, G. 2006, 'Effects of T-cell-mediated immune response on avian oxidative stress', *Comp Biochem Physiol A Mol Integr Physiol*, vol. 145, no. 1, pp. 137-42.
Costantini, D., Dell'Omo, G., Filippis, S. P. D., Marquez, C., Snell, H. L., Snell, H. M., Tapia, W., Brambilla, G. and Gentile, G. 2009, 'Temporal and spatial covariation of gender and oxidative stress in the Galápagos Land Iguana Conolophus subcristatus', *Physiological and Biochemical Zoology*, vol. 82, no. 5, pp. 430-437.
Costantini, D., Marasco, V. and Moller, A. P. 2011, 'A meta-analysis of glucocorticoids as modulators of oxidative stress in vertebrates', *J Comp Physiol B*, vol. 181, no. 4, pp. 447-56.
Costantini, D., Rowe, M., Butler, M. W. and McGraw, K. J. 2010, 'From molecules to living systems: historical and contemporary issues in oxidative stress and antioxidant ecology', *Functional Ecology*, vol. 24, no. 5, pp. 950-959.
Courchamp, F. and Macdonald, D. W. 2001, 'Crucial importance of pack size in the African Wild Dog *Lycaon pictus*', *Animal Conservation*, vol. 4, no. 2, pp. 169-174.
Creel, N. M., Creel, S. and Monfort, S. L. 1996a, 'Social stress and dominance', *Nature*, vol. 379, no. pp. 212.
Creel, S. 1992, 'Causes of Wild Dog deaths', *Nature*, vol. 360, pp. 633.
Creel, S. 1997a, 'Cooperative hunting and group size: assumptions and currencies', *Animal Behaviour*, vol. 54, no. 5, pp. 1319-1324.
Creel, S. 1997b, 'Handling of African Wild Dogs and chronic stress: Reply to East et al', *Conservation Biology*, vol. 11, no. 6, pp. 1454-1456.
Creel, S. 2001, 'Social dominance and stress hormones', *Trends in Ecology & Evolution*, vol. 16, no. 9, pp. 491-497.
Creel, S. and Creel, N. M. 1995, 'Communal hunting and pack size in African wild Dogs, *Lycaon pictus*', *Animal Behaviour*, vol. 50, no. 5, pp. 1325-1339.
Creel, S. and Creel, N. M. 1996, 'Limitation of African Wild Dog by competition with larger carnivores', *Conservation Biology*, vol. 10, no. 2, pp. 526-538.
Creel, S. and Creel, N. M. 2002, *The African Wild Dog: Behavior, ecology and conservation*, Priceton University Press, New Jersey, USA.
Creel, S., Creel, N. M., Mills, M. G. L. and Monfort, S. L. 1997a, 'Rank and reproduction in co-operatively breeding African Wild Dogs: Behavioral and endocrine correlates', *Behavioural Ecology*, vol. 8, no. 3, pp. 298-306.
Creel, S., Creel, N. M. and Monfort, S. L. 1996b, 'Social stress and dominance', *Nature*, vol. 379, no. 6562, pp. 212-212.
Creel, S., Creel, N. M. and Monfort, S. L. 1997b, 'Radiocollaring and stress hormones in African Wild Dogs', *Conservation Biology*, vol. 11, no. 2, pp. 544-548.
Creel, S., Creel, N. M. and Monfort, S. L. 1998, 'Birth order, estrogens and sex-ratio adaptation in African Wild Dogs (Lycaon pictus)', *Animal Reproduction Science*, vol. 53, pp. 315-320.
Creel, S., Creel, N. M., Munson, L., Sanderlin, D. and Appel, M. J. 1997c, 'Serosurvey for selected viral diseases and demography of African Wild Dogs in Tanzania', *Journal of Wildlife Disease*, vol. 33, no. 4, pp. 823-32.

Creel, S., Mills, M. G. L. and McNutt, J. W. 2004, 'Demography and population dynamics of African Wild Dogs in three critical populations', in *Biology and Conservation of Wild Canids*, ed. D. W. Macdonald and C. Sillero-Zubiri, Oxford University Press, Oxford, pp. 337-352.

Crooks, K. R. and Sanjayan, M. (ed), 2006, *Connectivity Conservation*, Conservation biology series, Cambridge University Press, Cambridge, UK.

Dantzer, B., Fletcher, Q. E., Boonstra, R. and Sheriff, M. J. 2014, 'Measures of physiological stress: a transparent or opaque window into the status, management and conservation of species?', *Conservation Physiology*, vol. 2, no. 1, cou023.

Davies-Mostert, H. T., Mills, M. G. L. and Macdonald, D. W. 2009, 'A critical assessment of South Africa's managed metapopulation recovery strategy for African Wild Dogs', in *Reintroduction of Top-Order Predators*, ed. M. W. Haywood and M. J. Somers, Blackwell Publishing Ltd, UK, pp. 10-42.

de Almeida Curi, N., Araújo, A., Campos, F., Lobato, Z., Gennari, S., Marvulo, M., Silva, J. and Talamoni, S. 2010, 'Wild canids, domestic dogs and their pathogens in southeast Brazil: Disease threats for canid conservation', *Biodiversity and Conservation*, vol. 19, no. 12, pp. 3513-3524.

De Castro, F. and Bolker, B. 2005, 'Mechanisms of disease-induced extinction', *Ecology Letters*, vol. 8, no. 1, pp. 117-126.

de Magalhaes, J. P. and Costa, J. 2009, 'A database of vertebrate longevity records and their relation to other life-history traits', *Journal of Evolutionary Biology*, vol. 22, no. 8, pp. 1770-1774.

De Villiers, M. S., Meltzer, D. G., van Heerden, J., Mills, M. G., Richardson, P. R. and van Jaarsveld, A. S. 1995, 'Handling-induced stress and mortalities in African Wild Dogs (*Lycaon pictus*)', *Proceedings of the Royal Society B: Biological Sciences*, vol. 262, no. 1364, pp. 215-20.

De Villiers, M. S., van Jaarsveld, A. S., Meltzer, D. G. A. and Richardson, P. R. K. 1997, 'Social dynamics and the cortisol response to immobilization stress of the African Wild Dog, *Lycaon pictus*', *Hormones and Behavior*, vol. 31, no. 1, pp. 3-14.

Decaro, N. and Buonavoglia, C. 2012, 'Canine parvovirus - A review of epidemiological and diagnostic aspects, with emphasis on type 2c', *Veterinary Microbiology*, vol. 155, pp. 1-12.

Decaro, N., Desario, C., Elia, G., Campolo, M., Lorusso, A., Mari, V., Martella, V. and Buonavoglia, C. 2007, 'Occurrence of severe gastroenteritis in pups after canine parvovirus vaccine administration: A clinical and laboratory diagnostic dilemma', *Vaccine*, vol. 25, no. 7, pp. 1161-1166.

DEFRA 2010, *Vaccination as a control tool for exotic animal disease - key considerations*, Department for Environment, Food and Rural Affairs, London, UK, [Online], Available: www.defra.gov.uk [28th December 2012].

Ditchkoff, S. 2006, 'Animal behavior in urban ecosystems: Modifications due to human-induced stress', *Urban ecosystems*, vol. 9, no. 1, pp. 5-12.

Dronjak, S., Jezova, D. and Kvetnansky, R. 2004, 'Different effects of novel stressors on sympathoadrenal system activation in rats exposed to long-term immobilization', *Annals of the New York Academy of Sciences*, vol. 1018, no. 1, pp. 113-123.

Durchfeld, B., Baumgärtner, W., Herbst, W. and Brahm, R. 1990, 'Vaccine-associated canine distemper infection in a litter of African Hunting Dogs (*Lycaon pictus*)', *Journal of Veterinary Medicine Series B*, vol. 37, no. 1-10, pp. 203-212.

East, M. L., Hofer, H. and Burrows, R. 1997, 'Stress hormones and radiocollaring of African Wild Dogs', *Conservation Biology*, vol. 11, no. 6, pp. 1451-1453.

EAZWV 2002, *Transmissible disease fact sheet, parvovirus infections*, Sheet No. 64, Kai Frölich, Institute for Zoo and Wildlife Research, Berlin, Germany.

Edwards, K. M. 2001, 'Development, acceptance, and use of immunologic correlates of protection in monitoring the effectiveness of combination vaccines', *Clinical Infectious Diseases*, vol. 33, no. Supplement 4, pp. S274-S277.

Estes, R. and Goddard, J. 1967, 'Prey selection and hunting behavior of the African Wild Dog', *Journal of Wildlife Management*, vol. 31, no. 1, pp. 52-70.

Ewen, J. G., Acevedo-Whitehouse, K., Alley, M. R., Carraro, C., Sainsbury, A. W., Swinnerton, K. and Woodroffe, R. 2012, 'Empirical consideration of parasites and health in reintroduction', in *Reintroduction Biology: Intergrating Science and Management*, ed. J. G. Ewen, D. P. Armstrong, K. A. Parker and J. Seddon, John Wiley & Sons, Inc., UK, pp. 290-335.

Ewer, R. 1973, *The carnivores (The world naturalist)*, Weidenfeld and Nicolson, London.

Excel 2010, *Software: Microsoft Office Professional Plus 2010 v14.07*, Microsoft Corporation, California, USA.

Fanshawe, J. H. and Fitzgibbon, C. D. 1993, 'Factors influencing the hunting success of an African Wild Dog pack', *Animal Behaviour*, vol. 45, no. 3, pp. 479-490.

Fanshawe, J. H., Frame, L. H. and Ginsberg, J. R. 1991, 'The Wild Dog: Africa's vanishing carnivore', *Oryx*, vol. 25, no. pp. 137-146.

Fanshawe, J. H., Ginsberg, J. R., Sillero-Zubiri, C. and Woodroffe, R. 1997, 'The status & distribution of remainin wild dog populations', in *The African Wild Dog - Status Survey and Conservation Action Plan*, ed. R. Woodroffe, J. R. Ginsberg, D. W. Macdonald and I. S. C. S. Group, IUCN, Gland, Switzerland.

Feldhamer, G. A., Drickamer, L. C., Vessey, S. H., Merritt, J. F. and Krajewski, C. 2007, *Mammalogy: Adaption, diversity, ecology*, 3rd Edition, The John Hopkins University Press, Baltimore, Maryland, USA.

Feldman, E. C. and Nelson, R. W. (ed), 2004, *Canine and feline endocrinology and reproduction*, Third Edition, Saunders, Missouri, USA.

Ferrari, E., Cravello, L., Muzzoni, B., Casarotti, D., Paltro, M., Solerte, S. B., Fioravanti, M., Cuzzoni, G., Pontiggia, B. and Magri, F. 2001, 'Age-related changes of the hypothalamic-pituitary-adrenal axis: pathophysiological correlates', *European Journal of Endocrinology*, vol. 144, no. pp. 319-329.

Fine, P. E. M. 1993, 'Herd immunity: History, theory, practice', *Epidemiologic Reviews*, vol. 15, no. 2, pp. 265-302.

Fiorello, C. V., Deem, S. L., Gompper, M. E. and Dubovi, E. J. 2004, 'Seroprevalence of pathogens in domestic carnivores on the border of Madidi National Park, Bolivia', *Animal Conservation*, vol. 7, pp. 45-54.

Flacke, G., Becker, P., Cooper, D., Szykman Gunther, M., Robertson, I., Holyoake, C., Donaldson, R. and Warren, K. 2013, 'An infectious disease and mortality survey in a population of free-ranging African Wild Dogs and sympatric domestic dogs', *International Journal of Biodiversity*, vol. 2013, pp. 9.

Fluss, R., Faraggi, D. and Reiser, B. 2005, 'Estimation of the Youden Index and its associated cutoff point', *Biom J*, vol. 47, no. 4, pp. 458-72.

Frame, L. H., Malcolm, J. R., Frame, G. W. and van Lawick, H. 1979, 'Social organisation of African Wild Dogs (*Lycaon pictus*) on the Serengeti Plains, Tanzania 1967-1978', *Zeitschrift für Tierpsychologie*, vol. 50, no. pp. 225-249.

Frantzen, M. A. J., Ferguson, J. W. H. and de Villiers, M. S. 2001, 'The conservation role of captive African Wild Dogs (*Lycaon pictus*)', *Biological Conservation*, vol. 100, no. 2, pp. 253-260.

Frölich, K., Streich, W. J., Fickel, J., Jung, S., Truyen, U., Hentschke, J., Dedek, J., Prager, D. and Latz, N. 2005, 'Epizootiologic investigations of parvovirus infections in free-ranging carnivores from Germany', *Journal of Wildlife Diseases*, vol. 41, no. 1, pp. 231-235.

Fuller, T. K. 1995, 'An international review of large carnivore conservation status', in *Integrating People and Wildlife for a Sustainable Future*, ed. J. A. Bissonette and P. R. Krausman, The Wildlife Society, Bethesda, Maryland, pp. 410-412.

Fuller, T. K. and Kat, P. W. 1993, 'Hunting success of African Wild Dogs in Southwestern Kenya', *Journal of Mammalogy*, vol. 74, no. 2, pp. 464-467

Fuller, T. K., Kat, P. W., Bulger, J. B., Maddock, A. H., Ginsberg, J. R., Burrows, R., McNutt, J. W. and Mills, M. G. L. 1992, 'Population dynamics of African Wild Dogs', in *Wildlife 2001: Populations*, ed. D. R. McCullough and R. H. Barrett, Elsevier Science Publishers, London, pp. 1125-1139.

Gascoyne, S. C., King, A. A., Laurenson, M. K., Borner, M., Schildger, B. and Barrat, J. 1993a, 'Aspects of rabies infection and control in the conservation of the African Wild Dog (*Lycaon pictus*) in the Serengeti Region, Tanzania', *Onderstepoort Journal of Veterinary Research*, vol. 60, no. 4, pp. 415-20.

Gascoyne, S. C., Laurenson, M. K., Lelo, S. and Borner, M. 1993b, 'Rabies in African Wild Dogs (Lycaon pictus) in the Serengeti region, Tanzania', *Journal of Wildlife Disease*, vol. 29, no. 3, pp. 396-402.

George Adamson Trust 1996, *Veterinary Report 1996*, [Online], Available: http://www.georgeadamson.org/downloads/vet_96.pdf [27 May 2012].

Gibert, P. 1993, 'Consequences de la capture et des manipulations sur la physiologie des ongules sauvages. Incidence pathologique. Bilan et connaissances.', in *Techniques de capture et de marquage des ongule´s sauvages*, ed. D. Dubray, FDC de l'He´ rault, Montpellier, France, pp. 169-177.

Ginsberg, J. R. 1993, 'Mapping Wild Dogs', *Canid News*, vol. 1, pp. 2-6.

Ginsberg, J. R. 1994a, 'Captive breeding, reintroduction and the conservation of canids', in *Creative conservation: Interactive Management of Wild and Captive Animals*, ed. P. J. S. Olney, G. M. Mace and A. T. C. Feistner, Chapman & Hall, London, pp.

Ginsberg, J. R. 1994b, 'Conservation biology and status of the African Wild Dog, *Lycaon pictus*', *Endangered Species Update*, vol. 11, no. 10, pp. 2-5.

Ginsberg, J. R., Alexander, K. A., Creel, S., Kat, P. W., McNutt, J. W. and Mills, M. G. L. 1995a, 'Handling and survivorship of African Wild Dog (*Lycaon pictus*) in five ecosystems', *Conservation Biology*, vol. 9, no. 3, pp. 665-674.

Ginsberg, J. R., Mace, G. M. and Albon, S. 1995b, 'Local extinction in a small and declining population: Wild Dogs in the Serengeti', *Proceedings of the Royal Society of London. Series B: Biological Sciences*, vol. 262, no. 1364, pp. 221-228.

Girman, D. J., Kat, P. W., Mills, M. G. L., Ginsberg, J. R., Borner, M., Wilson, V., Fanshawe, J. H., Fitzgibbon, C., Lau, L. M. and Wayne, R. K. 1993, 'Molecular genetic and morphological analyses of the African Wild Dog (Lycaon pictus)', *The Journal of Heredity*, vol. 84, no. 6, pp. 450-459.

Girman, D. J., Mills, M. G. L., Geffen, E. and Wayne, R. K. 1997, 'A molecular genetic analysis of social structure, dispersal, and interpack relationships of the African Wild Dog (*Lycaon pictus*)', *Behavioral Ecology and Sociobiology*, vol. 40, no. 3, pp. 187-198.

Girman, D. J., Vila, C., Geffen, E., Creel, S., Mills, M. G. L., McNutt, J. W., Ginsberg, J. R., Kat, P. W., Mamiya, H. and Wayne, R. K. 2001, 'Patterns of population subdivision, gene flow and genetic variability in the African Wild Dog (*Lycaon pictus*)', *Molecular Ecology*, vol. 10, pp. 1703-1723.

Girman, D. J. and Wayne, R. K. 1997, 'Genetic perspectives on Wild Dog conservation', in *The African Wild Dog - Status Survey and Conservation Action Plan*, ed. R. Woodroffe, J. R. Ginsberg, D. W. Macdonald and I. S. C. S. Group, IUCN, Gland, Switzerland, pp. 7-10.

Gittleman, J. L., Funk, S. M., Macdonald, D. W. and Wayne, R. K. (ed), 2001, *Carnivore Conservation*, Zoological Society of London, Cambridge University Press, Cambridge, UK.

Goddard, A. and Leisewitz, A. L. 2010, 'Canine Parvovirus', *The Veterinary clinics of North America. Small animal practice*, vol. 40, no. 6, pp. 1041-1053.

Goddard, R. D., Nicholas, R. A. and Luff, P. R. 1990, 'Inactivated canine parvovirus vaccines: an alternative method for assessment of potency', *Veterinary Record*, vol. 126, no. 20, pp. 497-499.

Gog, J., Woodroffe, R. and Swinton, J. 2002, 'Disease in endangered metapopulations: The importance of alternative hosts', *Proceedings of the Royal Society London B*, vol. 269, pp. 671-676.

Goller, K. V., Fyumagwa, R. D., Nikolin, V., East, M. L., Kilewo, M., Speck, S., Müller, T., Matzke, M. and Wibbelt, G. 2010, 'Fatal Canine Distemper Infection in a Pack of African Wild Dogs in the Serengeti Ecosystem, Tanzania', *Veterinary Microbiology*, vol. In Press, Corrected Proof, no. pp.

Gordon, J. C. and Angrick, E. J. 1986, 'Canine parvovirus: Environmental effects on infectivity', *American Journal of Veterinary Research*, vol. 47, no. 7, pp. 1464-7.

Goswami, A. and Friscia, A. 2010, *Carnivoran evolution: New views on phylogeney, form and function,* Cambridge University Press, UK.

Goy-Thollot, I., Decosne-Junot, C. and Bonnet, J. M. 2007, 'Influence of aging on adrenal responsiveness in a population of eleven healthy beagles', *Research in Veterinary Science*, vol. 82, pp. 195-201.

Goymann, W. 2005, 'Noninvasive monitoring of hormones in bird droppings: Physiological validation, sampling, extraction, sex differences, and the influence of diet on hormone metabolite levels', *Annals of the New York Academy of Sciences*, vol. 1046, no. 1, pp. 35-53.

Goymann, W. 2012, 'On the use of non-invasive hormone research in uncontrolled, natural environments: the problem with sex, diet, metabolic rate and the individual', *Methods in Ecology and Evolution*, vol. 3, no. 4, pp. 757-765.

Goymann, W., East, M. L., Hofer, H., Mostl, E. and Van'T Hof, T. 1999, 'Noninvasive fecal monitoring of glucocorticoids in spotted hyenas, Crocuta crocuta', *General and Comparative Endocrinology*, vol. 114, pp. 340-348.

Graham, L. H. and Brown, J. L. 1996, 'Cortisol metabolism in the domestic cat and implications for non-invasive monitoring of adrenocortical function in endangered felids', *Zoo Biology*, vol. 15, no. 1, pp. 71-82.

Graph Pad Software Inc. 2013, *Graph Pad Prism*, v. 6.03, USA

Greene, C. E. (ed), 2012, *Infectious diseases of the dog and Cat*, 4th Edition, Elsevier Saunders, Missouri, USA.

Greiner, M., Sohr, D. and Gobel, P. 1995, 'A modified ROC analysis for the selection of cut-off values and the definition of intermediate results of serodiagnostic tests', *J Immunol Methods*, vol. 185, no. 1, pp. 123-32.

Grissom, N. and Bhatnagar, S. 2009, 'Habituation to repeated stress: get used to it', *Neurobiology of learning and memory*, vol. 92, no. 2, pp. 215-224.

Gupta, P. K. 2007, *Cytogenetics*, Rakesh Kumar Rastogi, New Delhi, India. 305

Gusset, M., Stewart, G. B., Bowler, D. E. and Pullin, A. S. 2010, 'Wild Dog Reintroductions in South Africa: A systematic review and cross-validation of an endangered species recovery programme', *Journal for Nature Conservation*, vol. 18, no. 3, pp. 230-234.

Gusset, M., Swarner, M. J., Mponwane, L., Keletile, K. and McNutt, J. W. 2009, 'Human-wildlife conflict in Northern Botswana: Livestock predation by endangered African Wild Dog *Lycaon pictus* and other carnivores', *Oryx*, vol. 43, no. 1, pp. 67-72.

Halliwell, B. 2007, 'Biochemistry of oxidative stress', *Biochemical Society Transactions*, vol. 35, no. 5, pp. 1147-1151.

Hämäläinen, A., Heistermann, M., Fenosoa, Z. S. E. and Kraus, C. 2014, 'Evaluating capture stress in wild gray mouse lemurs via repeated fecal sampling: Method validation and the influence of prior experience and handling protocols on stress responses', *General and Comparative Endocrinology*, vol. 195, pp. 68-79.

Harper, J. M. and Austad, S. N. 2000, 'Fecal glucocorticoids: A noninvasive method of measuring adrenal activity in wild and captive rodents', *Physiological and Biochemical Zoology*, vol. 73, no. 1, pp. 12-22.

Haydon, D. T., Laurenson, M. K. and Sillero Zubiri, C. 2002, 'Integrating epidemiology into population viability analysis: Managing the risk posed by rabies and canine distemper to the Ethiopian wolf', *Conservation Biology*, vol. 16, pp. 1372-1385.

Haydon, D. T., Randall, D. A., Matthews, L., Knobel, D. L., Tallents, L. A., Gravenor, M. B., Williams, S. D., Pollinger, J. P., Cleaveland, S., Woolhouse, M. E. J., Sillero-Zubiri, C., Marino, J., Macdonald, D. W. and Laurenson, M. K. 2006, 'Low-coverage vaccination strategies for the conservation of endangered species', *Nature*, vol. 443, pp. 692-696.

Hayward, M. W., Kerley, G. I. H., Adendorff, J., Moolman, L. C., O'Brien, J., Sholto-Douglas, A., Bissett, C., Bean, P., Fogarty, A., Howarth, D. and Slater, R. 2007, 'The reintroduction of large carnivores to the Eastern Cape, South Africa: An Assessment', *Oryx*, vol. 41, pp. 205-214.

Hayward, M. W., O'Brien, J., Hofmeyr, M. and Kerley, G. I. 2006, 'Prey preferences of the African Wild Dog *Lycaon pictus* (Canidae: Carnivora): Ecological requirements for conservation', *Journal of Mammalogy*, vol. 87, no. 6, pp. 1122-1131.

Hayward, M. W. and Somers, M. 2009, *Reintroduction of Top-Order Predators*, Blackwell Publishing Ltd, UK.

Hennessy, M. B., Davis, H. N., Williams, M. T., Mellott, C. and Douglas, C. W. 1997, 'Plasma cortisol levels of dogs at a county animal shelter', *Physiology & Behavior*, vol. 62, no. pp. 485-490.

Hoelzer, K. and Parrish, C. R. 2010, 'The emergence of parvoviruses of carnivores', *Veterinary Research*, vol. 41, no. 6, pp. 39-52.

Hofmeyr, M., Bingham, J., Lane, E. P., Ide, A. and Nel, L. 2000, 'Rabies in African Wild Dogs (*Lycaon pictus*) in the Madikwe Game Reserve, South Africa', *Veterinary Record*, vol. 146, no. 2, pp. 50-52.

Hofmeyr, M., Hofmeyr, D., Nel, L. and Bingham, B. 2004, 'A second outbreak of rabies in African Wild Dogs *Lycaon pictus* in Madikwe Game Reserve, South Africa, Demonstrating the efficacy of vaccination against natural rabies challenge', *Animal Conservation*, vol. 7, no. 2, pp. 193-198.

Hogg, C., Pemberton, D., Fox, S. and Grueber, C. 2016, *Tasmanian Devil Metapopulation Framework 2016-2020*, Metapopulation Advisory Committee, Save the Devil Program, Hobart, Tamsmania. pp. 14.

Hõrak, P. and Cohen, A. 2010, 'How to measure oxidative stress in an ecological context: Methodological and statistical issues', *Functional Ecology*, vol. 24, no. 5, pp. 960-970.

Hõrak, P., Saks, L., Zilmer, M., Karu, U. and Zilmer, K. 2007, 'Do dietary antioxidants alleviate the cost of immune activation? An experiment with greenfinches', *The American Naturalist*, vol. 170, no. 4, pp. 625-635.

Houston, D. M., Ribble, C. S. and Head, L. L. 1996, 'Risk factors associated with parvovirus enteritis in dogs: 283 cases (1982-1991)', *Journal of American Vetinary Medicine Association*, vol. 208, no. 4, pp. 542-546.

IUCN 2002, *IUCN Technical Guidelines on the Management of Exsitu Population for Conservation*, 14th Meeting of the Programme Committee if Council, Gland Switzerland.

IUCN 2009, *Mammals on the IUCN Red List*, [Online], Available: http://www.iucnredlist.org/mammals [24th October 2009].

IUCN 2017a, *Carnivora on the IUCN Red List*, [Online], Available: http://discover.iucnredlist.org/ [23rd August 2017].

IUCN 2017b, *Mammals on the IUCN Red List*, [Online], Available: http://www.iucnredlist.org/initiatives/mammals/ [23rd August 2017].

IUCN/SSC 2007a, *Regional Conservation Strategy for the Cheetah and African Wild Dog in Eastern Africa*, Gland, Switzerland.

IUCN/SSC 2007b, *Regional Conservation Strategy for the Cheetah and African Wild Dog in Southern Africa*, Gland, Switzerland.

IUCN/SSC 2012, *Regional Conservation Strategy for the Cheetah and African Wild Dog in Western, Central and Northern Africa*, Gland, Switzerland.

Jackson, A. 2010, 'Role of oxidative stress in rabies virus infection of adult mouse dorsal root ganglion neurons', *Journal of virology*, vol. 84, no. 9, pp. 4697-705.

Jessup, D. A. 1999, 'Capture and handling of mountain sheep and goats', in *Zoo and Wildlife Animal Medicine: Current Therapy*, ed. M. E. Fowler and R. E. Miller, Volume 4, W. B. Saunders Company, Philadelphia, USA, pp. 681-687.

Joseph, M. B., Mihaljevic, J. R., Arellano, A. L., Kueneman, J. G., Preston, D. L., Cross, P. C. and Johnson, P. T. J. 2013, 'Taming wildlife disease: Bridging the gap between science and management', *Journal of Applied Ecology*, vol. 50, no. 3, pp. 702-712.

Jones, C. G. and Merton, D. V. 2012, 'A tale of two islands: The rescue and recovery of endemic birds in New Zealand and Mauritius', in *Reintroduction Biology: Integrating Science and Management*, ed. J. G. Ewen, D. P. Armstrong, K. A. Parker and P. J. Seddon, Blackwell Publishing Ltd, West Sussex, UK, pp. 33-72.

Joost, P. 2010, 'Vaccination of non-domestic carnivores: A review', in *Transmissible Diseases Handbook*, ed. J. Kaandorp, European Association of Zoo and Wildlife Veterinarians, Amsterdam, The Netherlands, pp. 30.

Juster, R. P., McEwen, B. S. and Lupien, S. J. 2010, 'Allostatic load biomarkers of chronic stress and impact on health and cognition', *Neuroscience Biobehavior Review*, vol. 35, no. 1, pp. 2-16.

Kamler, J. F., Davies-Mostert, H. T., Hunter, L. and Macdonald, D. W. 2007, 'Predation on black-backed jackals (*Canis mesomelas*) by African Wild Dogs (*Lycaon pictus*)', *African Journal of Ecology*, vol. 45, no. 4, pp. 667-668.

Kat, P. W., Alexander, K. A., Smith, J. S. and Munson, L. 1995, 'Rabies and African Wild Dogs in Kenya', *Proceedings of the Royal Society of London - B. Biological Sciences*, vol. 262, no. 1364, pp. 229-33.

Kat, P. W., Alexander, K. A., Smith, J. S., Richardson, J. D. and Munson, L. 1996, 'Rabies among African Wild Dogs (*Lycaon pictus*) in the Masai Mara, Kenya', *Journal of Veterinary Diagnostic Investigation*, vol. 8, pp. 420-426.

KAZA 2014, *Conservation Strategy and Action Plan for the African Wild Dog (Lycaon pictus) in the Kavango Zambezi Transfrontier Conservation Area*, KAZA TFCA Secretariat, Kasane, Botswana.

Keller-Wood, M. E., Shinsako, J. and Dallman, M. F. 1983, 'Integral as well as proportional adrenal responses to ACTH', *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, vol. 245, no. 1, pp. R53.

Kelly, W. R. 1978, 'An enteric disease of dogs resembling feline panleucopaenia', *Australian Veterinary Journal*, vol. 54, no. 12, pp. 593-593.

Kingdon, J. 1997, *The Kingdon Field Guide to African Mammals*, Academic Press, San Diego, USA.

Kirk, R. W., Bistner, S. I. and Ford, R. B. 1990, *Handbook of Veterinary Procedures & Emergency Treatment*, 5th Editiion, W. B. Saunders Company, Inc., USA.

Knobel, D. L., Liebenberg, A. and Du Toit, J. T. 2003, 'Seroconversion in captive African Wild Dogs (*Lycaon pictus*) following administration of a chicken head bait/SAG-2 oral rabies vaccine combination', *The Onderstepoort Journal of Veterinary Research*, vol. 70, no. 1, pp. 73-77.

Kolevská, J., Brunclík, V. and Svoboda, M. 2003, 'Circadian rhythm of cortisol secretion in dogs of different daily activities', *Acta Veterinaria Brno*, vol. 72, no. 4, pp. 599-605.

Koptopoulos, G., Papadopoulos, O., Papanastasopoulou, M. and Cornwell, H. J. 1986, 'Presence of antibody cross-reacting with canine parvovirus in the sera of dogs from Greece', *The Veterinary Record*, vol. 118, no. 12, pp. 332-333.

Kumar, R. and Indrayan, A. 2011, 'Receiver Operating Characteristic (ROC) curve for medical researchers', *Indian Pediatrics*, vol. 48, pp. 277-287.

Lamglait, B., Trunet, E. and Leclerc, A. 2015, 'Retrospective study of mortality of captive African Wild Dogs (*Lycaon pictus*) in a French Zoo (1974-2013)', *Journal of Zoo and Aquarium Research*, vol. 3, no. 2, pp. 47-51.

Lane, L. V. and Rizzi, T. E. 2010, Low-dose dexamethasone suppression testing for hyperadrenocorticism, *Consultant on Call, NAVC Clinician's Brief*, Oklahoma State University, USA. pp. 69-73.

Laurenson, K., Sillero-Zubiri, C., Thompson, H., Shiferaw, F., Thirgood, S. and Malcolm, J. 1998, 'Disease as a threat to endangered species: Ethiopian Wolves, domestic dogs and canine pathogens', *Animal Conservation*, vol. 1, pp. 273 - 280.

Laurenson, K., Van Heerden, J., Stander, P. and Van Vuuren, M. J. 1997, 'Seroepidemiological survey of sympatric domestic and Wild Dogs (*Lycaon pictus*) in Tsumkwe District, North-eastern Namibia', *Onderstepoort Journal of Veterinary Research*, vol. 64, pp. 313-316.

Laurenson, M. K., Cleaveland, S., Artois, M. and Woodroffe, R. 2004, 'Assessing and managing infectious disease threats to canids', in *Canids: Foxes, Wolves, Jackals and Dogs. Status Survey and Conservation Action Plan*, ed. C. Sillero-Zubiri, M. Hoffmann and D. W. Macdonald, IUCN/SSC Canid Specialist Group, Gland, Switerland and Cambridge, UK, pp. 246-256.

Lees, C., Andrew, P., Bradley, K. and Ford, C. 2016, *Greater Bilby (Macrotis lagotis) Metapopulation Plan - Draft*, National recovery plan for the Greater Bilby, Taronga Conservation Soiciety, NSW. pp. 12.

Lindsell, J. 2011, 'The impact of civil war on forest wildlife in West Africa: Mammals in Gola Forest, Sierra Leone', *Oryx*, vol. 45, no. 1, pp. 69-77.

Lindsey, P. A., Alexander, R., Du Toit, J. T. and Mills, M. G. L. 2005a, 'The cost efficiency of Wild Dog conservation in South Africa', *Conservation Biology*, vol. 19, no. 4, pp. 1205-1214.

Lindsey, P. A., du Toit, J. T. and Mills, M. G. L. 2005b, 'Attitudes of ranchers towards African Wild Dogs *Lycaon pictus*: Conservation implications on private land', *Biological Conservation*, vol. 125, no. 1, pp. 113-121.

Ling, M., Norris, J. M., Kelman, M. and Ward, M. P. 2012, 'Risk Factors for Death from Canine Parvoviral-related Disease in Australia', *Veterinary Microbiology,* vol. 158, no. 3-4, pg. 280-290.

Linneaus, C. 1758, *Systema Naturae per regna tri naturae, secundum classis, ordines, genera, species cum characteribus, differentiis, synonymis locis*, 10th Ed, Stockholm.

Lubroth, J., Rweyemamu, M. M., Viljoen, G., Diallo, A., Dungu, B. and Amanfu, W. 2007, 'Veterinary vaccines and their use in developing countries', *Revue scientifique et technique (International Office of Epizootics)*, vol. 26, no. 1, pp. 179.

Macbeth, B. J., Cattet, M. R. L., Stenhouse, G. B., Gibeau, M. L. and Janz, D. M. 2010, 'Hair cortisol concentration as a noninvasive measure of long-term stress in free-ranging Grizzly Bears (*Ursus arctos*): Considerations with Implications for other wildlife', *Canadian Journal of Zoology*, vol. 88, pp. 935.

Macdonald, D. W. 1993, 'Rabies and wildlife: a conservation problem?', *Onderstepoort J Vet Res*, vol. 60, no. 4, pp. 351-355.

Macdonald, D. W., Artois, M., Aubert, M., Ginsberg, J. R., Kock, N. and Perry, B. D. 1992, 'Cause of wild dog deaths', *Nature*, vol. 360, pp. 633-34.

Macdonald, D. W. and Sillero-Zubiri, C. 2004, *Biology and Conservation of Wild Canids*, Oxford University Press, Oxford.

Macintire, D. K. and Smith-Carr, S. 1997, 'Canine parvovirus. Part II. Clinical signs, diagnosis, and treatment', *Compendium on Continuing Education for the Practicing Veterinarian*, vol. 19, no. 3, pp. 291-302.

Maia, O. B. and Gouveia, A. M. 2001, 'Serologic response of Maned Wolves (*Chrysocyon brachyurus*) to canine and canine parvovirus vaccination distemper virus', *Journal of Zoo and Wildlife Medicine*, vol. 32, pp. 78-80.

Malcolm, J. R. 1999, 'African Wild Dog', in *The Encyclopedia of Mammals*, ed. D. W. Macdonald, 2nd Edition, Andromeda Oxford Limited, Oxford, UK, pp. 56-61.

Malcolm, J. R. and Marten, K. 1982, 'Natural selection and the communal rearing of pups in African Wild Dogs (*Lycaon pictus*)', *Behavioral Ecology Sociobiology*, vol. 10, no. 1,pp. 1-13

Malcolm, J. R. and Sillero-Zubiri, C. 2001, 'Recent records of African Wild Dog (*Lycaon pictus*) from Ethiopia', *Canid News*, vol. 4 [online] URL: http://www.canids.org/canidnews/4/ethiopian_wild_dog.htm.

Mandelker, L. 2011, 'Oxidative stress, free radicals and cellular damage', in *Studies on Veterinary Medicine: Oxidative Stress in Applied Research and Clinical Practice*, ed. D. Armstrong, Springer Science, New York, USA, pp. 1-18.

Mann, P. C., Bush, M. and Appel, M. J. G. 1980, 'Canine parvovirus infection in South American canids', *Journal of the American Veterinary Medical Association*, vol. 177, pp. 779-783.

Marsden, C. D., Mable, B. K., Woodroffe, R., Rasmussen, G. S. A., Cleaveland, S., McNutt, J. W., Emmanuel, M., Thomas, R. and Kennedy, L. J. 2009, 'Highly endangered African Wild Dogs (*Lycaon pictus*) lack variation at the major histocompatibility complex', *The Journal of Heredity*, vol. 100, no. 1, pp. 54-65.

Marsden, C. D., Verberkmoes, H., Thomas, R., Wayne, R. K. and Mable, B. K. 2013, 'Pedigree, MHC and microsatellites: An intergrated approach for genetic management of captive populations', *Conservation Genetics*, vol. 14, no. 1, pp. 171-183.

Marsden, C. D., Woodroffe, R., Mills, M. G. L., McNutt, J. W., Creel, S., Groom, R., Emmanuel, M., Cleaveland, S., Kat, P., Rasmussen, G. S. A., Ginsberg, J., Lines, R., Andre, J., Begg, C., Wayne, R. K. and Mable, B. K. 2012, 'Spatial and temporal patterns of neutral and adaptive genetic variation in the endangered African Wild Dog (*Lycaon pictus*)', *Molecular Ecology*, vol. 21, no. 6, pp. 1379-1393.

Martella, V., Decaro, N., Elia, G. and Buonavoglia, C. 2005, 'Surveillance activity for canine parvovirus in Italy', *Journal of Veterinary Medicine, Series B*, vol. 52, no. 7-8, pp. 312-315.

Martínez-Navarro, B. and Rook, L. 2003, 'Gradual evolution in the African Hunting Dog lineage systematic implications', *Comptes Rendus Palevol*, vol. 2, no. 8, pp. 695-702.

McCandlish, I. A. P., Thompson, H., Fisher, E. W., Cornwell, H. J. C., Macartney, J. and Walton, I. A. 1981, 'Canine parvovirus infection', *In Practice*, vol. 3, no. 3, pp. 5-14.

McCormick, A. E. 1983, 'Canine distemper in African Cape Hunting Dogs (*Lycaon pictus*) possibly vaccine induced', *Journal of Zoo Animal Medicine*, vol. 14, pp. 66-71.

McCreery, E. K. 2000, 'Spatial relationships as an indicator of successful pack formation in free-ranging african wild dogs', *Behaviour*, vol. 137, no. 5, pp. 579-590.

McEwen, B. S. 2008, 'Central effects of stress hormones in health and disease: Understanding the protective and damaging effects of stress and stress mediators', *European Journal of Pharmacology*, vol. 583, no. 2–3, pp. 174-185.

McEwen, B. S. and Gianaros, P. J. 2011, 'Stress- and allostasis-induced brain plasticity', *Annual review of medicine*, vol. 62, pp. 431-445.

McEwen, B. S. and Stellar, E. 1993, 'Stress and the individual: Mechanisms leading to disease', *Archives of Internal Medicine*, vol. 153, no. 18, pp. 2093-2101.

McGibbon, W. H. 1944, 'Cellular antigens in species and species hybrids in ducks', *Genetics*, vol. 29, no. 5, pp. 407.

McGowan, P. J. K., Traylor-Holzer, K. and Leus, K. 2017, 'IUCN Guidelines for determining when and how ex situ management should be used in species conservation', *Conservation Letters*, vol. 10, no. 3, pp. 361-366.

McNutt, J. W. 1996a, 'Adoption in African Wild Dogs, *Lycaon pictus'*, *Journal of Zoology*, vol. 240, pp. 163-173.

McNutt, J. W. 1996b, 'Sex-biased dispersal in African Wild Dogs, *Lycaon pictus*', *Animal Behaviour*, vol. 52, pp. 1067-1077.

McNutt, J. W., Mills, M. G. L., McCreery, K., Rasmussen, G. S. A., Robbins, R. L. and Woodroffe, R. 2008, *Lycaon pictus*, In: IUCN 2009. IUCN Red List of Threatened Species. Version 2009.2, [Online], Available: www.iucnredlist.org [14th December 2009].

McNutt, J. W. and Silk, J. 2008, 'Pup production, sex ratios, and survivorship in African Wild Dogs, *Lycaon pictus'*, *Behavioral Ecology and Sociobiology*, vol. 62, no. 7, pp. 1061-1067.

Mech, D. 1995, 'Effects of canine parvovirus on Grey Wolves in Minnesota', *Journal of Wildlife Management*, vol. 59, no. 3, pp. 565.

Mech, L., Goyal, S. M., Paul, W. and Newton, W. 2008, 'Demographic effects of canine parvovirus on a free-ranging wolf population over 30 years', *Journal of Wildlife Diseases*, vol. 44, no. 4, pp. 824-836.

Mech, L. D. and Goyal, S. M. 1993, 'Canine parvovirus effect on wolf population change and pup survival', *Journal of Wildlife Diseases*, vol. 29, pp. 330-333.

Meers, J., Kyaw-Tanner, M., Bensink, Z. and Zwijnenberg, R. 2007, 'Genetic analysis of canine parvovirus from dogs in Australia', *Australian Veterinary Journal*, vol. 85, no. 10, pp. 392-396.

Meeusen, E. N. T., Walker, J., Peters, A., Pastoret, P. and Jungersen, G. 2007, 'Current status of veterinary vaccines', *Clinical Microbiology Revies*, vol. 20, no. 3, pp. 489-510.

Mendoza, S. P., Capitanio, J. P. and Mason, W. A. 2000, 'Chronic social stress: Studies in non-human primates', in *The Biology of Animal Stress: Basic Principals and Implications for Animal Welfare*, ed. G. P. Moberg and J. A. Mench, CAB International, Wallingford, pp. 227-248.

MET 2013, *National Conservation Action Plan for the African wild dog in Namibia*, Ministry of Environment and Tourism, Government of Namibia, Namibia.

Miller, B., Dugelby, B., Foreman, D., Martinez del Rio, C., Noss, R. F., Phillips, M., Reading, R. P., Soule, M., Terborgh, J. and Willcox, L. 2001, 'The importance of large carnivores to healthy ecosystems', *Endangered Species Update*, vol. 18, no. 5, pp. 202-211.

Miller, P. S. 2007, 'Tools and techniques for disease risk assessment in threatened wildlife conservation programmes', *International Zoo Yearbook*, vol. 41, no. 1, pp. 38-51.

Miller, R. E. 1996, 'Quarantine protocols and preventive medicine procedures for reptiles, birds and mammals in zoos', *Scientific and Technical Review of the Office International des Epizooties*, vol. 15, no. 1, pp. 183-189.

Miller, R. E. and Fowler, M. E. 2012, *Fowler's Zoo and Wildlife Animal Medicine: Current Therapy*, Volume 7, Saunders Elsevier Inc., Missouri, USA. 662

Mills, M. 2002, *Is a Sixth Extinction Looming?*, African Conservation Foundation, [Online], Available: http://96.30.21.87/forum/research-articles-reports-talks/6377-is-a-sixth-extinction-looming [24th November 2012].

Mills, M. G. L. and Biggs, H. C. 1993, 'Prey apportionment and related ecological relationships between large carnivores in Kruger National Park', *Symposia of the Zoological Society of London*, vol. 65, pp. 253–268.

Mills, M. G. L., Ellis, S., Woodroffe, R., Maddock, A., Stander, P., Rasmussen, A. P., Fletcher, P., Bruford, M., Wildt, D., Macdonald, D. W. and Seal, U. 1998, *Population and Habitat Viability Assessment for the African Wild Dog (Lycaon pictus) in Southern Africa*, Final Report from the Workshop, 13-17 October 1997 in Pretoria, South Africa, IUCN/SSC Conservation Breeding Specialist Group, Apple Valley, USA.

Mills, M. G. L. and Gorman, M. L. 1997, 'Factors affecting the density and distribution of Wild Dogs in the Kruger National Park', *Conservation Biology*, vol. 11, no. 6, pp. 1397-1406.

Millspaugh, J. J. and Washburn, B. E. 2004, 'Use of fecal glucocorticoid metabolite measures in conservation biology research: considerations for application and interpretation', *General and Comparative Endocrinology*, vol. 138, no. 3, pp. 189-99.

Mivart, S. G. J. 1890, *Dogs, Jackals, Wolves and Foxes: A Monograph of the Canidae*, London, pp. 216.

Moberg, G. P. 2000, 'Biological responses to stress: Implications for animal welfare', in *The Biology of Animal Stress: Basic Principals and Implications for Animal Welfare*, ed. G. P. Moberg and J. A. Mench, CAB International, Wallingford, pp. 1-21.

Monfort, S. L., Mashburn, K. L., Brewer, B. A. and Creel, S. 1998, 'Evaluating adrenal activity in African Wild Dogs (*Lycaon pictus*) by fecal corticosteriod analysis', *Journal of Zoo Wildlife Medicine*, vol. 29, no. 2, pp. 129-133.

Monfort, S. L., Wasser, S. K., Mashburn, K. L., Burke, M., Brewer, B. A. and Creel, S. 1997, 'Steroid metabolism and validation of noninvasive endocrine monitoring in the African Wild Dog (*Lycaon pictus*)', *Zoo Biology*, vol. 16, no. 6, pp. 533-548.

Mongillo, P., Prana, E., Gabai, G., Bertotto, D. and Marinelli, L. 2014, 'Effect of age and sex on plasma cortisol and dehydroepiandrosterone concentrations in the dog (*Canis familiaris*)', *Research in Veterinary Science*, vol. 96, no. 1, pp. 33-38.

Montali, R. J., Bartz, C. R. and Bush, M. 1987, 'Canine distemper virus', in *Virus Infections in Carnivores*, ed. M. Appel, Elservier Science, Amsterdam, pp. 437-443.

Montanha, J., Silva, S. and Boere, V. 2009, 'Comparison of Salivary cortisol concentrations in jaguars kept in captivity with differences in exposure to the public', *Ciencia Rural*, vol. 39, no. 6, pp. 1745-1751.

Morgan, K. N. and Tromborg, C. T. 2007, 'Sources of stress in captivity', *Applied Animal Behaviour Science*, vol. 102, pp. 262-302.

Morrison, J. C., Sechrest, W., Dinerstein, E., Wilcove, D. S. and Lamoreux, J. F. 2007, 'Persistence of large mammal faunas as indicators of global human impacts', *Journal of Mammalogy*, vol. 88, no. 6, pp. 13-63-1380.

Mostl, E. and Palme, R. 2002, 'Hormones as indicators of stress', *Domestic Animal Endocrinology*, vol. 23, pp. 67-74.

Murray, D. L., Kapke, C. A., Evermann, J. F. and Fuller, T. K. 1999, 'Infectious disease and the conservation of free-ranging large carnivores', *Animal Conservation*, vol. 2, pp. 241-254.

Nass, S. J. 2009, *Beyond the HIPAA privacy rule enhancing privacy, improving health through research*, Washington, D.C. National Academies Press, Washington, D.C.

Nauta, J. 2010, *Statistics in Clinical Vaccine Trials*, Springer-Verlag, Netherlands.

Ndeereh, D., Obanda, V., Mijele, D. and Gakuya, F. 2012, 'Medicine in the wild: Strategies towards healthy and breeding wildlife population in Kenya', *The George Wright Forum*, vol. 29, no. 1, pp. 100-108.

Newell-Fugate, A. E., Noethling, J. O. and Bertschinger, H. J. 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*, vol. 178, no. 2, pp. 272-281.

NNF 2006, *African Wild Dog Conservation in Namibia, Phase 1 Final Report*, Namibian Nature Foundation: Wild Dog Project, Windhoek, Namibia.

NNF 2009, *Background Information and Species Management Guidelines for Namibia's Rare and Valuable Wildlife*, Namibian Nature Foundation: Wild Dog Project, Windhoek, Namibia.

Novak, M. A., Hamel, A. F., Kelly, B. J., Dettmer, A. M. and Meyer, J. S. 2013, 'Stress, the HPA axis and nonhuman primate well-being: A review', *Applied Animal Behaviour Science*, vol. 143, no. 2, pp. 135-149.

Nowak, R. M. 1999, *Walker's Mammals of the World*, Johns Hopkins University Press, Baltimore.

Nowak, R. M. and Federoff, N. E. 1998, 'Validity of the red wolf: Response to Roy et al', *Conservation Biology*, vol. 12, no. 3, pp. 722-725.

Nunomura, A. 2006, 'Involvement of oxidative stress in Alzheimer disease', *Journal of neuropathology and experimental neurology*, vol. 65, no. 7, pp. 631-41.

Okitsu, S. 2010, 'Vegetation structure of the biomes in southwestern africa and their precipitation patterns', *African Study Monographys*, vol. 40, pp. 77-89.

Olney, P. J. S., Mace, G. M. and Feistner, A. T. C. (ed), 1994, *Creative Conservation: Interactive managment of wild and captive animals*, Springer Science, Salisbury.

Palme, R. 2005, 'Measuring fecal steroids: Guidelines for practical application', *Annals of the New York Academy of Sciences*, vol. 1046, no. 1, pp. 75-80.

Palme, R., Rettenbacher, S., Touma, C., El-Bahr, S. M. and Mostl, E. 2005, 'Stress hormones in mammals and birds: comparative aspects regarding metabolism, excretion, and noninvasive measurement in fecal samples', *Annals of the New York Academy of Science*, vol. 1040, pp. 162-71.

Palmieri, B. and Sblendorio, V. 2007, 'Oxidative stress tests: Overview on reliability and use. Part I', *European Review for Medical and Pharmacological Sciences*, vol. 11, no. 5, pp. 309-42.

Panda, D., Patra, R. C., Nandi, S. and Swarup, D. 2009, 'Oxidative stress indices in gastroenteritis in dogs with canine parvoviral infection', *Research in Veterinary Science*, vol. 86, no. 1, pp. 36-42.

Parrish, C. R., Aquadro, C. F., Strassheim, M. L., Evermann, J. F., Sgro, J. Y. and Mohammed, H. O. 1991, 'Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus', *Journal of Virology*, vol. 65, no. 12, pp. 6544-52.

Parrish, C. R., Have, P., Foreyt, W. J., Evermann, J. F., Senda, M. and Carmichael, L. E. 1988, 'The global spread and replacement of canine parvovirus strains', *Journal of General Virology*, vol. 69, pp. 1111-1116.

Parsons, P. A. 1995, 'Evolutionary response to drought stress: Conservation implications', *Biological Conservation*, vol. 74, no. 1, pp. 21-27.

Pedersen, A. B., Jones, K. E., Nunn, C. L. and Altizer, S. 2007, 'Infectious diseases and extinction risk in wild mammals', *Conservation Biology*, vol. 21, no. 5, pp. 1269-1279.

Pereira, R. J., Granzinolli, M. A. and Duarte, J. M. 2010, 'Annual profile of fecal androgen and glucocorticoid levels in free-living male American kestrels from southern mid-latitude areas', *General Comparative Endocrinology*, vol. 166, no. 1, pp. 94-103.

Peterson, M. E. 2007, 'Diagnosis of hyperadrenocorticism in dogs', *Clinical Techniques in Small Animal Practice*, vol. 22, no. 1, pp. 2-11.

Pfizer 2005, *Parvac™ Vaccine*, Material Safety Data Sheet, Pfizer Australia Pty Ltd, West Ryde, NSW.

Pfizer 2010, *Parvac™ product label*, Pfizer Animal Health, [Online], Available: http://services.apvma.gov.au/PubcrisWebClient/search.do

Plowman, A. B. 2008, 'BIAZA statistics guidelines: Toward a common application of statistical tests for zoo research', *Zoo Biology*, vol. 27, no. 3, pp. 226-233.

Plumb, G., Babiuk, L., Mazet, J., Olsen, S., Pastoret, P., Rupprecht, C. and Slate, D. 2007, 'Vaccination in conservation medicine', *Review of Science and Technology Office International Epizootiology*, vol. 26, no. 1, pp. 229-241.

Pohanka, M. 2013, 'Role of oxidative stress in infectious diseases. A review', *Folia Microbiol (Praha)*, vol. 58, no. 6, pp. 503-13.

Pollock, R. V. H. and Carmichael, L. E. 1983, 'Canine viral enteritis', *Veterinary Clinics of North America: Small Animal Practice*, vol. 13, no. 3, pp. 551-566.

Pollock, R. V. H. and Coyne, M. J. 1993, 'Canine parvovirus', *The Veterinary Clinics of North America. Small Animal Practice*, vol. 23, no. 3, pp. 555-568.

Popelin, F. 2010, Vaccination Des Canides Sauvages en Parc Zoologique, PhD, La Faculte de Medecine de Creteil, Enquête dans les Parcs Zoologiques Européens, France, pp. 276.

Posten, H. O. 1984, 'Robustness of the two-sample T-Test', in *Theory and Decision Library: Robustness of Statistical Methods and Nonparametric Statistics*, ed. D. Rasch and M. L. Tiku, Springer Netherlands, pp. 92-99.

Pratelli, A., Cavalli, A., Martella, V., Tempesta, M., Decaro, N., Carmichael, L. E. and Buonavoglia, C. 2001, 'Canine parvovirus (CPV) vaccination: Comparison of neutralizing antibody responses in pups after inoculation with CPV2 or CPV2b modified live virus vaccine', *Clinical and Diagnostic Laboratory Immunology*, vol. 8, no. 3, pp. 612-615.

Rafacz, M. L. and Santymire, R. M. 2014, 'Using odor cues to elicit a behavioral and hormonal response in zoo-housed African Wild Dogs', *Zoo Biology*, vol. 33, no. 2, pp. 144-9.
Rasmussen, G. S. A. 1996, 'Predation on Bat-eared Foxes *Otocyon megalotis* by Cape Hunting Dogs *Lycaon pictus'*, *Koedoe*, vol. 39, pp. 127-129.
Ray, J. C., Hunter, L. and Zigouris, J. 2005a, *Setting Conservation and Research Priorities for Larger African Carnivores*, WCS Working Paper No. 24. Wildlife Conservation Society, New York.
Ray, J. C., Redford, K. H., Steneck, S. R. and Berger, J. 2005b, *Large Carnivores and the Conservation of Biodiversity*, Island Press, USA.
Reeder, D. 2005, 'Stress in free-ranging Mammals: Integrating physiology, ecology, and natural hstory', *Journal of Mammalogy*, vol. 86, no. 2, pp. 225.
Reich, A. 1981, The behaviour and ecology of the African Wild Dog (*Lycaon pictus*) in the Kruger National Park, PhD Thesis, Faculty of the Graduate School, Yale University, New Haven, USA, pp. 425.
Reul, J. M. H. M., Rothuizen, J. and de Kloet, E. R. 1991, 'Age-related changes in the dog hypothalamic-pituitary-adrenocortical system: Neuroendocrine activity and corticosteroid receptors', *The Journal of Steroid Biochemistry and Molecular Biology*, vol. 40, no. 1–3, pp. 63-69.
Ripple, W. J., Estes, J. A., Beschta, R. L., Wilmers, C. C., Ritchie, E. G., Hebblewhite, M., Berger, J., Elmhagen, B., Letnic, M., Nelson, M. P., Schmitz, O. J., Smith, D. W., Wallach, A. D. and Wirsing, A. J. 2014, 'Status and ecological effects of the world's largest carnivores', *Science*, vol. 343, no. 6167, 1241484.
Robbins, C. T., Schwartz, C. C. and Felicetti, L. A. 2004, 'Nutritional ecology of Ursids: A review of newer methods and management implications', *Ursus*, vol. 15, pp. 161-171.
Romero, L. M. 2004, 'Physiological stress in ecology: Lessons from biomedical research', *Trends in Ecology & Evolution*, vol. 19, no. 5, pp. 249-255.
RWCP 2016, *Greetings from the RWCP*, Newsletter No. 2, May, Range wide Conservation Program for Cheetah and Afrcian Wild Dogs.
RWCP 2017a, *Range wide Conservation Program for Cheetah and African Wild Dogs - Home page*, [Online], Available: http://www.cheetahandwilddog.org/ [8th November 2017].
RWCP 2017b, *Working together to make space for Cheetah and African Wild Dogs*, Range wide Conservation Program for Cheetah and African Wild Dogs, [Online], Available: http://www.cheetahandwilddog.org/ [26th April 2017].
Sabeta, C. T., Mansfield, K. L., McElhinney, L. M., Fooks, A. R. and Nel, L. H. 2007, 'Molecular epidemiology of rabies in bat-eared foxes (*Otocyon megalotis*) in South Africa', Virus Res, vol. 129, no. 1, pp. 1-10.
Sands, J. and Creel, S. 2004, 'Social dominance, aggression and faecal glucocorticoid levels in a wild population of Wolves, *Canis lupus*', *Animal Behaviour*, vol. 67, no. 3, pp. 387-396.
Santymire, R. M. and Armstrong, D. M. 2010, 'Development of a field-friendly technique for fecal steroid extraction and storage using the African Wild Dog (*Lycaon pictus*)', *Zoo Biology*, vol. 29, no. 3, pp. 289-302.
Sapolsky, R. M. 1985, 'Stress-induced suppression of testicular function in the wild Baboon: Role of glucocorticoids', *Endocrinology*, vol. 116, no. 6, pp. 2273-2278.
Sapolsky, R. M. 1990, 'Stress in the wild', *Scienific American*, vol. 262, pp. 106-113.
Sarma, A. 2010, 'Free radicals and their role in different clinical conditions: An overview', *International journal of pharma sciences and research*, vol. 1, no. 3, pp. 185.
Sarrazin, F. and Barbault, R. 1996, 'Reintroduction: Challenges and lessons for basic ecology', *Trends in Ecology & Evolution*, vol. 11, no. 11, pp. 474-478.
Saunders, D. A. 1990, 'The landscape approach to conservation,: Community involvement, the only practical solution', *Australian Zoologist*, vol. 26, no. 2, pp. 49-53.

Schaller, G. B. 1972, *The Serengeti Lion: A Study of Predator-Prey Relations*, University of Chicago Press, Chicago.

Schatz, S. and Palme, R. 2001, 'Measurement of faecal cortisol metabolites in cats and dogs: A non-invasive method for evaluating adrenocortical function', *Veterinary Research Communications*, vol. 25, no. 4, pp. 271-287.

Scheepers, J. L. and Venzke, K. A. E. 1995, 'Attempts to reindroduce African Wild Dogs *Lycaon pictus* into Etosha National Park, Namibia', *South African Journal of Wildlife Research* vol. 25, pp. 138-140.

Schultz, D. J., Rich, B. G., Rohrig, W., McCarthy, P. J., Mathews, B., Schultz, T. J., Corrigan, T. and Taggart, D. A. 2011, 'Investigations into the health of brush-tailed rock-wallabies (*Petrogale penicillata*) before and after reintroduction', *Australian Mammalogy*, vol. 33, no. 2, pp. 235-244.

Schultz, R. D. 2006, 'Duration of immunity for canine and feline vaccines: A review', *Veterinary Microbiology*, vol. 117, no. 1, pp. 75-79.

Schwarzenberger, F. 2007, 'The many uses of non-invasive faecal steroid monitoring in zoo and wildlife species', *International Zoo Yearbook*, vol. 41, pp. 52-74.

Schwarzenberger, F., Palme, R., Bamberg, E. and Mostl, E. 1997, 'A review of faecal progesterone metabolite analysis for non-invasive monitoring of reproductive function in mammals', *International Journal of Mammalian Biology*, vol. 62, no. 2, pp. 214-221.

Scott, F. W. 1980, 'Virucidal disinfectants and feline viruses', *American Journal of Veterinary Research*, vol. 41, no. 3, pp. 410-4.

Selye, H. 1946, 'The general adaption syndrome and the diseases of adaption', *Journal of Clinical Endocrinology*, vol. 6, pp. 117-230.

Sergio, F., Caro, T., Brown, D., Clucas, B., Hunter, J., Ketchum, J., McHugh, K. and Hiraldo, F. 2008, 'Top predators as conservation tools: Ecological rationale, assumptions, and efficacy', *Annual Review of Ecology, Evolution, and Systematics*, vol. 39, no. 1, pp. 1-19.

Shackelton, L. A., Parrish, C. R., Truyen, U. and Holmes, E. C. 2005, 'High rate of viral evolution associated with the emergence of carnivore parvovirus', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 2, pp. 379-384.

Sharma, S. N. and Adlakha, S. C. 2009, *Textbook of Veterinary Virology*, International Book Distributing Co., Lucknow, India.

Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R. and Boonstra, R. 2011a, 'Measuring stress in wildlife: Techniques for quantifying glucocorticoids', *Oecologia*, vol. 166, no. 4, pp. 869-887.

Sheriff, M. J., Krebs, C. J. and Boonstra, R. 2011b, 'From process to pattern: how fluctuating predation risk impacts the stress axis of snowshoe hares during the 10-year cycle', *Oecologia*, vol. 166, no. 3, pp. 593-605.

Shultz, E. K. 1995, 'Multivarate receiver operating characteristic curve analysis: Prostate cancer screening as a example', *Clinical Chemistry*, vol. 41, no. 8, pp. 1248-1255.

Sillero-Zubiri, C., Hoffmann, M. and Macdonald, D. W. (ed), 2004, *Canids: Foxes, Wolves, Jackals and Dogs. Status Survey and Conservation Action Plan*, IUCN/SSC Canid Specialist Group, Gland, Switerland and Cambridge, UK. 430 pp.

Sinclair, A. R. E. 2003, 'Mammal population regulation, keystone processes and ecosystem dynamics', *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 358, no. 1438, pp. 1729-1740.

Skinner, J. D. and Chimimba, C. T. 2005, *The Mammals of Southern Africa*, Cambridge University Press, Singapore.

Smith-Carr, S., Macintire, D. K. and Swango, L. J. 1997, 'Canine parvovirus. Part I. pathogenesis and vaccination', *Compendium on Continuing Education for the Practicing Veterinarian*, vol. 19, no. 2, pp. 125-33.

Soulé, M. E. and Terborgh, J. 1999, 'Conserving nature at regional and continental scales—a scientific program for North America', *BioScience*, vol. 49, no. 10, pp. 809-817.
Spencer, J. and Burroughs, R. 1992, 'Antibody response to canine distemper vaccine in African Wild Dogs', *Journal of Wildlife Disease*, vol. 28, no. 3, pp. 443-444.
Spencer, J. A. 1991, 'Antibody response to modified-live canine adenovirus vaccine in African Hunting Dogs (Lycaon pictus)', *Zentralblatt fur Veterinarmedizin. Reihe B*, vol. 38, pp. 477-479.
Spencer, J. A., Bingham, J., Heath, R. and Richards, B. 1999, 'Presence of antibodies to canine distemper virus, canine parvovirus and canine adenovirus type 1 in free-ranging Jackals (*Canis adustus* and *Canis mesomelas*) in Zimbabwe', *Onderstepoort Journal of Veterinary Research*, vol. 66, pp. 251-253.
Spencer, J. A. and Burroughs, R. E. J. 1990, 'Antibody response in Wild Dogs to canine parvovirus vaccine', *South African Journal of Wildlife Research*, vol. 20, no. 1, pp. 14-15.
Spielman, D., Brook, B. W., Briscoe, D. A. and Frankham, R. 2004, 'Does inbreeding and loss of genetic diversity decrease disease resistance', *Conservation Genetics*, vol. 5, pp. 439-448.
Spiering, P. A., Somers, M. J., Maldonado, J. E., Wildt, D. E. and Gunther, M. S. 2010, 'Reproductive sharing and proximate factors mediating cooperative breeding in the African Wild Dog (*Lycaon pictus*)', *Behavioral Ecology and Sociobiology*, vol. 64, no. 4, pp. 583-592.
Steinel, A., Munson, L., van Vuuren, M. and Truyen, U. 2000, 'Genetic characterization of feline parvovirus sequences from various carnivores', *Journal of General Virology*, vol. 81, no. 2, pp. 345-350.
Steinel, A., Parrish, C. R., Bloom, M. E. and Truyen, U. 2001, 'parvovirus infections in wild carnivores', *Journal Wildlife Disease*, vol. 37, no. 3, pp. 594-607.
Steinel, A., Venter, E. H., Van Vuuren, M., Parrish, C. R. and Truyen, U. 1998, 'Antigenic and genetic analysis of canine parvoviruses in Southern Africa', *Onderstepoort Journal of Veterinary Research*, vol. 65, pp. 239-242.
Sterling, P. and Eyer, J. 1988, 'Allostasis: A new paradigm to explain arousal pathology', in *Handbook of Life Stress, Cognition and Health*, ed. S. Fisher and J. Reason, John Wiley & Sons, USA, pp. 629-650.
Stuart, C. and Stuart, T. 2001, *Field Guide to Mammals of Southern Africa*, 3rd Edition, Struik Publishers, Cape Town, South Africa.
Taylor, C. R., Schmidt-Nielsen, K., Dmi'el, R. and Fedak, M. 1971, 'Effect of hyperthermia on heat balance during running in the African Hunting Dog', *American Journal of Physiology*, vol. 220, no. 3, pp. 823-827.
Terborgh, J., Estes, J., Paquet, P. C., Ralls, K., Boyd-Heger, D., Miller, B. J. and Noss, R. F. 1999, 'The role of top carnivores in regulating terrestrial ecosystems', in *Continential Conservation: Scienific Foundations of Regional Reserve Networks*, ed. M. E. Soule and J. Terborgh, Island Press, Washington, USA, pp. 39-64.
Terborgh, J. and Freeley, K. 2010, *Trophic Cascades: Predators, Prey, and the Changing Dynamics of Nature*, Island Press, Washington, DC. serg
Terio, K. A., Brown, J. L. and Citino, S. B. 1999, 'Fecal cortisol metabolite analysis for noninvasive monitoring of adrenocortical function in the cheetah (Acinonyx jubatus)', *Journal of Zoo and Wildlife Medicine*, vol. 30, pp. 484-491.
Tizard, I. 2013, *Veterinary Immunology*, 9th Edition, Elsevier, China.
Tompkins, D. M., Dunn, A. M., Smith, M. J. and Telfer, S. 2011, 'Wildlife diseases: From individuals to ecosystems', *Journal of Animal Ecology*, vol. 80, no. 1, pp. 19-38.
Touma, C. and Palme, R. 2005, 'Measuring fecal glucocorticoid metabolites in mammals and birds: the importance of validation', *Annals of the New York Academy of Sciences*, vol. 1046, no. 1, pp. 54-74.

Treves, A. and Karanth, K. U. 2003, 'Human-carnivore conflict and perspectives on carnivore management worldwide', *Conservation Biology*, vol. 17, no. 6, pp. 1491-1499.

Treves, A. and Naughton-Treves, L. 1999, 'Risk and opportunity for humans coexisting with large carnivores', *Journal of Human Evolution*, vol. 36, no. 3, pp. 275-82.

Tribe, A. and Spielman, D. 2009, 'Restraint and handling of captive wildlife, fact sheet', *ANZCCART News*, vol. 9, no. 1, pp. 1-8.

Truyen, U. 2006, 'Evolution of canine parvovirus—A need for new vaccines?', *Veterinary Microbiology*, vol. 117, no. 1, pp. 9-13.

Tsao, J., Chapman, M. S., Agbandje, M., Keller, W., Smith, K., Wu, H., Luo, M., Smith, T. J., Rossmann, M. G., Compans, R. W. and Parrish, C. R. 1991, 'the three-dimensional structure of canine parvovirus and its functional implications', *Science*, vol. 251, no. 5000, pp. 1456-1464.

Valdivia, P. A., Zenteno-Savín, T., Gardner, S. C. and Aguirre, A. A. 2007, 'Basic oxidative stress metabolites in eastern Pacific green turtles (Chelonia mydas agassizii)', *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, vol. 146, no. 1–2, pp. 111-117.

Van de Bildt, M. W. G., Kuiken, T., Visee, A. M., Lema, S., Fitzjohn, T. R. and Osterhaus, D. M. E. 2002, 'Distemper outbreak and its effect on African Wild Dog conservation', *Emerging Infectious Diseases*, vol. 8, no. 2, pp. 212-213.

Van den Berghe, F., Paris, D. B., Van Soom, A., Rijsselaere, T., Van der Weyde, L., Bertschinger, H. J. and Paris, M. C. 2012, 'Reproduction in the endangered African Wild Dog: Basic physiology, reproductive suppression and possible benefits of artificial insemination', *Animal Reproduction Science*, vol. 133, no. 1-2, pp. 1-9.

Van der Weyde, L. 2013, Reproductive biology of the endangered African Wild Dog (*Lycaon pictus*) in captive and free-ranging populations: An endocrine, behavioural and demographic approach, Masters Thesis, School of Animal Biology, University of Western Australia, Australia, pp. 118.

Van der Weyde, L. K., Martin, G. B., Blackberry, M. A., Gruen, V., Harland, A. and Paris, M. C. J. 2015, 'Reproductive hormonal patterns in pregnant, pseudopregnant and acyclic captive African wild dogs (Lycaon pictus)', *Animal Reproduction Science*, vol. 156, no. pp. 75-82.

Van der Weyde, L. K., Martin, G. B. and 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*, vol. 226, pp. 50-55.

van Heerden, J., Bainbridge, N., Burroughs, R. E. and Kriek, N. P. 1989, 'Distemper-like disease and encephalitozoonosis in Wild Dogs (*Lycaon pictus*)', *Journal of Wildlife Disease*, vol. 25, no. 1, pp. 70-75.

van Heerden, J., Bingham, J., van Vuuren, M., Burroughs, R. E. and Stylianides, E. 2002, 'Clincal and serological response of Wild Dogs (*Lycaon pictus*) to vaccination against canine distemper and parvovirus', *Journal of the South African Veterinary Association*, vol. 73, no. 1, pp. 8-12.

van Heerden, J. and Kuhn, F. 1985, 'Reproduction in captive Hunting Dogs *Lycaon pictus'*, *South African Journal of Wildlife Research*, vol. 15, no. 3, pp. 80-84.

van Heerden, J., Mills, M. G. L., Van Vuuren, M. J., Kelly, P. J. and Dreyer, M. J. 1995, 'An investigation into the health status and diseases of Wild Dogs (*Lycaon pictus*) in the Kruger National Park', *Journal of the South African Veterinary Association*, vol. 66, pp. 18-27.

van Heerden, J., Swart, W. H. and Meltzer, D. G. A. 1980, 'Serum antibody levels before and after administration of live canine distemper vaccine to the Wild Dog *Lycaon pictus*', *Journal of the South African Veterinary Association*, vol. 51, pp. 283-284.

Van Meter, P., French, J., Dloniak, S., Watts, H., Kolowski, J. and Holekamp, K. 2009, 'Fecal glucocorticoids reflect socio-ecological and anthropogenic stressors in the lives of wild Spotted Hyenas', *Hormones and Behavior*, vol. 55, no. 2, pp. 329-337.

van Valkenburgh, B. 2006, *Lycaon pictus*, [Online], Available: http://digimorph.org/specimens/Lycaon_pictus/male/ [23rd April 2010].
Ven, C. C. 2009, Non-invasive assessment of adrenocortical endocrine function in African Wild Dogs (*Lycaon pictus*) by measurement of glucocorticoid metabolites in faeces, Faculty of Veterinary Medicine, Utrecht University, The Netherlands, pp. 12.
Viranta, S. 2009, 'Geographic and temporal ranges of middle and late miocene carnivores', *Journal of Mammalogy*, vol. 84, no. 4, pp. 1267-1278.
Visee, A. M. 2001, *Veterinary Report 1999-2001*, George Adamson Trust, [Online], Available: http://www.georgeadamson.org/downloads/vet_99-01.pdf [27 May 2012].
Walker, B. G., Dee Boersma, P. and Wingfield, J. C. 2006, 'Habituation of adult magellanic penguins to human visitation as expressed through behavior and corticosterone secretion', *Conservation Biology*, vol. 20, no. 1, pp. 146-154.
Walsh, P. D. 2013, 'Disease control', in *Key Topics in Conservation Biology 2*, ed. D. W. Macdonald and K. J. Willis, Wiley-Blackwell, West Sussex, pp. 472-486.
Wang, G. 2000, The role of vegetation dynamics in the climate of West Africa, PhD Thesis, Department of Civil and Environmental Engineering, Massachusetts Institute of Technology, Massachusetts, pp. 225.
Wang, X., Tedford, R. H. and Anton, M. 2008, *Dogs: Their Fossil Relatives and Evolutionary History*, Columbia University Press, USA.
Wayne, R. K. 1989, 'The phylogeny of the recent carnivore', in *Carnivore Behavior, Ecology and Evolution*, ed. J. L. Gittleman, Chapman and Hall Ltd, USA, pp. 465-494.
Wayne, R. K., Geffen, E., Girman, D. J., Koepfli, K. P., Lau, L. M. and Marshall, C. R. 1997, 'Molecular systematics of the canidae', *Systematic Biology*, vol. 46, no. 4, pp. 622–653.
Wang, X., Tedford, R. H., Van Valkenburgh, B. and Wayne, R. K. 2004, 'Evolutionary history, molecular systematics, and evolutionary ecology of Canidae', in Biology and Conservation of Wild Canids, ed. D. W. Macdonald and C. Sillero-Zubiri, Oxford University Press, USA, pp. 39-54.
WAZA 2013, *Towards Integrated Species Conservation*, World Association of Zoos and Aquariums magazine, Vol. 14, pp. 1-46. [Online] URL: http://www.waza.org/files/webcontent/1.public site/5.conservation/conservation breeding programme/integrated species conservation/WAZA%20Magazine%2014.pdf [29 August 2013].
Weigl, R. 2005, *Longevity of Mammals in Captivity; From the Living Collections of the World*, Kleine Senckenberg-Reihe 48, Stuttgart.
Welbourn, L. C., DeVries, J. G., Ford, R., Franklin, R. T., Hurley, K. F., McClure, K. D., Paul, M. A. and Schultz, R. D. 2011, 'Canine vaccination guidelines', *Journal of the American Animal Hospital Association*, vol. 47, no. 5, pp. 1-42.
Wilkening, J. L., Ray, C. and Sweazea, K. L. 2013, 'Stress hormone concentration in Rocky Mountain populations of the American Pika (*Ochotona princeps*)', *Conservation Physiology*, vol. 1, no. 1, cot027.
Wilmers, C. C., Crabtree, R. L., Smith, D. W., Murphy, K. M. and Getz, W. M. 2003, 'Trophic facilitation by introduced top predators: grey wolf subsidies to scavengers in Yellowstone National Park', *Journal of Animal Ecology*, vol. 72, pp. 909-916.
Winrow, V. R., Winyard, P. G., Morris, C. J. and Blake, D. R. 1993, 'Free radicals in inflammation: Second messengers and mediators of tissue destruction', *British Medical Bulletin*, vol. 49, no. 3, pp. 506-522.
Winterbach, H. E. K., Winterbach, C. W., Somers, M. J. and Hayward, M. W. 2012, 'Key factors and related principles in the conservation of large African carnivores', *Mammal Review*, vol. 43, no. 2, pp. 89-110.
Wobeser, G. A. 2007, *Disease in Wild Animals: Investigation and Management*, 2nd Edition, Springer-Verlag, Berlin, Heidelberg.

Woodford, M. H. (ed), 2000, *Quarantine and Health Screening Protocols for Wildlife prior to Translocation and Release into the Wild*, IUCN Species Survival Commission's Veterinary Specialist Group, Gland, Switzerland, the Office International des Epizooties (OIE), Paris, France, Care for the Wild, U.K., and the European Association of Zoo and Wildlife Veterinarians, Switzerland.,

Woodford, M. H. and Kock, R. A. 1991, 'Veterinary considerations in re-introduction and translocation projects', *Symposia of the Zoological Society of London*, vol. 62, pp. 101-110.

Woodroffe, R. 1999, 'Managing disease threats to wild mammals', *Animal Conservation*, vol. 2, no. 3, pp. 185-193.

Woodroffe, R. 2001, 'Assessing the risks of intervention: Immobilization, radio-collaring and vaccination of African Wild Dogs', *Oryx*, vol. 35, no. 3, pp. 234-244.

Woodroffe, R., André, J. M., Andulege, B., Bercovitch, F., Carlson, A., Coppolillo, P., Davies-Mostert, H., Dickman, A., Fletcher, P., Ginsberg, J., Hofmeyr, M., Laurenson, L. K., Leigh, K., Lindsey, P., Lines, R., Mazet, J., McCreery, K., McNutt, J., Mills, M. G. L. and Gunther, M. S. 2005, *Tools for the conservation of African Wild Dogs. Do we know enough? What more do we need to know? Report of a Workshop on Research for Conservation of the African Wild Dog Kruger National Park, South Africa*,

Woodroffe, R., Chapman, K. and Lemusana, E. 2009, 'Solitary breeding in an African Wild Dog (*Lycaon pictus*)', *African Journal of Ecology*, vol. 47, no. 4, pp. 790-791.

Woodroffe, R., Cleaveland, S., Courtenay, O., Laurenson, M. K. and Artois, M. 2004a, 'Infectious disease in the management and conservation of wild canids ', in *Biology and Conservation of Wild Canids*, ed. D. Macdonald and C. Sillero-Zuberi, Oxford University Press, New York, pp. 123-142.

Woodroffe, R., Davies-Mostert, H. T., Ginsberg, J., Graf, J., Leigh, K., McCreery, K., Robbins, R., Mills, M. G. L., Pole, A., Rasmussen, G. S. A., Somers, M. J. and Szykman, M. 2007, 'Rates and causes of mortality in Endangered African Wild Dogs *Lycaon pictus*: Lessons for management and monitoring', *Oryx*, vol. 41, pp. 215-223

Woodroffe, R. and Donnelly, C. A. 2011, 'Risk of contact between Endangered African Wild Dogs *Lycaon pictus* and domestic dogs: Opportunities for pathogen transmission', *Journal of Applied Ecology*, vol. 48, no. 6, pp. 1345-1354.

Woodroffe, R. and Ginsberg, J. R. 1999, 'Conserving the African wild dog *Lycaon pictus*. II. Is there a role for reintroduction?', *Oryx*, vol. 33, no. 2, pp. 143-151.

Woodroffe, R., Ginsberg, J. R., Macdonald, D. W. and IUCN/SSC Canid Specialist Group 1997, *The African Wild Dog - Status Survey and Conservation Action Plan*, IUCN, Gland, Switzerland. 166 pg.

Woodroffe, R., Groom, R. and McNutt, J. W. 2017, 'Hot dogs: High ambient temperatures impact reproductive success in a tropical carnivore', *Journal of Animal Ecology*, vol. 86, no. 6, pp.1329-1338.

Woodroffe, R., McNutt, J. W. and Mills, M. G. L. 2004b, 'Species status accounts: African Wild Dog', in *Canids: Foxes, Wolves, Jackals and Dogs - 2004 Status Survey and Conservation Action Plan*, ed. C. Sillero-Zubiri, M. Hoffman and D. Macdonald, IUCN, Gland, Switzerland, pp. 174-183.

Woodroffe, R. and Sillero-Zubiri, C. 2012, *Lycaon pictus*, IUCN Red List of Threatened Species. Version 2013.1. Date accessed 28th August 2013. [Online], Available: www.iucnredlist.org [28 August 2013].

Woods, M. S., Stephens, N. C., Alison, B. K. and Howell, C. I. 2001, *Plantations of Australia - A Report from the National Plantation Inventory and National Farm Forest Inventory*, National Forest Inventory, Bureau of Rural Science, Canberra.

Woolford, L., Crocker, P., Bobrowski, H., Baker, T. and Hemmatzadeh, F. 2017, 'Detection of the canine parvovirus 2c subtype in Australian dogs', *Viral Immunology*, vol. 30, no. 5, pp. 371-376.

Wozencraft, W. C. 1989, 'The phylogeny of the recent carnivore', in *Carnivore Behavior, Ecology and Evolution*, ed. J. L. Gittleman, Chapman and Hall Ltd, USA, pp. 495-535.

Wroe, S., McHenry, C. and Thomason, J. 2005, 'Bite club: Comparative bite force in big biting mammals and the prediction of predatory behaviour in fossil taxa', *Proceedings of the Royal Society B*, vol. 272, pp. 619-625.

WWF 2011, *Conservation and Management of the African Wild Dog and its Habitat in Eastern Zambia*, [Online], Available: http://wwf.panda.org/about_our_earth/teacher_resources/best_place_species/current_top_10/african_wild_dog.cfm?uProjectID=ZM0039 [4th March 2011].

Youden, W. J. 1950, 'Index for rating diagnostic tests', *Cancer*, vol. 3, no. 1, pp. 32-5.

Young-Joon, S. 2005, 'Transcriptional regulation of cellular antioxidant defense mechanisms', in *Oxidative Stress, Inflammation and Health*, ed. S. Young-Joon and P. Lester, Taylor & Francis Group, LLC, USA, pp.

Young-Joon, S. and Lester, P. (ed), 2005, *oxidative stress, inflammation and health*, Taylor & Francis Group, LLC, USA.

Young, K. M., Walker, S. L., Monfort, S. L., Brown, J. L., Lanthier, C. and Waddell, W. T. 2004, 'Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses', *General and Comparative Endocrinology*, vol. 137, no. 2, pp. 148-165.

ZAA 2017, *Annual Report and Recommendations: African Wild Dog, Lycaon pictus*, Australasian Species Management Program, Zoo Aquarium Assosication, Mosman, NSW. pp. 1-22.

ZCP 2011, *Habitat Corridor Conservation*, Zambian Carnivore Program, [Online], Available: http://www.zambiacarnivores.org/conservation/habitat-corridor-conservation/ [4th March 2011].

ZIMS 2017, *Lycaon*, Zoological Information Management System, [Online], Available: https://zims.species360.org/Main.aspx [11th October 2017].

ZoosSA 2009, *Monarto Zoo - General Information*, Zoo South Australia, [Online], Available: http://www.zoossa.com.au/home [7th December 2009].