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Appeasing pheromones, vocalisations and faecal antibodies to monitor and improve social cohesion and immune function in the African wild dog (*Lycaon pictus*)

Thesis submitted by

Pia Riddell, BA, BSc (Hons I) in June 2024

For the degree of

Doctor of Philosophy

College of Public Health, Medical and Veterinary Sciences, James Cook University, Australia

DECLARATIONS

(i) The extent of any collaborations with others has been stated clearly and fully in the thesis. To the best of my knowledge and belief, the thesis contains no material previously published by any other person.

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Pia Riddell

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STATEMENT OF CONTRIBUTION

Nature of assistance	contribution	Names, Titles & Affiliations
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STATEMENT OF THE USE OF GENERATIVE AI

Generative AI technology was not used in the preparation of any part of this thesis.

ABSTRACT

Wild and captive management approaches have been established to safeguard one of Africa's most endangered large carnivores, the African wild dog (*Lycaon pictus*), from extinction. These management approaches require frequent translocation of individuals between institutions/habitats with the aim of establishing new breeding packs (also termed, artificial pack formation). Translocation and artificial pack formations allow for the maintenance of genetically viable captive and wild populations. Despite many successful translocations and artificial pack formations, animals experience high levels of stress during these procedures, which increases anti-social behaviours and carries health and welfare risks to individuals. With 33% of deaths attributed to diseases such as canine distemper and rabies virus, and low efficacy of vaccinations in this species, stressful interventions could compromise vulnerable animals' resistance to pathogens. Additionally, stress-induced increases in anti-social behaviours and vocalisations could increase the time it takes to establish stable social hierarchies during artificial pack formation. Such prolonged aggression and instability can increase morbidity and mortality while in captivity and result in increased risk of pack annulment or dispersal away from designated reintroduction sites. Dog appeasing pheromone (DAP) have been shown to reduce stress and aggression related behaviours in captive African wild dogs and reduce aggression-related hormones and improve social-behaviours, however a new pharmacophore dog appeasing pheromone (phDAP) was recently developed with the aim of improving the modulation of stress and aggression-related hormones and behaviours in canines. As such, non-invasive strategies are needed to investigate the deleterious impact of management-induced stress and aggression on immune function, behaviour and vocalisations in African wild dogs. Moreover, naturally occurring appeasing pheromone strategies need to be tested to reduce the experience of stress and aggression during conservation interventions and thereby improve their welfare. This thesis aimed to develop tools to measure and improve

immunity and social cohesion in African wild dogs. This was achieved through the following objectives:

(i) *Develop a non-invasive, field-applicable assay for immune function (Chapter 3):* Faecal samples were collected from domestic dogs (n=10; Private owners & Moccarott Rottweilers, Queensland, Australia), dingoes (n=9; Wildlife Surrounds, Billabong Wildlife Sanctuary & private owners, Queensland, Australia), and African wild dogs (n=10; Perth Zoo, Western Australia, Australia). Pre-vaccination samples were used to validate an enzyme-linked immunosorbent assays (ELISA) for measuring faecal immunoglobulins M (fIgM), G (fIgG), and canine distemper virus-specific G (fCDV-IgG). Samples collected post-vaccination (using a Protech C3 or C4 vaccine) from domestic dogs and dingoes were used to track an increase in vaccination-related immunoglobulin concentrations, while African wild dog samples were used to determine the stability of IgM and IgG at ambient temperatures post-defaecation. In this study, fIgM and fIgG were measurable in all canids and fCDV-IgG were measurable in vaccinated domestic dogs and dingoes. Unvaccinated African wild dogs had significantly lower fIgG concentrations than vaccinated domestic dogs. A significant rise in fIgG and fCDV-IgG occurred six weeks and five or seven weeks after vaccination in domestic dogs and dingoes respectively. Finally, African wild dog fIgM and fIgG are stable for five to nine days respectively, post-defaecation under laboratory conditions at 20-25°C.

(ii) *Determine how African wild dog vocalisations change with different behaviours, contexts and housing (Chapter 4):* Video and audio data were collected opportunistically from one free-roaming pack before (Waterberg Wild Dog Initiative managed TOOG pack, n=15) and after male dispersal (n=10), one open-range zoo pack (Bothongo Rhino & Lion Nature Reserve, n=15), and three traditional zoo packs (Johannesburg Zoo, n=2; Ann Van Dyke Cheetah Centre pack 1, n=4, and pack 2, n=7) for three days per pack. Vocalisations and associated behaviours were recorded in the contexts of social interaction, feeding, animal threat, and human threat to determine how communication was influenced by environmental and social factors. During pro-social behaviours, a reduced range of multifunctional vocalisations were produced and there was an increase in the rate of sneezes associated with affiliation, vocalisations emitted with pro-social behaviours also tended to have shorter syllables and higher fundamental frequency. During anti-social behaviours, a larger range of vocalisations were emitted and there was an increase in the rate of social growls, alarm barks and alarm growls associated with dominance, vocalisations emitted with anti-social behaviours also tended that tended to have longer syllables and lower fundamental frequencies. Additionally, alarm barks, threat barks, attack barks, social growls, alarm growls, buzz moans and full moans were exclusive or predominantly associated with anti-social behaviours and, as such, were identified as anti-social vocalisations. This shows that behaviours associated with positive and negative emotional states, affect the rate and acoustic properties of vocalisation used by African wild dog packs. Vocalisation and behaviour use within different contexts showed that anti-social contexts (animal and human threats) are associated with increasing rates of anti-social vocalisations that have longer syllables and lower fundamental frequency than during more pro-social contexts (social interaction and feeding). When enclosure types were compared, traditional zoo and open-range zoo packs used a number of vocalisations in all behaviour categories more often than free-roaming packs and had longer syllables with lower frequencies, suggesting that zoo-based packs may experience increased negative emotional states, possibly due to increased stress and a reduced ability to display natural behaviours such as dispersal. Vocalisations appear to be a sensitive tool to measure changes in the emotional state and social cohesion of African wild dogs and, as such, could be used to monitor the impact of stress on this species.

(iii) *Determine how African wild dog vocalisations change with season. stress and dog appeasing pheromone mitigation (Chapter 5):* Retrospective data from five captive packs in the US (n=17; Albuquerque BioPark, Albuquerque, NM; Topeka Zoo, Topeka, KS; Brookfield Zoo, Chicago, IL; Binder Park Zoo, Battle Creek, MI; and Oklahoma City Zoo, Oklahoma City, OK) were analysed during the pre-breeding and breeding seasons, as well as during a separation, immobilisation, and reintroduction event. Vocalisation rates and acoustic parameters were measured during the pre-breeding and breeding seasons for three days before, during and for 3 days after the intervention. Packs were treated with either DAP or a placebo at the time of immobilisation to compare their effect on the rate and acoustic properties of vocalisations. Anti-social vocalisations increased in the breeding season and tended to become longer and lower in frequency compared to the pre-breeding season. During the stressful intervention, placebo-treated packs showed an increase in anti-social vocalisations which were longer and of lower frequency, while DAP-treated packs showed an increased rate of pro-social vocalisations that had a higher frequency but remained longer following the intervention. These results suggest that stress and aggression may induce an increase in the rate, lengthening of syllables and lowering of frequency of certain vocalisations, and that DAP may alleviate some of these detrimental changes during stressful events such as breeding or conservation intervention, making vocalisations a sensitive tool to detect emotional state in African wild dogs.

(iv) *Compare the perception of DAP vs. phDAP by African wild dogs (Chapter 6):* Behavioural responses of n=46 animals across seven captive packs (Ann Van Dyke Cheetah Centre, packs 1 - 4; SANBI National Zoological Gardens Pretoria; Hoedspruit Endangered Species Centre; Bothongo Rhino & Lion Reserve) were recorded for one hour daily for three days before treatments and for one hour after DAP or phDAP were applied to separate 1m^2 patches of ground within their enclosure. Enclosures were rested for several days before treatments were reapplied in reversed locations. DAP was perceived more favourably than phDAP, eliciting significantly higher investigative behaviours. Concerningly, phDAP caused

a significant decrease in play and a declining trend in affiliative behaviours in African wild dogs. These results suggest that phDAP has reduced perception and may decrease pro-social behaviours in this species.

In conclusion, the findings of this thesis demonstrate that immune markers can be measured non-invasively, vocalisations and their acoustic properties change in response to emotional state; stress and season significantly increase and modulate anti-social vocalisations and DAP may mitigate these effects, and finally that the original DAP formulation is more readily detected and more likely to alleviate stress and aggression-related behaviours and vocalisations in African wild dogs than the newly-developed phDAP. This study provides essential tools to monitor and enhance African wild dog social cohesion, welfare and conservation through improved management practices.

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LIST OF ABBREVIATIONS

ALB: Albuquerque Biopark

ANOVA: analysis of variance

AVDCC: Ann Van Dyke Cheetah Centre

BIN: Binder Park Zoo

BOT: Bothongo Rhino and Lion Nature Reserve

BRK: Brookfield Zoo

CDV: Canine Distemper Virus

CDV-IgG: Canine Distemper Virus specific Immunoglobulin G

CPV: Canine Parvovirus

CRH: Corticotrophin Releasing Hormone

dB: decibel

DAP: Dog Appeasing Pheromone

DHEA: dehydroepianderosterone

ELISA: Enzyme-Linked Immunosorbent Assay

Fc: Functional chain

fCDV-IgG: faecal Canine Distemper Virus specific Immunoglobulin G

fGCM: faecal Glucocorticoid Metabolite

fAM: faecal androgen metabolite

fIgG: faecal Immunoglobulin G

fIgM: faecal Immunoglobulin M

GnRH: Gonadotrophic releasing hormone

GLMM: Generalised linear mixed model

HESC: Hoedspruit Endangered Species Centre

h:m: hours:minutes

H-P-A: Hypothalamic Pituitary Adrenal Axis

H-P-G: Hypothalamic Pituitary Gonadal Axis

h: hour

HRP: Horse Radish Peroxidase

IgA: Immunoglobulin A

IgD: Immunoglobulin D

IgE: Immunoglobulin E
IgG: Immunoglobulin G
IgM: Immunoglobulin M
IUCN: International Union for the Conservation of Nature
JOB: Johannesburg Zoo
kHz: kilohertz
Max: maximum
Min: minimum
MOB: Main Olfactory Bulb
MOE: Main Olfactory Epithelium
MP4: MPEG-4, part 14 format
ms: millisecond
MS: Motivational-Structure
No.: number
OKL: Oklahoma City Zoo
PBS: Phosphate Buffered Saline
PBST: Phosphate Buffered Saline Tween
phDAP: pharmacophore Dog Appeasing Pheromone
PRET: National Zoological Gardens of Pretoria
SEM: Standard error of the mean
SIR: separation, immobilisation, reintroduction
TOOG: TOOG pack
TOOG$_{BD}$: TOOG pack before dispersal
TOOG$_{AD}$: TOOG pack after dispersal
TOP: Topeka Zoo and Conservation Centre
V1R: Vomeronasal type-1 receptors
VNO: Vomeronasal Organ

PREFACE

The current thesis consists of seven chapters, five of which (Chapters 2, 3, 4, 5 and 6) have been written as stand-alone publications prior to incorporation. Resulting from this there may be some overlap within the thesis. These chapters are either currently published or in preparation for publication in peer-reviewed journals and have been presented at international scientific conferences (see publications arising from the thesis).

PUBLICATIONS ARISING FROM THE THESIS

Journal articles

Riddell P, Paris MCJ, Joonè CJ, Pageat P and Paris DBBP (2021) Appeasing Pheromones for the Management of Stress and Aggression during Conservation of Wild Canids: Could the Solution Be Right under Our Nose? *Animals* **11,** 1574.

Paris DBBP, **Riddell P**, Joonè CJ, de la Rey M, Ganswindt A and Paris MCJ (2024) Cold Dogs: sperm freezing artificial insemination and non-invasive monitoring tools to facilitate a hybrid conservation management approach for endangered African wild dogs. *Theriogenology Wild* ***4**, 100073*.

Riddell P, Paris MCJ, Joone C, Bertshinger H and Paris DBBP. Non-invasive measurement of immunoglobulin concentrations in the faeces of healthy domestic dogs (*Canis familiaris*), Australian dingoes (*Canis familiaris dingo*) and African wild dogs (*Lycaon pictus*). *Vaccine* (In prep).

Riddell P, Malone K, Joone C, Paris MCJ and Paris DBBP. African wild dog (*Lycaon pictus*) vocalisation changes in response to behaviours, contexts and captive status. *Animal Behaviour* (In prep).

Riddell P, Paris MCJ, Joone C, Van den Berghe F, Ganswindt A and Paris DBBP. Dog appeasing pheromones increase pro-social and reduce the rate, lower the pitch and lengthen syllables of anti-social vocalisations during the breeding season and stressful interventions of captive African wild dogs (*Lycaon pictus*). *Animal Behaviour* (In prep).

Riddell P, Paris MCJ, Joone CJ, Ganswindt A, Pageat P and Paris DBBP. Pheromone perception behaviours increase but some pro-social behaviours decline when African wild dog (*Lycaon pictus*) enclosures are treated with different appeasing pheromone analogues. *Applied Animal Behaviour Science* (In prep).

Conferences

Riddell P, Paris MCJ, Joonè C, Pageat P, Ganswindt A, Parker DM, du Plessis C and Paris DBBP. (2020) Novel appeasing pheromones to minimise stress during metapopulation management of African wild dogs. 33rd AWMS Annual Conference, Online, Australia, December 9, 2020. AWMS Conference Proceedings

Riddell P, Paris MCJ, Joonè CJ, Lopata AL and Paris DBBP. (2021) Measurement of faecal antibodies to assess immune function in African wild dogs. 50th Southern African Wildlife Management Association Annual Conference, Online, South Africa, September 9, 2021. SWAMA Conference Program 2021

Riddell P, Paris MCJ, Joonè CJ, Lopata AL and Paris DBBP. (2021) Faecal antibody measurement to assess immunity in African wild dogs and other canids. EAZA Online Annual Conference, Online, Global, September 22, 2021. EAZA Conference Program 2021

Riddell P, Paris MCJ, Joonè CJ, Lopata AL, Bertschinger H and Paris DBBP. (2022) Immune response to vaccination can be measured non-invasively in the faeces of wild canids using enzyme linked immunosorbent assays. African Wild Dogs United Virtual Conference, Online, February 15, 2022. Wild Dogs United 2022 Conference Program

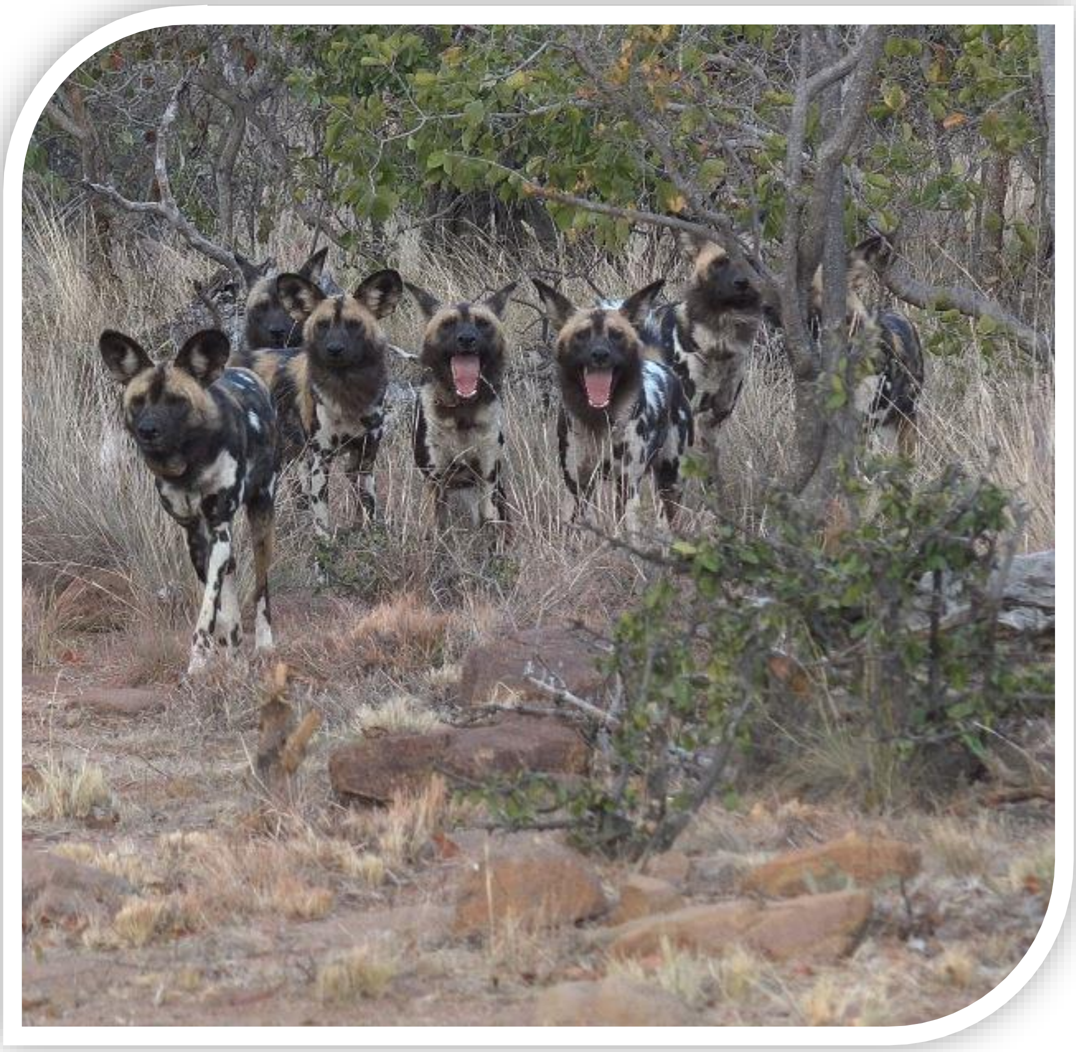
Riddell P, Paris MCJ, Joonè CJ, Van den Berghe F and Paris DBBP. (2022). What did the dog say? Dog appeasing pheromones reduce social twitters and contact dominance during the reintroduction of African wild dogs (*Lycaon pictus*). South African Wildlife Management Association (SAWMA) Hybrid conference, Hluhluwe, South Africa, September 8, 2022. SAWMA 2022

Riddell P, Paris MCJ, Joonè CJ, Van den Berghe F and Paris DBBP. (2022). What did the dog say? Dog appeasing pheromones increase social cohesion during African wild dog reintroductions. European Association of Zoos and Aquaria (EAZA) Hybrid conference; Canid TAG, Online, September 30, 2022.

Poster presentations

Riddell P, Paris M.C.J, Joonè C.J, Bertschinger H, Ganswindt A and Paris D.B.B.P. (2023). Non-invasive detection of faecal antibodies to assess immune response following vaccination in wild canids. 12th Oppenheimer Research Conference, Johannesburg, South Africa, October 5th, 2023. ORC 2023

Chapter 1: General introduction



Abstract

With less than 6,600 individuals and a declining population, the African wild dog (*Lycaon pictus*) remains one of the most endangered canids in Africa. One promising strategy implemented within highly fragmented populations in South Africa is meta-population management, involving the translocation of animals from two different areas to artificially form a new pack. However, this approach can cause chronic stress and heightened aggression within African wild dog packs, leading to increased rates of morbidity and mortality and decreased animal welfare. Chronic stress and aggression are known to negatively affect overall animal health in other species, but little is known about their effects on African wild dogs. The management of stress and aggression in domestic animals has been achieved with the identification and application of appeasing pheromones. Dog appeasing pheromone (DAP) has been used successfully during stressful intervention within established and stable captive wild dog packs. However, a newly developed pharmacophore dog appeasing pheromone may be a more potent natural behaviour modifier able to reduce stress and aggression during translocation and artificial pack formation initiatives. This literature review provides a general background on African wild dogs, the impact of stress and aggression on this species and the potential application of pheromones to manage deleterious behaviours.

1. An overview of the African wild dog

1.1 Species: characteristics, distribution and conservation status

The African wild dog (*Lycaon pictus;* also known as painted wolf, painted hunting dog or cape hunting dog) is a unique carnivorous African mammal. It is a member of the Canidae, which consists of 36 different species (Wayne et al., 1997). The African wild dog is the only species in the *Lycaon* genus but is most closely related to the bush dog (*Speothos venaticus*) and other wolf-like carnivores, including wolves (*Canis lupus*), coyotes (*Canis latrans*), golden jackals *(Canis aures*) and the dhole (*Cuon alpinus*; Wayne et al., 1997).

Distinguishing characteristics of African wild dogs include large rounded ears, long legs with four toes per foot, and a distinctive mottled coat consisting of yellow, brown, black and white colour, patterned uniquely for each individual (Fig. 1.1). Height and weight range from 75 – 110 cm and 18 – 36 kg respectively, with females generally smaller and lighter than males.

Figure 1.1. African wild dog (*Lycaon pictus*). Photo credit Pia Riddell, 2024

Once abundant over much of sub-Saharan Africa, the African wild dog is now one of the most vulnerable carnivores in Africa, being listed as endangered by the International Union for Conservation of Nature (IUCN) Red List of threatened species for the past thirty years (Woodroffe & Sillero-Zubiri, 2020). In the wild, the estimated population size is only 6,600 individuals (of which approximately 1,400 are mature) with a declining trend (Woodroffe & Sillero-Zubiri, 2012). Since 1997, the global population of African wild dogs has declined by 17%, with a 50%, 27%, 26% and 3% population decrease reported in West Africa, Southern Africa, Central Africa and East Africa respectively (Woodroffe & Sillero-Zubiri, 2012). In South Africa only an estimated 601 animals remain in 76 packs across 16 fragmented subpopulations (Nicholson et al., 2020; Wild Dog Advisory Group, 2023). African wild dogs live in low population densities (Creel & Creel, 1998; Sillero-Zubiri et al., 2004) and occupy home ranges as large as 2,500 km^2 (Woodroffe et al., 1997). Currently, free-roaming African wild dog populations are found mainly within fenced protected areas throughout South Africa (Hayward et al., 2007; Lindsey et al., 2004; Paris et al., 2024), though wild populations also exist in reduced numbers outside these protected zones. Populations within other parts of Southern and Eastern Africa largely persist outside of fenced areas and as a result increased threat of human persecution where they range close to human used lands (Jordan et al., 2023).

1.2 Social pack structure

African wild dogs are a highly social species, with a hierarchical cooperative pack structure consisting typically of between 5–15 adult animals and yearlings in separate male and female dominance hierarchies (Creel & Creel, 1995; Creel & Creel, 2002; Creel et al., 1997a; Girman et al., 1997). The alpha male and female of the pack have almost exclusive reproductive privileges, with only around 8% of pups born to subordinate bitches and 10% sired by subordinate males (Girman et al., 1997; Moueix, 2007; Spiering et al., 2010). Established packs produce an annual litter of pups, which are raised over a three-month period in a den (Malcolm & Marten, 1982). The rearing of young, hunting, and protection of den sites and territories is performed cooperatively by pack members (Creel et al., 1997a; Paris et al., 2024). Pack size is positively correlated with reproductive success, hunting success and survival (Creel et al., 1997a; Courchamp & Macdonald, 2001; Buettner et al., 2006). The critical threshold for pack survival is five animals, which permits a minimum number of individuals to both guard/raise pups as well as hunt efficiently (Courchamp & Macdonald, 2001).

Young dogs break away from their natal pack as single-sex groups. Female groups disperse at a mean age of 21.8 months while male groups disperse at a mean age of 28.1 months (McNutt, 1996). Dispersal of young single-sex groups facilitates the formation of new packs, which occurs when two opposite sex groups from different natal packs join as a new stable reproductive unit (McNutt, 1996; Gusset et al., 2006). Group size, mate choice and intra-pack competition are known to affect the success of newly formed packs (McCreery & Robbins, 2001; Van den Berghe et al., 2019b; Marneweck et al., 2019a). Failure to form and maintain a pack of five or more animals with a mixture of sexes may occur when competition for resources is high, or there is limited availability of habitable land that prohibits successful dispersal (Van den Berghe et al., 2012). In both zoo-based captive populations and wild populations fenced within Private Reserves and National parks of African wild dogs, natural dispersal is disrupted.

Communication between canids includes physical, scent and vocal behaviours (Robbins 2000; Walker et al., 2017; Van den Berghe et al., 2019a; Claase et al., 2022; Apps et al., 2022; Jordan et al., 2023), with most canid species using a diverse repertoire of both short- and long-range vocalisations to communicate territory, hierarchy and social bonds with conspecifics and other animal species within their habitat (Robbins, 2000; Robbins & McCreery, 2003; Hallberg, 2007; Déaux & Clarke, 2013; Mazzini et al., 2013). The African wild dog's high dependence on the cooperation of pack members to hunt, guard territory, raise pups and care for sick, injured and elderly animals means that pack coordination requires sophisticated communication. As such, they possess a diverse vocal repertoire which consisting of 11 vocal classes and 18 subclasses (Matern, 1981; Robbins, 2000). Seven of these vocalisations are unique to the African wild dog (Robbins, 2000). Despite their classification, there is very little known about how African wild dogs use their vocalisations. One vocalisation that is relatively well understood, the long distance hoo-call, is used to locate separated pack mates and has been identified as a good indicator of social cohesion during pack bonding (Hartwig, 2005; Potgieter et al., 2015; Marneweck et al., 2019a). The remainder of their vocal repertoire are predominantly short-range vocalisations which are likely to have a role in intra-pack communication (Robbins, 2000; Webster, 2009; Jordan et al., 2023). However, in other canids vocalisations may be good indicators of social cohesion and emotional states. The howls emitted by wolves, coyotes and dingoes differ between packs in a range of parameters and are thought to be used as a way for isolated members to recognise their pack from others in adjacent territories (Hallberg, 2007; Deaux and Clarke, 2013). Playback and individual animal removal studies have also confirmed that the howls of wolves facilitate recognition of individuals (Mazzini et al., 2013; Palacios et al., 2015). In domestic dogs, disturbance barks are harsh, unmodulated and of low frequency, while play barks are rich, modulated and of high pitch (Yin and McCowan, 2004). Together, these findings suggest that canid vocalisations can be modulated to infer changes in emotional state and have distinctive individually recognisable traits (Farago et al., 2010; Kershenbaum et al., 2016). Despite emerging evidence of the importance vocalisations play in canid communication, very little is known about how vocalisations are used during social interactions in most species.

1.3 Threats to species survival

Free-living African wild dogs face numerous threats to survival including habitat loss anthropogenic factors, intra- and interspecies competition and infectious diseases (Woodroffe et al., 2007; Nicholson et al., 2020). Such threats contribute significantly to the decline of African wild dog populations and are all amplified by human encroachment on their habitat (Woodroffe & Sillero-Zubiri, 2020).

1.3.1 Habitat loss

African wild dogs are particularly vulnerable to habitat fragmentation due to their natural dispersal patterns, large home ranges and preference for low population densities (Fanshawe & Fitzgibbon, 1993). Dispersal and exploratory patterns of African wild dog packs are influenced by landscape structure (Jackson et al., 2017). Unfavourable areas usually include landscapes altered for human use, and settlement of packs in these areas may result in increased human-wildlife conflict (Jackson et al., 2017). African wild dogs are habitat generalists, meaning they can thrive in a variety of different environments and utilise a range of resources (Pomilia et al., 2015). During dispersal, African wild dogs prefer woodland habitats, and to a lesser extent dense bushland areas, mirroring the habitats of preferred prey species (Whittington-Jones et al., 2014). Transient packs tend to remain within close proximity to both food and water sources, but models show that large areas of highly suitable land, which could sustain African wild dogs, are isolated and separated by areas that are extremely unsuitable (Jackson et al., 2014). Such unsuitable land may not facilitate dispersal into highly suitable habitats, decreasing dispersal success and possibly reducing the success of new packs forming (Jackson et al., 2014). Dispersing packs of young adults are thought to transit through unfavourable areas to more high-quality habitat where they will establish themselves (Jackson et al., 2017).

When dispersal success is low, declines in population size, subdivision of populations and genetic isolation occur (Marsden et al., 2012). Isolation of subpopulations and reduced genetic variability increase the risk of extinction (Flacke et al., 2013; Whittington-Jones et al., 2014) and increase susceptibility to disease and infection. Most African wild dogs are found in state-owned national parks in South Africa which are often fenced and made up of natural land cover with low human densities (Lindsey et al., 2004; Hayward et al., 2007; Whittington-Jones et al., 2014). National parks provide highly suitable, and relatively safe, habitat for African wild dogs but do not facilitate dispersal, leading to genetic bottlenecks in these areas. As such, careful management of African wild dog populations is required to maintain genetic diversity. Outside protected areas, African wild dogs may come into close contact with human settlements leading to increased human-wildlife conflict.

1.3.2 Human-wildlife conflict

A large proportion of African wild dog mortalities occur because of conflict with humans (Creel & Creel, 1998; Woodroffe et al., 2007; Gusset et al., 2008). Human activity often results in reduced quality of habitat available to native animals, due to alteration of the landscape to make it more suitable for human habitation and industry. Human encroachment has increased contact between African wild dogs and humans. In 1998 within the Selous region of Tanzania, human factors caused 12% of wild dog deaths (Creel & Creel, 1998). However, the majority of African wild dog deaths were recorded as of unknown causes, suggesting this figure may be an underestimate of the true human impact. A more recent review indicated that humans caused 37% of all African wild dog mortalities across Africa (Woodroffe et al., 2007). Considered separately, 27% of these deaths were accidental (road/train accidents) and 73% were attributed to deliberate illegal activity (hunting, poisoning and snaring; Woodroffe et al., 2007). African wild dogs were often hunted and killed by farmers where their presence overlapped with livestock farming, due to a real or perceived threat to livelihoods (Gusset et al., 2008). Moreover, persecution, illegal poaching and roads may isolate and restrict population dispersal.

1.3.3 Intra- and interspecies competition

Large carnivores can limit the number of smaller carnivores within a habitat (Johnson & Franklin, 1994; Raath et al., 1998; Palomares et al., 1999). Lions (*Panthera leo*) and spotted hyenas (*Crocuta crocuta*) negatively influence the ability of African wild dogs to thrive in suitable habitats through kleptoparasitism (the stealing of prey; Fanshawe & Fitzgibbon, 1993; Gorman et al., 1998; Jongeling & Koetsier, 2014; Speakman et al., 2016), partial exclusion from preferred habitats (Mills & Gorman, 1997; Creel, 2001a) and through predation (Ginsberg et al., 1995; van Heerden et al., 1995). Predation by larger carnivores accounts for between 15% and 19% of African wild dog mortality (Creel & Creel, 1998; Woodroffe et al., 2007) and when competition is intense, local extinctions of African wild dogs can occur (Vucetich & Creel, 1999). Furthermore, high carnivore densities may inhibit the use of habitats by dispersing and transient African wild dogs, due to increased competition for prey, thereby reducing the likelihood of pack persistence in these areas (Webster et al., 2012; Darnell et al., 2014; Marneweck et al., 2022).

Intra-species aggression accounts for between 10% and 69% of mortalities (Creel & Creel, 1998; Woodroffe et al., 2007), with incidences largely occurring in areas where there is reduced access to resources (Jackson et al., 2017). Direct encounters between African wild dog packs often result in aggressive interactions (Creel & Creel, 2002). The degree to which African wild dog home ranges overlap varies greatly in studied populations (Mills & Gorman, 1997; Creel & Creel, 2002). The most extensive overlap of home ranges occurs between natal packs and new packs consisting of related dispersing female groups, likely due to related male groups dispersing further to form new packs (Jackson et al., 2017).

African wild dogs display relatively high levels of infanticide (Creel, 2001b; Creel & Creel, 2002; Woodroffe et al., 2007), which is most common when subordinate females whelp alongside the dominant female – under these circumstances, the dominant female will usually kill the pups of the subordinate female (Creel & Creel, 1998). Due to the difficulty of observing litters during denning, little is known about why infanticide occurs in African wild dogs when only the alpha female whelps (Creel & Creel, 2002). Pup survival in African wild dogs and other canids is positively associated with pack size (Creel et al., 2004; Ausband et al., 2017;

Marneweck et al., 2022), reduced breeder turnover (Ausband et al., 2017), previous experience (Borg et al., 2015; Yordy and Mossotti, 2016), familiarity with territory and resource defence (Borg et al., 2015; Yordy and Mossotti, 2016) and pack cohesion (Ausband et al., 2017). More experienced African wild dog females are more likely to raise pups beyond one year of age (Creel & Creel, 2002). Pups are also more likely to survive beyond this age during denning seasons that are drier and cooler (Buettner et al., 2006), in packs that are larger (Creel & Creel, 1995; Buettner et al., 2006), and when packs encounter increased numbers of prey (Creel & Creel, 2002).

1.3.4 Infectious disease

Proximity to humans and domestic animals increases exposure of African wild dogs to infectious diseases and pathogens (Woodroffe et al., 2012). The maintenance of generalist pathogens (those that can infect a range of species) in abundant reservoir species, may pose a serious threat to more susceptible species. Local extinction events and population crashes of wild dogs have been linked to a number of known pathogens that can infect canids. Known pathogens of concern to wild dogs include canine distemper virus (CDV), canine parvovirus (CPV) and rabies virus (Alexander et al., 2010). Encounters between packs are generally rare as a result of territorial behaviours and low population densities; as such, it is likely that transmission of these diseases occurs due to proximity with domestic dogs and other host species (Woodroffe *et al.,* 2012; Jordan et al., 2017).

Historical evidence of CDV in African wild dogs has been linked to contact with domestic dogs due to their proximal location and evidence of interactions between these species. Genetic sequencing has shown that in a number of different CDV outbreaks, the CDV variant was most closely related to that described in domestic dogs (Goller et al., 2010). However, this pathogen is able to infect a range of other wildlife species (Prager et al., 2012;

Beineke et al., 2015). Variants closely related to those from infected lions, spotted hyena and bat-eared foxes have also been identified in African wild dog outbreaks across different conservation areas throughout Africa (Goller et al., 2010), suggesting this disease may be transmitted by a range of species to African wild dogs. In 1972, fatal illness in a Serengeti National Park pack was identified as CDV (Woodroffe et al., 1997), and between 1970 and 1991 that park's African wild dog population was completely eradicated due to recurrent outbreaks of both CDV and rabies virus (Creel et al., 1997b). Further localised population crashes due to CDV have occurred in Tswalu Kalahari Reserve in South Africa, Chobe National Park in Botswana and Loliondo Game Controlled Area in Kenya (Goller et al., 2010; Flacke et al., 2013; Woodroffe, 2022). The extremely high mortality in these cases highlights the risk CDV can pose to wild dogs, especially in small, fragmented populations.

Creel et al., (1997b) found that 59% of African wild dogs sampled in Selous Game Reserve in Tanzania had antibodies to CDV across 10 of 12 packs, but no wild dogs under the age of two years had CDV antibodies. This pathogen can survive within exposed host species and shed for extended periods (Creel et al., 1997b). Thus, young animals may be more susceptible to CDV infection due to a lack of previous exposure and a lack of immunity against this pathogen when passive maternal protection declines (Creel et al., 1997b). Additionally, serological surveys show that African wild dogs can have high exposure to CDV, either from interactions with other species or wild dog populations, without evident signs of fatality (Creel et al., 1997b; Alexander et al., 2010). As such, this disease may pose a chronic threat in some populations but not in others depending on age, location, immune health, and proximity of wild dogs to other host species (Beineke et al., 2015). The survival of young animals plays a key role in the viability of the populations (Cross & Beissinger, 2001; Creel, 2005). Thus, it is important to target young, immunologically naïve animals when considering vaccination programs that aim to control this disease.

CPV appears to have low rates of mortality in adult African wild dogs but could pose a major risk to juvenile animals. Creel et al., (1997b) found 68% of African wild dogs that were surveyed were seropositive for CPV and exposure was widespread across packs in Serengeti National Park, Tanzania. However, no CPV antibodies were found in African wild dogs from Kruger National Park, South Africa (van Heerden et al., 1995; Prager et al., 2012; Flacke et al., 2013;). Although no direct evidence of CPV mortality exists thus far (Fuller et al., 1992), it is reasonable to assume that it may be the cause of high pup mortality in dens (Creel et al., 1997b; Woodroffe et al., 1997). In Serengeti National Park in 1994, CPV titres within adult African wild dogs suggested an epidemic in the area; this coincided with increased juvenile mortality and a 38% decrease in normal litter size (Creel et al., 1997b; Paris et al., 2024). During outbreaks, juvenile survival can decrease to less than 40% (Fuller et al., 1992; Burrows et al., 1994). It remains unknown whether CPV spills over from domestic dogs to African wild dogs or whether it is able to persist within African wild dog populations. However, there is some evidence of disease persistence in African wild dogs without domestic dog contact (Creel et al., 1997b). Comprehensive knowledge of how CPV effects African wild dog populations remains unknown. It is reasonable to suggest that it is an important cause of juvenile mortality. Studies in grey wolves suggest CPV does not appear to affect recruitment, but it inhibits recovery of populations by limiting pup survival (Mech & Goyal, 1993).

Rabies virus poses a threat to all mammals. In Africa, the domestic dog and yellow mongoose appear to be the principal host of this pathogen (Appel, 1987; Nel et al., 2005). In canids, rabies virus epidemics have large-scale effects on global species populations (Sillero-Zubiri et al., 1996). Rabies causes high levels of mortality in all mammals when infection occurs. Populations in Masai Mara National Reserve in Kenya, Serengeti National Park in Tanzania and Madikwe Game Reserve in South Africa all experienced extinction events due to rabies between the 1980s and early 2000s (Gasgoyne et al., 1993b; Kat et al., 1995; Hofmyer et al., 2000). Rabies virus is primarily transmitted through biting behaviour, which is indiscriminate when rabies infections occur (Hampson et al., 2009). Outbreaks of rabies virus within African wild dog packs are similar to those in domestic dogs (Baer & Walander, 1987). The rapid spread of the disease within African wild dog packs and high mortality rate makes the persistence of the virus within wild dog populations unlikely and spill-over into this species probably occurs from reservoir hosts in the environment (Woodroffe et al., 1997; Prager et al., 2012).

Due to the wide range of pressures this endangered species faces, there is a need to actively conserve the remaining populations within Africa and around the world. Conservation strategies should aim to remove or reduce the pressures of habitat fragmentation, anthropogenic mortalities, competition from other large carnivores, and decrease the chance of death from intra-species aggression and disease outbreaks.

1.4 Past and current conservation strategies

The low density of African wild dogs, especially outside protected areas, coupled with few dispersal routes between preferred habitat types, creates considerable obstacles for the survival of free-roaming packs. The development of programs across Africa aims to aid the conservation of African wild dogs. Cooperation between the IUCN and several African countries, has resulted in the development of regional conservation strategies which aim to reduce human-wildlife conflict, improve conservation, increase biological knowledge and reduce the negative impacts of continuing land development on African wild dogs (IUCN/SSC, 2007a, 2007b). These regional plans inform the development of specific national action plans by different countries.

1.4.1 Community awareness and education

Direct human persecution of African wild dogs has been the most substantial cause of population declines throughout Africa. African wild dogs were considered vermin until the late 1900s. As a result of increased knowledge about African wild dog ecology and behaviour, active persecution has declined (Creel & Creel, 1998). However, the suppression of African wild dog population growth due to human-wildlife conflict remains a significant obstacle.

The main issue faced by wild dogs is the perceived *vs.* real threat to farmers livelihoods in some communities. Preventative killing, negative attitudes and risk perception occur in agricultural and pastoral communities (Kissui, 2008; Mkonyi et al., 2017). African wild dogs do not preferentially predate on livestock but farmers' attitudes towards them remain negative (Davies & du Toit, 2004; Mkonyi et al., 2017). Nevertheless, increasing levels of formal education tend to correlate with increased positive views towards large carnivores (Mkonyi et al., 2017), highlighting the importance of community education to improve attitudes and tolerance to wildlife (Paris et al., 2024). A number of wildlife protection organisations, including the World Wildlife Fund, Wildlife Conservation, The Waterberg Wild Dog Initiative and the African Wildlife Foundation, are involved in community awareness and education programs aimed at protecting African wild dogs.

1.4.2 Captive breeding

Captive populations of African wild dogs exist in zoos around the world and are important to help maintain population numbers and genetic diversity of the species (Woodroffe et al., 1997). Captive populations safeguard against species extinction and can be used to reinstate or supplement wild populations (Woodroffe et al., 1997). Captive populations also play a key role in understanding the basic biology of the species, in which the lack of existing knowledge currently impacts their conservation management in the wild. Captive animals can be used to develop non-invasive monitoring tools, evaluate the safety and efficacy of vaccines, and perfect techniques and protocols for immobilisation, equipment design and stress management (Frantzen et al., 2001; Paris et al., 2024). Moreover, they serve to raise public awareness and funding for the species (Frantzen et al., 2001).

High rates of stress in captive African wild dogs (Crossey et al., 2020) may impact their reproduction and the social stability of packs (Paris et al., 2024). Despite vast improvements in welfare of captive African wild dogs, relatively high rates of pup mortality occur (Yordy & Mossotti, 2016; Riddell et al., 2021). Between 1993 and 2014 in captive North American packs, pup mortality was 53% (Williamson, 2013). In comparison, pup mortality was 63% in captive packs in South Africa, between 1954 and 1997 (Frantzen et al., 2001). These mortality rates are slightly higher than the 48% reported in free-roaming South African populations (Marneweck et al., 2022). The age and experience of the bitch and genetic relatedness of parents have been identified as contributing factors, with younger, primiparous/low experience and high degrees of genetic relatedness increasing the risk (Yordy & Mossotti, 2016). This high mortality may indicate a high level of inbreeding within captive populations (Wayne et al., 1997), but could also be the result of increased stress due to the captive environment. Female free-roaming African wild dogs experience significantly less stress than their captive counterparts (Van der Weyde et al., 2015; Crossey et al., 2020). This can be the result of a limited ability for captive African wild dogs to exhibit natural behaviours, such as dispersal from natal packs, which could lead to a destabilised social structure, and thus reduced co-operation and cohesion within the pack (Crossey et al., 2020; Riddell et al., 2021). These results highlight the need for captive management programs to consider natural drivers needed for successful breeding by the female, such as mate selection, genetic relatedness, age and experience.

Release of African wild dogs into new areas can increase local populations and aid the recovery of packs in ecosystems from which they have been lost. Captive-bred African wild dogs in South Africa have been used to supplement wild populations by releasing them into protected areas (Lindsey & Davies-Mostert, 2009). Initial reintroduction attempts in 1975 were unsuccessful and remained so for many years, largely due to captive-bred animals lacking necessary survival skills for the wild (Woodroffe & Ginsberg, 1998). Attempts to release packs composed of captive-bred coupled with wild-caught African wild dogs has resulted in greater success rates (Woodroffe & Ginsberg, 1998).

Large, fenced reserves provide a more natural free-living experience for African wild dogs while preventing human-wildlife conflict. However, only the largest reserves are able to provide adequate numbers of African wild dogs to be self-sustaining. Even within larger reserves, African wild dogs are known to range outside reserve boundaries and experience substantial edge effects (Woodroffe et al., 1997; Davies & du Toit, 2004). As such, it is important to establish cross-border parks and dispersal corridors to link reserves and buffer zones around national park borders (Jackson et al., 2017). It is also important to consider how populations can be managed in a way that facilitates natural dispersal and encourages both reproductive success and maximum pup survival in a free-roaming setting.

2. Translocation and artificial (new) pack formation strategies

The translocation and reintroduction of endangered animals has become a useful and effective conservation tool (Seddon et al., 2007). When translocating and reintroducing group-living species, it is important to consider the impact of social behaviour on success (Fischer & Lindenmayer, 2000). Holding group-living carnivores in captivity for a period of time prior to reintroduction may allow social integration within artificial groups (Gusset et al., 2006), and aid in acclimatization to and minimize dispersal from reintroduction zones (Miller et al., 1999; Briers-Louw et al., 2019). These management techniques are extremely useful to maintain genetic diversity and increase the numbers of African wild dogs, but are not without challenges and welfare concerns which need to be improved (Marneweck et al., 2019b).

2.1 The cost of genetic loss in endangered animals

Genetic variation refers to naturally occurring differences in gene alleles among individuals of the same species. This variation permits flexibility and adaptation in populations in the face of environmental change and is often considered an advantage that aids survival in the face of adversity (Charlesworth & Willis, 2009). In cases where populations are small and/or isolated, genetic divergence between different populations of the same species increases (Templeton et al., 1990; Frankham, 1996; Marsden, 2010). This leads to a reduction in genetic diversity due to high rates of genetic drift (a random decline in allele frequency within a population as a result of population bottlenecks or founder effects), inbreeding and lower rates of gene flow (the transfer of genetic variation between populations; Templeton et al., 1990; Frankham, 1996). When deciding management options for endangered species, it is important to consider the level of genetic diversity in the population and conditions that might limit gene flow.

Climatic changes in the mid-Holocene period corresponded to major genetic declines in many African mammals (Heller et al., 2008; Okello et al., 2008; Gebremedhin et al., 2009; Marsden et al., 2012). However, large genetic reductions have only occurred within the last 100 years in African wild dogs and have led to divergence of alleles according to location within Africa (Marsden et al., 2012). These genetic declines occurred concurrently with extensive habitat loss and deliberate hunting, highlighting the negative effect of unregulated anthropogenic factors on wildlife (Woodroffe et al., 1997). These human-related factors have limited the current African wild dog population to just 7% of their former range (Woodroffe et al., 1997; IUCN/SSC, 2009). The reduction in African wild dog habitat and subsequent development of population bottlenecks has led to declines in both neutral and adaptive gene alleles (Marsden et al., 2009; Leigh et al., 2012; Marsden et al., 2012; Paris et al., 2024). Not surprisingly then, genetic variation of African wild dogs is below that in other free-living canids (Marsden et al., 2009). Historical genetic drift has caused differentiation of neutral genetic markers between packs, with minimal gene exchange between Southern and Eastern African populations now evident, which has led to clear genetic structuring in these populations (Girman et al., 2001; Marsden et al., 2012). Despite this, meta-population management in Southern Africa may overturn the effects of genetic drift, showing that management of the species can help restore genetic diversity (Tensen et al., 2024).

When populations of African wild dogs exist in geographic proximity and in large continuous areas, there is a reduced likelihood of separate genetic clustering (Marsden et al., 2012). Despite inbreeding avoidance behaviour in African wild dogs, evidence for this is apparent based on genetic evaluations (Spiering et al., 2011; Leigh et al., 2012). In populations where there is inadequate dispersal success and little to no natural immigration into the area, reduced fitness may occur (Tensen et al., 2016). Reduced fitness as a result of genetic depletion has been demonstrated in other canids, through reductions in litter size and fertility, longevity, juvenile weight, occurrence of blindness and high rates of infection and disease (Laikre & Ryman, 1991; Peterson et al., 1998; Liberg et al., 2005; Fredrickson et al., 2007). It has recently been suggested that to maintain genetic diversity at 95% heterozygosity, and thus avoid inbreeding depression, in managed populations within South Africa at least two individuals would need to infuse their genetics into each fragmented population every two years (Tensen et al., 2024). Given the complexity of the African wild dog's social system, the limited habitat available, and the fact that most reserves are already at carrying capacity for African wild dogs, this would not be feasible for the majority of smaller reserves, which can often only sustain a maximum of one or two packs (Paris et al., 2024). Thus, there is a need to maximise genetic diversity through active management of populations where there are no easy dispersal corridors connecting wild dogs, in order to avoid genetic drift and inbreeding as well as promote continued species fitness. This presents several challenges in countries which have high human growth rates and where human-used lands encroach on natural habitats for this species which need to be tackled through conservation management initiatives.

2.2 Translocation and artificial pack formation in African wild dogs

The IUCN has defined translocation as “the deliberate and mediated movement of wild individuals from one part of their range to another” (IUCN, 1998). Translocation, which aims to create self-sustaining populations, as a conservation tool is increasingly used to augment declining or restore extirpated populations of animals (Armstrong & Seddon, 2008; Dickens et al., 2010). Translocations should aim to promote animal survival post-release and establish reproductive packs at the release location (Gosling & Sutherland, 2000; Letty et al., 2003; Teixeira et al., 2007). Translocations are often performed in conjunction with reintroductions into previously occupied habitats and form part of metapopulation management strategies for species whose habitat exhibits a high level of fragmentation.

Translocation can reinforce populations that are in decline to avoid genetic drift (Leigh et al., 2012). During translocation, familiarity between newly introduced animals reduces the chance of pack annulment after release (Woodroffe et al., 1997). When translocating animals that live in social groups, such as the African wild dog, it is necessary to consider strategies to form successful cohesive packs that will eventually lead to functional breeding groups (Marneweck et al., 2019a; Riddell et al., 2021).

Similarly, an important conservation strategy for African wild dogs in South Africa is the artificial formation of new packs for reintroduction into small-protected areas that can also supplement populations living outside protected reserves (Davies-Mostert et al., 2009).

Unfortunately, too few protected areas throughout Africa are large enough to maintain a viable African wild dog population (Woodroffe et al., 1997). As such, where natural dispersal corridors are lacking, reintroduction of artificially formed packs remains an important conservation tool to maintain genetic diversity among metapopulations (Davies-Mostert et al., 2009). This strategy requires the translocation of single-sex groups to release sites where they are joined with an unrelated opposite-sex group, then released together after the pack forms (Gusset et al., 2010). This aims to mimic the natural dispersal pattern observed in free-living African wild dogs (Creel & Creel, 2002).

Translocation and artificial pack formation of African wild dogs is believed to have begun in 1975, though early attempts were generally unsuccessful (Woodroffe et al., 1997). The captive-bred individuals involved lacked cohesive pack hunting skills (Woodroffe et al., 1997; Jule et al., 2008) and had no immunity to diseases present in the wild (Woodford & Rossiter, 1994; Cunningham, 1996). Wild-caught animals have greater success in reintroductions. However, overall success of this technique remains relatively low in African wild dogs outside fenced reserves where human-wildlife conflict exists (Gusset et al., 2008). Formation of packs of unfamiliar females with males occurs when animals test their compatibility with each other and, if incompatible, pack annulment occurs (McCreery, 2000). When social integration occurs, affiliative behaviours are observed between sexes. Formation of a stable unit may be indicated by an increase in animals resting in close proximity as well as a decrease in the rate of hoo-calls (long distance contact calls; McCreery, 2000; O'Riain et al., 2015). Artificial pack formation in African wild dogs involves confining unfamiliar animals in a boma (a temporary animal holding enclosure) prior to release. Time and reproduction within the boma can improve long-term stability of packs post release (Gusset et al., 2008). Group size can affect the amount of time required for stable packs to form within the boma, with smaller groups bonding faster than larger groups (O'Riain et al., 2015; Marneweck, et al., 2019). The cohesive formation of these new packs prior to release is critical to the success of metapopulation management.

2.3 Problems associated with translocation/new pack formation

The success of translocation, artificial pack formation and reintroduction is reliant on wildlife managers utilising evidence-based conservation practices. In a natural setting, when groups of dispersing African wild dogs meet, aggression can occur between or within opposite-sex groups of animals due to poorly established hierarchies (Creel et al., 1997). The use of bomas can increase social integration, with increased time spent bonding within an enclosure being correlated with pack cohesion (Gusset et al., 2008). Marneweck, et al., (2019) found that integration time did not differ between packs of males and females introduced immediately or after holding in adjacent pens. Integration time was more closely associated with group size and sex-ratio. When the proportion of females was higher than males, successful integration occurred with over a shorter time in the boma (Marneweck, et al., 2019). Negative interactions also decreased when animals were more familiar with the odour of new pack mates (Marneweck, et al., 2019). However, captivity is known to cause chronic stress in African wild dogs especially when captivity is prolonged, resulting in negative interactions (Crossey et al., 2020). During translocation of the Australian eastern bettong, delayed release resulted in increased faecal glucocorticoid metabolite concentration (Batson et al., 2016). Similarly, both temporarily and permanently captive African wild dogs experience significantly higher faecal glucocorticoid metabolite levels than their free-living counterparts (Van der Weyde et al., 2016; Crossey et al., 2020). Establishment of hierarchies is often accompanied by increased dominance and aggression which may be exacerbated and prolonged when animals are stressed (Creel et al., 1997a; Engelhard et al., 2000). Additionally, disruption of hierarchical bonds and temporary pack separation during translocation of established packs of captive African wild dogs often results in pack instability and thus could exacerbate the experience of chronic stress and heightened aggression (Scheepers & Venzke, 1995; Johnston et al., 2007; Vlamings, 2011). As such, it is evident that stress may intensify anti-social behaviour in this species which could lead to increased morbidity and mortalities. Furthermore, It is thought that prolonged confinement and artificial pack formation may result in chronic stress to such an extent that reproductive success post-release could be compromised (Marneweck, et al., 2019). While efforts have been made to understand how stress is experienced by African wild dogs, this remains a problem during artificial pack formations. As such research into strategies to mitigate stress and related aggression is warranted.

3. Stress and Aggression

Stress is a stimulus that disrupts the body's internal balance. Stress occurs as a pattern of events beginning with a stressful stimulus perceived by the brain, which in turn activates physiological mechanisms in the body as a response (Dhabhar & McEwen, 1997, 1999). Stress acts immediately through the sympathetic nervous system, initiating the release of catecholamines to mediate a fight-or-flight response (Dickens et al., 2010). This is followed by a hormonal stress response, which diverts energy stores and suppresses a number of physiological pathways (Dickens et al., 2010; Sheriff et al., 2011). Stress may be acute (lasting minutes to hours) or chronic (lasting days to months; Dhabhar, 2000; Riddell et al., 2021). Acute stress leads to the release of glucocorticoids, which are beneficial in the short term as they may enhance immunity at the level of the skin (Dhabhar, 2000), promote anti-inflammatory cytokines that prevent the immune system from mounting an over-reactive immune response (Robles et al., 2005), suppress aggression (Yohe et al., 2012), and divert physiological and behavioural body processes toward immediate survival requirements (Romero, 2004). However, these adaptations become detrimental during chronic stress, as a result of prolonged elevated concentrations of glucocorticoids.

Social species often experience stress during the establishment and maintenance of social hierarchies (Creel et al., 1997a; Engelhard et al., 2000). The response of an individual to stressful interactions, such as dominance and aggression, has important ramifications for survival and reproduction of that animal. Historically, it was thought that subordination in African wild dogs was accompanied by chronic stress leading to suppression of reproduction by cortisol-mediated negative feedback on the hypothalamic-pituitary-gonadal (H-P-G) axis causing physiological castration (Creel, 2005; Van den Berghe et al., 2012). A two-year study showed that dominant African wild dogs may have elevated cortisol levels compared to their subordinate pack mates (Creel et al., 1997a). Comparatively, several short-term and long-term studies have found no difference in cortisol levels between dominant and subordinate animals (de Villiers et al., 1997; Van der Weyde, 2013; Van den Berghe et al., 2019a). These conflicting observations could be explained by differences in pack structures, behaviour, captive status or threats encountered by dogs between the studies (Paris et al., 2024). Differences in baseline cortisol concentrations do not appear to affect sperm quality through suppression of the H-P-G axis, providing evidence against the physiological castration theory (Van den Berghe et al., 2019a). Glucocorticoid and androgen concentrations increase during the breeding season in African wild dogs and other social species, leading to decreased social unity and increased dominance and aggression from the dominant male or female that reduces mating opportunities by subordinates (Creel et al., 1997a; Dloniak et al., 2006). However, a year-round pattern of elevated glucocorticoids in dominant individuals across social species is not well understood but may be due to dominance, aggression, captivity, environmental disturbance or pack instability (Dickens et al., 2010; Teixeira et al., 2007). One inadvertent cost associated with African wild dog translocation is chronic stress resulting from human intervention (Fig. 1.2; Dickens et al., 2010), which may lead to increased aggression during artificial pack formation.

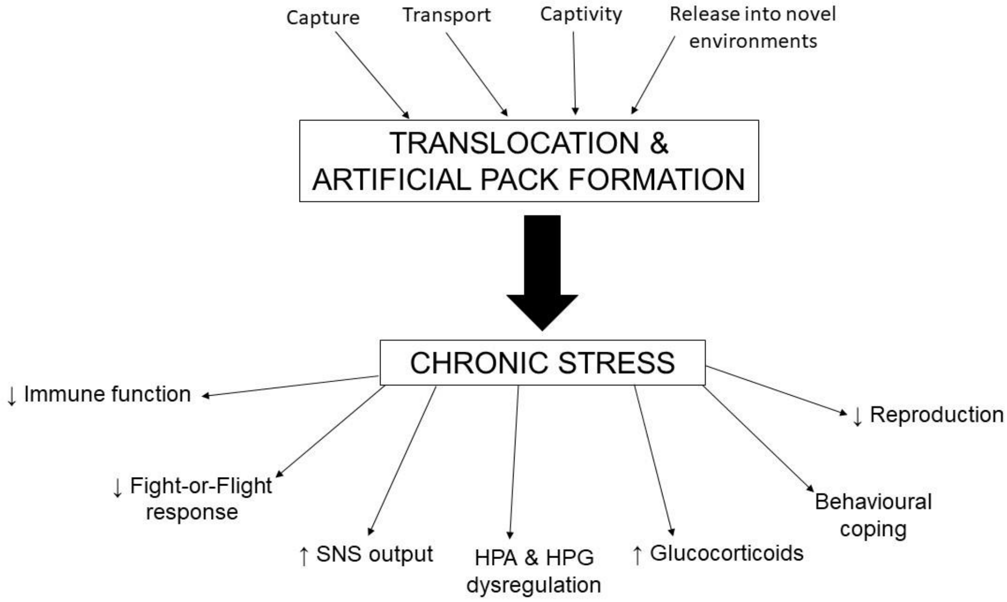


Figure 1.2. The main components of translocation and artificial pack formation can each lead to chronic stress in African wild dogs. Chronic stress in turn leads to a range of deleterious physiological, immunological and behavioural responses. SNS, sympathetic nervous system; HPA, hypothalamic-pituitary-adrenal axis; HPG, hypothalamic-pituitary-gonadal axis.

In most social group-living species, it is expected that negative interactions will occur during hierarchy establishment and maintenance. Increased aggression is often accompanied by an increase in androgens, including testosterone and androstenedione (Riddell et al., 2021). In female baboons, elevated androgens and concurrent increased aggressive behaviours necessary for hierarchy stability may place limits on fertility (Packer et al., 1995); whereby high androgen levels and aggression may lead to higher rank but might also interfere with reproduction and maternal behaviour (Creel, 2005). Furthermore, elevated androgens may interfere with the secondary antibody response to antigen exposure, leading to compromised immunity and fitness (Wingfield & Ramenofsky, 1999). The frequency of negative interactions could indicate levels of stress in animals. Aggression is essential to establish and maintain dominance hierarchies in African wild dog packs (Chase et al., 2002). During artificial pack formation in African wild dogs, stress induced by prolonged captivity of free-roaming animals could intensify aggression between unfamiliar groups resulting in injury or death (Marneweck, et al., 2019; Van den Berghe et al., 2019b). To understand the role chronic stress plays in aggression and its deleterious effect on immune function, it is important to understand the physiological mechanisms behind the stress response.

3.1 The H-P-A axis and cortisol

The hypothalamic-pituitary-adrenal (HPA) axis is one of a number of important mechanisms involved in the response to stress. In the presence of a stressor, neurons in the paraventricular nucleus of the hypothalamus release hormones; corticotrophin-releasing hormone (CRH) and arginine vasopressin, which are transported to the pituitary gland (Pasquali, 2012). This stimulates the anterior pituitary gland to secrete adrenocorticotrophic hormone (ACTH) into the circulation. ACTH induces glucocorticoid (cortisol and/or corticosterone) synthesis and release from the adrenal glands (Kirschbaum & Hellhammer, 1994). During acute stress, this mechanism is controlled by a negative feedback system (Fig. 1.3).

When the HPA axis is functioning normally, elevated glucocorticoids (GC) act by negative feedback to saturate hypothalamic receptors and terminate further CRH release (de Kloet et al., 1998; Wingfield & Sapolsky, 2003; Creel, 2005). During chronic stress, the negative feedback system becomes impaired, due to decreased glucocorticoid receptors on target cells in the hypothalamus and pituitary and does not effectively prevent the release of further glucocorticoids (Sapolsky, 1992). This persistence of elevated glucocorticoids in the peripheral circulation and prolonged binding to receptors on downstream target tissue throughout the body causes compromised energy storage, suppression of digestion and growth, as well as pathological suppression of the immune and reproductive systems (Sapolsky, 1992; Sapolsky et al., 2000).

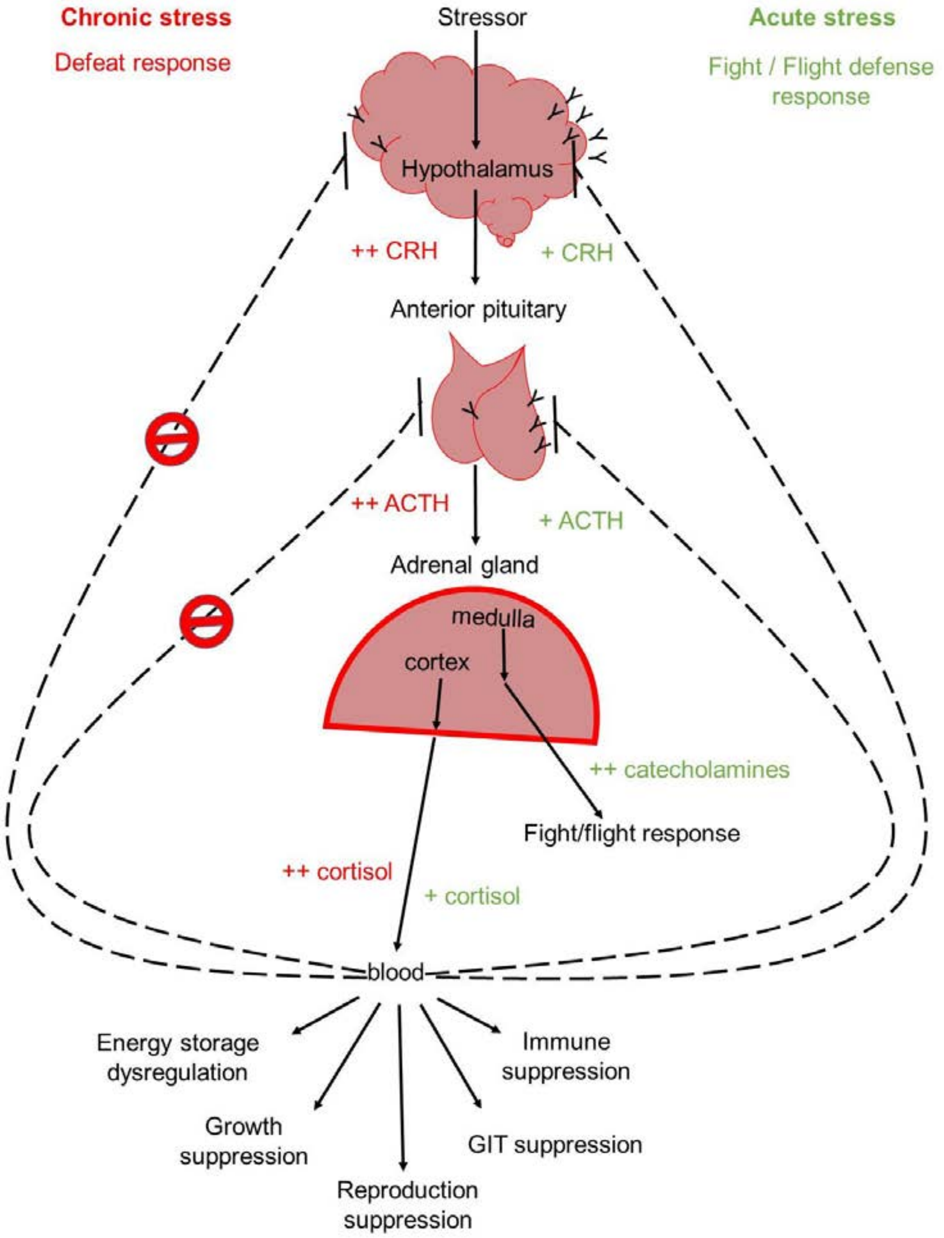


Figure 1.3. The hypothalamic-pituitary-adrenal (HPA) axis, its negative feedback response, and its effect on downstream body processes in response to stress. Activation of the HPA axis causes upregulation of hormones during acute stress (+) which is exacerbated during chronic stress (++). Furthermore, inhibition (θ) of negative feedback occurs during chronic stress caused by decreased glucocorticoid receptors (Y) on target cells in the hypothalamus and pituitary gland. CRH, corticotropic releasing hormone; ACTH, adrenocorticotropic hormone.

3.2 The H-P-G axis stress and aggression

A body of evidence exists showing that reproductive suppression in wild dogs is behaviourally mediated. Subordinate male wild dogs have spermatozoa of similar quality to dominant males and copulate, although at a lower frequency (Creel et al., 1997a; Van den

Berghe et al., 2019a), with multiple paternity litters relatively common (Girman et al., 1997; Spiering et al., 2010; Van den Berghe et al., 2012b). Subordinate females ovulate and have been known to produce litters both in captive and free-living populations (Van der Weyde et al., 2015). Mate guarding is observed in wild dogs where the dominant male uses aggression to guard the dominant female from subordinate males (Van Heerden and Kuhn, 1985). Reproduction is controlled by the hypothalamic pituitary gonadal (HPG) axis. The hormones produced in this physiological pathway are also known to regulate aggression in many mammals (Engelhard et al., 2000; Dloniak et al., 2006; Van den Berghe et al., 2019).

The HPG axis normally functions by secreting gonadotrophin-releasing hormone (GnRH) from the hypothalamus, which in turn stimulates the release of luteinising hormone (LH) and follicle stimulating hormone (FSH) from the pituitary gland (Plant & Zeleznik, 2014). These hormones are then transported via the blood to the gonads where they stimulate gametogenesis as well as the synthesis and release of sex hormones including testosterone, oestrogen and progesterone (Fig. 1.4; Plant & Zeleznik, 2014).

Increased activity of the H-P-G axis is associated with elevated androgen output (from the testes of males and ovaries and adrenals of females) that is strongly linked to aggression, especially around the breeding season (Engelhard et al., 2000; Dloniak et al., 2006; Van den Berghe et al., 2019; Riddell et al., 2021). During the breeding season, dominant male African wild dogs may exhibit higher levels of testosterone than subordinates (Creel et al., 1997a; Monfort et al., 1997; Johnston et al., 2007; Newell-Fugate et al., 2012; Riddell et al., 2021), although Van den Berghe et al., (2019a) found no difference in cortisol, androgens or fertility between captive males of different social rank.

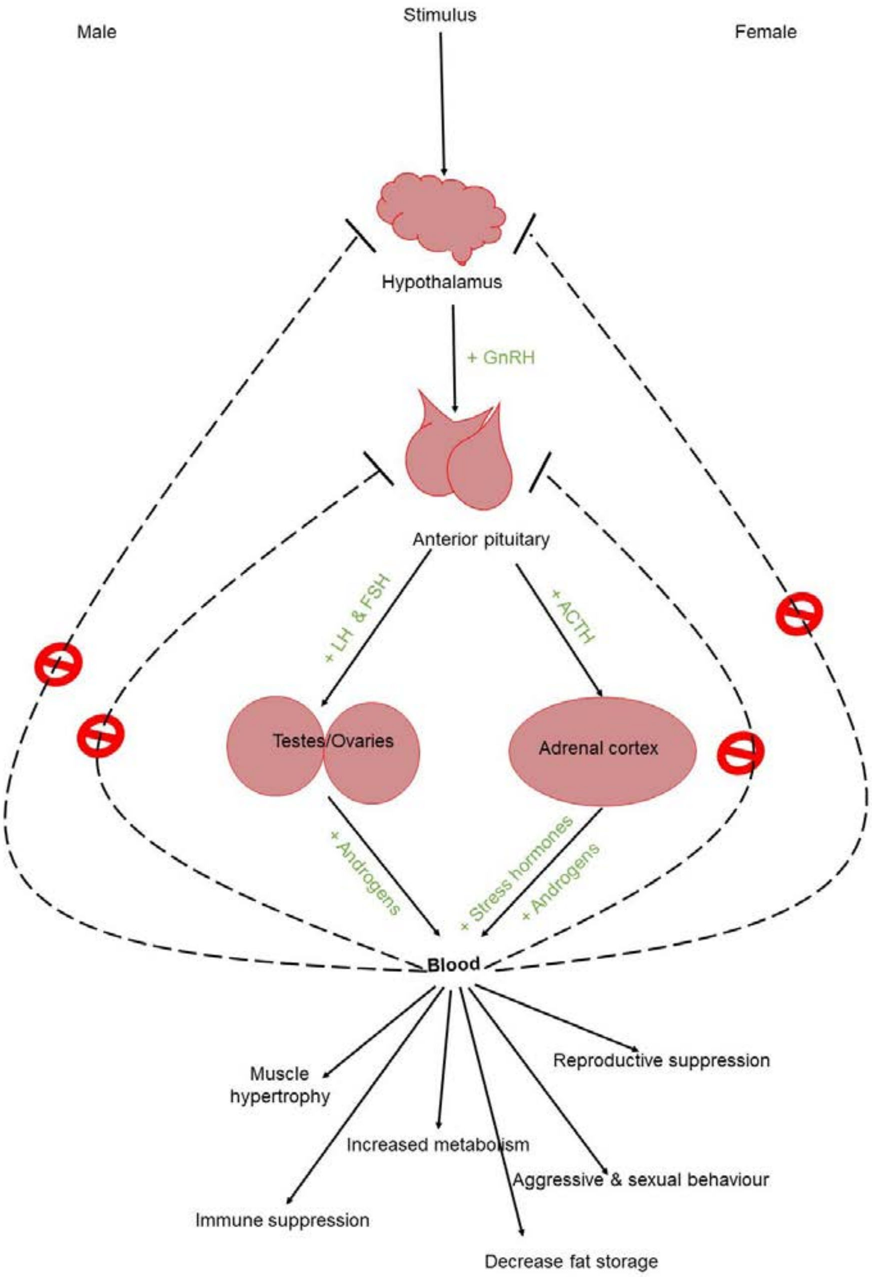


Figure 1.4. Upregulation of the hypothalamic-pituitary-gonadal axis leads to the synthesis of male and female sex hormones (+ androgens that also influence aggression) through a cascading effect that starts with upregulation (+) of GnRH from the hypothalamus which acts on the pituitary gland to upregulate LH and FSH or ACTH that activate androgen release from the reproductive organs or adrenal cortex respectively into the blood stream alongside increased stress hormones. Under acute aggression this process is controlled by negative feedback (⊥), which is inhibited (θ) during chronic aggression. GnRH, gonadotropin-releasing hormone; LH, luteinising hormone; FSH, follicle stimulating hormone; ACTH, adrenocorticotropic hormone.

There is a lack of research on the role of androgens in stress, aggression and reproduction of female wild dogs. In females, the ovaries and adrenal glands produce testosterone. The majority of testosterone produced by the ovaries is converted to oestradiol, the principal female sex hormone. Circulating androgens influence female aggression (Glickman et al., 1992). High testosterone in female mammals leads to increased aggression and is directly related to dominance ranking (Joslyn, 1973; Bouissou, 1983). During the breeding season, increased rates of conflict between female lemurs were associated with a two-fold increase in testosterone concentrations (Engelhard et al., 2000). In spotted female hyena, circulating testosterone is lower than in males, although the concentration of other androgens, such as androstenedione, may be higher (Glickman et al., 1987; Glickman et al., 1992). Androstenedione is a pro-hormone that is converted mostly to testosterone and oestrogen and in females is released into the bloodstream prior to conversion. Female ovarian steroids have been implicated in facilitating both reproductive function and aggressive behaviour and dominance (Glickman et al., 1992; Dloniak et al., 2006). However, most research in this field has compared male to female steroid concentrations, and comparison of steroid concentrations between female animals may be more informative.

There is a prevailing view that the HPA and HPG axes negatively feedback on each other, causing concurrent inactivation (Viau, 2002; Montoya et al., 2012). In some social species, elevated cortisol in subordinates is a common mechanism for reproductive suppression via the HPG axis (Creel, 2001; Van den Berghe et al., 2012a). However, African wild dog glucocorticoid concentrations do not differ between animals of different social rank in captivity (De Villiers et al., 1995; de Villiers et al., 1997; Van den Berghe et al., 2019a), and elevated cortisol levels reported in some studies of dominant males (Creel et al., 1997) do not appear to impair reproductive potential of African wild dogs (Van den Berghe et al., 2019a). In a limited number of human studies, both HPA and HPG hormones have been found to react at the same time (Bateup et al., 2002; Marceau et al., 2012). Co-elevation of cortisol and testosterone occurs in human males actively seeking a romantic partner (Gettler et al., 2011), and outcomes of competitive interactions may result in concurrent rise or fall of cortisol in the presence or absence of testosterone (Mehta et al., 2008). Elevated baseline levels of cortisol and concurrent elevation of testosterone during the breeding season may not impair reproduction in captive African wild dogs (Van den Berghe et al., 2019a), but chronic stress and aggression may cause reproductive suppression in this species (Van den Berghe et al., 2019b). As such, concurrent activation of these hormones may reflect complex social interactions rather than inhibition of either hormone production pathway (Van den Berghe et al., 2019a).

Elevated cortisol may increase the amount of risky decision making (Putman et al., 2010), while elevated testosterone leads to risk taking and anti-social dominant behaviour aimed at gaining high social status in humans (Eisenegger et al., 2010). The delicate interplay of behaviours and hormones in African wild dogs requires more research to properly understand the potential effects of translocation on these behavioural and physiological interactions. During the breeding season, African wild dogs experience stress more often but despite this mating behaviour and pregnancy rates remain high (Creel et al., 1997a). During translocations, breeding in the boma can often dictate pack outcomes (Gusset et al., 2006). However, first litters following new pack formation frequently have high pup mortality (Yordy & Mossotti, 2016), possibly due to the impact of chronic stress and reproductive inexperience of females.

3.3 The effects of chronic stress and/or aggression on the immune system

The immune system of mammals works on two levels, an innate and an adaptive immune response (Barnard et al., 1996; Baumgarth et al., 2005; Curno et al., 2009). The innate immune response is the body’s first line of pathogen defence (Medzhitov & Janeway, 1998; Mogensen, 2009). Cells that are activated during an innate immune response provide a general or non-specific defence action that occur within minutes or hours. These cells include granulocytes, natural killer cells and complement proteins (Sergestrom & Miller, 2004). Adaptive immunity produces a pathogen-specific, learned immune response. The activation of cells involved in adaptive immunity is much slower than the innate immune response and is triggered when pathogens persist for a longer duration or are encountered again after an initial exposure event (Maier & Watkins, 1998). Activated cells include lymphocytes such as T-helper cells, T-cytotoxic cells and B cells, which are antigen-specific but must proliferate over several days before mounting a full immune response.

The immune system responds to signals from multiple body systems, particularly from the nervous and endocrine systems (Sergestrom & Miller, 2004). During a stress response, the H-P-A system secretes brain peptides and pituitary and adrenal hormones that bind to specific receptors on white blood cells to regulate their distribution and function (Ader et al., 2001; Sergestrom & Miller, 2004). During chronic stress, the regulation and distribution of immune cells become dysfunctional. Stress-related immunosuppression can lead to increased vulnerability to disease (Ben-Eliyahu et al., 2000; Quan et al., 2001). Stressors that become chronic reduce the potential for the immune system to mount adaptive defences (Sergestrom & Miller, 2004).

Chronic stressors are associated with systemic immunosuppression and causes a decrease in almost all functional immune measures (Sergestrom & Miller, 2004). This increases an individual's susceptibility to bacterial and viral infections (Chao et al., 1990; Cohen et al., 1991b), delays wound healing (Marucha et al., 1998), and impairs immune response to vaccination (Glaser et al., 1992; Kiecolt-Glaser et al., 1996; Stammen et al., 2018a). Stress and/or negative emotional states, such as anger, activate the H-P-A axis to induce the release of GCs, which in turn strongly suppress immune cells such as pro-inflammatory cytokines and leukocytes (Lew et al., 1988; Angeli et al., 1999). The extent of cortisol induced suppression of the immune system may vary depending on whether acute or chronic stress is experienced (Sergestrom & Miller, 2004).

The association between aggression and immune system function is still unclear, however research has found that behavioural aggression may be positively correlated with immune cell function under certain conditions (Marsland et al., 2007). Acute episodes of anger in mice, induce pro-inflammatory cytokines, suggesting that the onset of aggression induces upregulation of the immune system (Takahashi et al., 2018). Aggression has adaptive significance for animals and can be critical for territory protection, food acquisition, maintenance of hierarchies and reproductive privileges. Mammals tend to exhibit increased immune function in more aggressive individuals. In wild olive baboons (*Papio Anubis*) of higher social standing, wound healing is faster than subordinates (Archie et al., 2012). More aggressive cynomolgus monkeys (*Macaca fascicularis*) have higher lymphocyte numbers when faced with threat of infection (Line et al., 1996), with low-ranked males having higher infection rates (Cohen et al., 1997b).

Adaptation of the immune system may be advantageous to animals that display acute levels of aggression and dominance but may become maladaptive in cases where aggression and dominance behaviours are chronic (Takahashi et al., 2018). The effect of continued aggressive behaviour from dominant animals on subordinates may result in over-activation of the immune system. In mouse models, repeated aggressive encounters caused increased serum pro-inflammatory cytokines in the loser as a result of chronic stress and anti-inflammatory cytokines in winners which had significantly lower cortisol levels (Stewart et al., 2015). When aggression is pathological and prolonged, elevated androgens can compromise immune function (Wingfield & Ramenofsky, 1999). In humans experiencing chronic stress, pathological aggression was common and related to delayed wound healing and increased activity of pro-inflammatory cytokines (Kiecolt-Glaser et al., 2005; Coccaro et al., 2014; Das et al., 2016; Takahashi et al., 2018). In African wild dogs, dominant animals tend to have higher baseline cortisol than subordinates as reported in some studies (Creel, 2001b, 2005). The way in which stress and aggression impact immune function is still not well understood for the African wild dog. Moreover, the effect chronic levels of cortisol have on immune function requires further investigation. Lastly, since behavioural aggression is an important social feature of this species, there is a need to further study the impact of this behaviour on animal health and wellbeing in order to increase the welfare of animals during the artificial formation of captive and wild packs (Takahashi et al., 2018).

3.4 The effect of stress and aggression on canid vocalisations

In domestic dogs increased rates of howling, barking and whining may be associated with human-related separation anxiety (Pongracz et al., 2017). Hearing the whines of familiar conspecifics also increases alertness and stress-related behaviours in domestic dogs; dogs may also offer more affiliative behaviours towards the whining individual (Quervel-Chaumette et al., 2016). In addition to changes in the rate of vocalisations during periods of separation anxiety, vocal parameters have also been found to increase in spectral noise (Marx et al., 2021). Similarly, in wolves, separation of pack members causes stress which is accompanied by increased howling that was elevated further when animals lost visual contact with each other (Mazzini et al., 2013). As such these results and those found in domestic dogs suggest that vocalisations would appear to be driven by the emotional and/or physical state of animals.

The breeding season of most canids is thought to be a time of increased stress, especially for species that use behaviours to mediate access to mates, such as the African wild dog and wolf. This season often brings about an increase in aggressive behaviours which may be accompanied by changes in the rate of vocalisations. In grey wolves there is an increase in the rate of howling during the pre-breeding season (McIntyre et al., 2017), while the rate of howling in Mexican wolves and golden jackals increase in the breeding season and during establishment of territories (Jaeger et al., 1996; Servin, 2000). Changes in the rate of howling may indicate changes in behaviours that accompany the transition into and during the breeding season such as increased aggression and social conflict in pack-living canids. As such, vocalisations may be a sensitive tool to measure social cohesion/disruption as well as seasonal changes.

The only vocalisation of African wild dogs that is well understood is the hoo-call, a long-distance vocalisation used to locate separated individuals and mediate willingness of dispersing groups to form a new reproductive pack (Robbins, 2000; Maneweck et al., 2019). This vocalisation may also be indicative of distress during separation (Robbins, 2000). Additionally, based on observations during vocal classification, some vocalisations may be context specific, while others appear to be used across a range of contexts. For example, attack twitters have been observed during the context of animal threats, while begging cries were observed during both social interactions within packs as well as during intraspecific threats (Robbins, 2000). Furthermore, African wild dogs exhibit the ability to negotiate with sufficient consensus (Walker et al., 2017). For example, significant input by subordinates into sneeze rallies alongside high energy greeting ceremonies is sufficient to override dominant animal preferences to initiate group departure for hunting or territory maintenance (Walker et al., 2017). Additionally, changes in physical behaviours of pack members have been noted to occur when other specific vocalisations are expressed, such as submissive behaviours being emitted with yelps during greetings, or alarm growls being given to warn pack members of potential threats (Robbins, 2000; Potgeiter et al., 2015). As such, it would appear that vocal communication is very important within African wild dog packs and may be a good indicator of pack cohesion and welfare during stressful interventions.

3.5 Current drugs/strategies to alleviate stress and aggression

A range of treatments are used to alleviate stress and aggression in domestic dogs (Riddell et al., 2021). These include behavioural modification training with or without pharmaceutical treatment (Ciribassi & Ballantyne, 2014), and appeasing pheromone treatment (Pageat & Gaultier, 2003). The use of these treatments may be applicable to varying degrees in captive and free-living wild canids, although behavioural modification is generally not feasible (for a comprehensive overview of these topics see Chapter 2; Riddell et al., 2021).

4. Pheromones and their effect on behaviour and physiology

In mammals, pheromones are natural chemicals that are released by one animal that modulate the behaviour and/or physiology of another animal of the same or related species (Karlson & Lüscher, 1959; Liberles, 2014). Pheromones modulate endocrine status, signal identity; and evoke a range of behaviours (Wyatt, 2017). In mammals, pheromones are detected by the olfactory system in sensory substructures that include the main olfactory epithelium (MOE), vomeronasal organ (VNO), Grueneberg ganglion and the septal organ (Tirindelli et al., 2009). The way in which these olfactory organs detect pheromones is explained in detail in Chapter 2.

Chemical components of a pheromone are highly specific to chemoreceptors, with each activating a single glomerulus, causing precise excitation of interneurons to initiate a specific behavioural response (Wyatt, 2017). The effect different pheromones have on neuroendocrine status are facilitated by neurons in the hypothalamus (Fleischer et al., 2007) and this is further explored in Chapter 2. Pheromones associated with stress and aggression act on paraventricular or GnRH neurons respectively, causing upregulation of stress and aggression hormones through the HPA and HPG axis (Tirindelli et al., 2009; Riddell et al., 2021).

4.1 Dog appeasing pheromone

Appeasing pheromones, secreted from glands located between the mammary glands of adult female domestic dogs shortly after parturition, appear to elicit an appeasing action in both pups and adults (Pageat & Gaultier, 2003). Biologically, this pheromone functions to calm and reassure newborn pups and is secreted until weaning occurs (Pageat & Gaultier, 2003; Mills et al., 2006). Appeasing pheromones have been isolated from a number of different species and are commercially available in synthetic form in some species to modify behaviour (Reviewed by Riddell et al., 2021).

Dog appeasing pheromone (often referred to as DAP and commercially sold as ADAPTIL®; Ceva Sante Animale, Libourne, France), has been one of a few canine pheromones successfully isolated and synthesised. This artificial congener of the natural dog appeasing pheromone acts to calm and reassure dogs in stressful situations. DAP has been reported to reduce behaviours associated with fear in puppies in new environments (Taylor & Mills, 2007), anxiety and stress in adult dogs during transportation (Pageat & Gaultier, 2003; Gandia Estellés & Mills, 2006), anxiety and fear in veterinary settings (Mills et al., 2006), sound-induced fear and anxiety (Landsberg et al., 2015), and reduce the stress-related behaviours of dogs in rescue shelters (Hermiston et al., 2018). DAP has been found to be as effective as pharmacological treatment for stress behaviours induced by separation anxiety in dogs (Gaultier et al., 2005). Dogs treated with clomipramine or DAP over a month-long period with a behavioural modification plan, all experienced a marked decrease in separation-related problems and over-attachment (Gaultier et al., 2005). Interestingly, anxiety-related aggression in dogs within veterinary practices was reduced when DAP collars (Mills & Hargrave, 2004), but not DAP diffusers were used (Mills et al., 2006). However, Mills et al., (2006) did not indicate the dosage of DAP released from diffusers nor the duration of DAP exposure in dogs, making it difficult to compare their results. This may suggest that DAP is only effective at a certain dosage, and that collars imbibed with DAP elicit a stronger effect than aerosolised DAP. While it has been shown that DAP has an effect on domestic dog behaviours associated with stress and aggression, most research is based on subjective owner reporting. To date, there are limited reports investigating the underlying physiological mechanisms influenced by this pheromone in domestic dogs, with one of two reports finding that cortisol, white blood cell counts, serum glucose, haptoglobin and C-reactive proteins during stressful veterinary stays were not modulated with application of DAP, but prolactin was decreased (Siracusa et al., 2010). In the second report, during veterinary visits heart rate, blood pressure, respiratory rate, rectal temperature, and cortisol were not modulated by application of DAP to dogs (Puglisi et al., 2022). Collectively though, the body of evidence in the domestic dog suggests that appeasing pheromones could potentially offer a non-pharmacological treatment for the alleviation of stress and aggression in African wild dogs, particularly during translocation and new pack formation in both captive and free-living populations.

4.2 Species-specific appeasing pheromones

Appeasing pheromones have been isolated in pigs, dogs, horses, cows, sheep and cats and contains the same three fatty acids at a ratio of 1:1:1: oleic acid, palmitic acid and linoleic acid – believed to be the core mammary appeasing message (Pageat & Gaultier, 2003). In addition to the core message other components, which are believed to be species-specific, act to excite selective sensory neurons in a species-specific manner to cause behaviour changes. These species-specific components always begin with myristic acid, but the ratio of fatty acids differs between species (Riddell et al., 2021). In domestic dogs, the species-specific component is made up of myristic acid, lauric acid, pentadecanoic acid and stearic acid (Pageat & Gaultier, 2003). It has been hypothesised that highly concentrated solutions of appeasing pheromone specific for one species may be expected to affect closely related species (Gaultier et al., 2005). In this regard, African wild dogs in captivity showed a higher rate of behavioural responses, including resting, directed towards DAP rather than placebo-treated areas of their enclosure (Vlamings, 2011). Despite these results suggesting that African wild dogs can recognise and react favourably towards DAP, they were not statistically significant, possibly due to a small sample size. A follow-up study performed with a larger sample size demonstrated that African wild dogs perceive DAP. Moreover, DAP significantly reduced contact-dominance behaviours associated with elevated aggression rates (Van den Berghe et al., 2019b). These results suggest that appeasing pheromones are not only attractive to African wild dogs but may ameliorate interactions associated with aggression.

The few published studies to date suggest that synthetic appeasing pheromones have little or no effect on the H-P-A axis. In domestic dogs, DAP exposure prior to surgery does not significantly reduce salivary cortisol concentrations in response to stress (Siracusa et al., 2010). Vlamings (2011) reported reduced faecal glucocorticoid metabolite (fGCM) levels in captive female African wild dogs after application of DAP collars. Compared to mean pre-treatment levels, reductions of 32% and 18% fGCM concentration after treatment occurred in two out of three females (Vlamings, 2011). In a follow-up study, DAP administered to male African wild dogs had no effect on rising fGCM associated with the stressful separation, immobilisation and reintroduction of individuals to packs (Van den Berghe et al., 2019b). However, DAP was found to suppress the rise of faecal androgen metabolites (fAM) compared to placebo-treated males during this stressful event. Moreover, in DAP-treated African wild dog packs, there was a significant shift toward lower rates of contact dominance and higher rates of non-contact dominance behaviour (Van den Berghe et al., 2019b). These findings suggest that there is an effect of DAP on the H-P-G axis and testosterone production, and that DAP may be a useful tool for decreasing agonistic interaction in African wild dogs during management interventions (Riddell et al., 2021).

Separation of African wild dog pack members in both captivity and free-living situations is sometimes required for management, although this practice can result in aggression between individuals when animals are reunited (Scheepers & Venzke, 1995; Johnston et al., 2007; Vlamings, 2011). This conflict can result in injuries and sometimes death (Vlamings, 2011). Reducing the testosterone surge experienced during reintroduction may help to mitigate problem behaviours during management practices such as veterinary interventions, translocations and artificial pack formations in both captive and wild meta-population management approaches. However, the high specificity of pheromones to each target species may reduce the effectiveness of domestic DAP in the African wild dog, as DAP may not excite all of the MOE and VNO selective sensory neurons required to create a complete pheromone information profile and thus would elicit an incomplete behavioural response.

A new pharmacophore DAP analogue has been developed using a bioinformatics approach and matching specific ratios of DAP chemical components to specificity of chemoreceptors in the domestic dog (Patrick Pageat, 2022, *personal communication*). It is expected that this new analogue will replace the current commercial ADAPTIL® formulation that has been shown to be effective at aggression management in African wild dogs. As such, this enhanced pheromone design modelled on dog olfactory receptors is thought to induce a more robust response in dogs and possibly African wild dogs. However, the way in which African wild dogs react to perceiving this pheromone requires investigation before it can be used as a tool to improve welfare of this species during stressful conservation interventions.

4.3 Testing the pheromone for behavioural modification effects

The measurement of changes in behaviours and hormones is an effective method of determining the effect of synthetic pheromones on animals, which can be achieved through direct observations and the quantification of hormones in body fluids and excretions (Wyatt, 2017). Some pheromones elicit both a releaser and primer effect requiring the measurement of both behavioural and physiological parameters respectively (Tirindelli et al., 2009). By contrast, primer pheromones generally require different bioassay techniques that focus specifically on the measurement of physiological responses such as hormone analysis (Wyatt, 2017).

In the case of appeasing pheromones, it can be expected that both an observable behaviour effect, such as decrease in dominance, aggression and submission behaviours, will occur in conjunction with physiological effects, such as reduced androgens and/or cortisol, similar to those observed by Van den Berghe et al., (2019b) and Vlamings (2011). Additionally, noticeable changes in vocalisations could occur alongside specific stress and aggression-related behaviours, which could be significantly alleviated by using appeasing pheromones. Blinded placebo-controlled perception trials and stress manipulation studies may help validate the effectiveness of phDAP relative to DAP for its subsequent application in free-living African wild dogs during translocations and artificial pack formation initiatives.

5. Implications of appeasing pheromones to improve immunity in African wild dog

When animals experience chronic stress, cortisol levels remain elevated and immune suppression may occur. Translocation and artificial pack formation cause chronic stress in African wild dogs (Creel, 2005; Gusset et al., 2010; Marneweck, et al., 2019). The link between chronic stress, immune suppression and increased susceptibility to infectious disease has been shown in numerous studies (Edwards & Dean, 1977; Chao et al., 1990; Cohen & Williamson, 1991a; Glaser et al., 1992; Kiecolt-Glaser et al., 1996; Stammen et al., 2018b). In a number of translocated African wild dog populations, increased mortality has been attributed to disease (Gusset et al., 2010). Environmental conditions may play a large role in pathogen presence, as most translocations and artificial pack formations in free-living African wild dogs occur into small, fragmented environments in South Africa (Davies-Mostert et al., 2009). Risk of exposure to pathogens is increased in these environments. Interaction with other wildlife and domestic dogs, which may act as disease hosts, is increased in small, fragmented protected zones due to proximity (Woodroffe et al., 2012). Releasing animals that are experiencing chronic stress and that have naïve immune systems or are vaccinated but have suppressed immunity may increase the risk of disease outbreak and mortality.

phDAP and/or DAP may bolster immunity by reducing physiological suppression of immune function caused by elevated androgens and/or cortisol. The reduced androgen surge found by Van den Berghe et al., (2019b) suggests that appeasing pheromones may decrease aggressive behaviours by acting on the H-P-G axis. Dysregulation of the H-P-A and H-P-G axis can lead to dysfunctional immune regulation as explained previously. To date, evidence that appeasing pheromones can directly modulate immune function has not yet been reported. Moreover, there is the added difficulty that current monitoring methods that require regular invasive blood samples to measure changes in antibody levels, are not viable in wild canids. Such repeated invasive sampling can cause additional stress that can both confound results and raise additional welfare concerns about conducting these studies. As such, non-invasive sampling methods are needed to monitor immune function in wild canids like the African wild dog.

6. Summary & thesis aims

Continued human intervention is required to maintain endangered African wild dog populations in captivity, and many protected areas and free-living meta-populations due to highly fragmented habitats (Paris et al., 2024). Human-assisted translocation and artificial pack formation strategies mimic natural dispersal patterns of African wild dogs across fragmented landscapes, where natural dispersal is unlikely to occur. These strategies help to increase genetic fitness of isolated populations and aim to increase the global African wild dog population, though these increases are slow (Davies-Mostert et al., 2009; Tensen et al., 2024). Despite encouraging results, translocation and artificial pack formation induce high levels of stress, aggression and risk pack annulment on release; these remain significant limiting factors to this conservation effort (Marneweck, et al., 2019). Furthermore, the stress of translocation and artificial pack formation may cause social disunity and dysregulation of immune function, making animals prone to disease. A newly developed pharmacophore dog appeasing pheromone (phDAP) is an enhanced formulation modelled on domestic dog olfactory receptors and is predicted to induce a more robust beneficial response, which may also apply to African wild dogs and is worth exploring given the promising effects of DAP in this species.

The overall aim of this thesis was to develop tools to measure and improve social cohesion and immunity in African wild dogs to improved welfare during stressful but necessary ongoing human management of this species. The aim of this thesis was achieved via a number of specific objectives that included:

(i) Develop a non-invasive, field-applicable assay for immune function;

(ii) Determine how African wild dog vocalisations change with different behaviours, contexts and housing;

(iii) Determine how African wild dog vocalisations change with season, stress and dog appeasing pheromone mitigation;

(iv) Compare the response to the perception of DAP *vs.* phDAP by African wild dogs.

Chapter 2: Literature review of appeasing pheromones for stress and aggression management in canids

Chapter No.	Details of publication(s) on which chapter is based	Nature and extent of the intellectual input of each author, including the candidate
2	Riddell, P.; Paris M.C.J..; Joone, C.J.; Pageat P.; & Paris D.B.B.P. Appeasing pheromones for the management of stress and aggression during conservation of wild canids: Could the solution be right under our nose?. *Animals* **2021**, *11*(6). https://doi.org/10.3390/ani11061574	The authors co-developed the research question. Riddell, P. wrote the first draft of the paper and developed the figures. The draft and figures where then revised with assistance from Paris M.C.J., Paris D.B.B.P., Joone C.J., and Pageat P.

Abstract

Thirty six species of canid exist globally, two are classified as critically endangered, three as endangered and five as near-threatened. Human expansion and coinciding habitat fragmentation necessitate conservation interventions and concurrent population deterioration. Current conservation management of wild canids includes animal translocation and artificial pack formation. These actions often cause chronic stress, leading to increased aggression and suppression of the immune and reproductive systems. Castration and pharmaceutical treatments are currently used to reduce stress and aggression in domestic and captive canids. Undesirable side effects make such treatments inadvisable during conservation management of wild canids. Pheromones are naturally occurring chemical messages which modulate behaviour between conspecifics; as such, they offer a natural alternative for behaviour modification. Animals are able to distinguish between pheromones of closely related species through small compositional differences but are more likely to have greater responses to pheromones from individuals of the same species. Appeasing pheromones have been found to reduce stress- and aggression-related behaviours in domestic species including dogs. Preliminary evidence suggests that dog appeasing pheromones (DAP) may be effective in wild canids. However, identification and testing of species-specific derivatives could produce more pronounced and beneficial behavioural and physiological changes in target species. In turn, this could provide a valuable tool to improve the conservation management of many endangered wild canids.

1. Introduction

Of the thirty-six species of Canidae, two are critically endangered (Darwin's fox, *Lyvalopex fulvipes*; and red wolf, *Canis rufus*), three are endangered (Ethiopian wolf, *Canis simensis*; African wild dog, *Lycaon pictus*; and dhole, *Cuon alpinus*) and five are near threatened (bush dog, *Speothos venaticus*; maned wolf, *Chryoscyon brachyurus*; sechuran fox, *Lycolapex sechurae*; short-eared dog, *Atelocynus microtis*; and island fox, *Urocyon litteralis*). Many other species of canid are rare with most populations in decline (Sillero-Zubiri et al., 2004). Increased human expansion is directly correlated with loss of natural habitat for wildlife (Farris et al., 2015). Most species of carnivore, including wild canids, naturally disperse long distances to find mates, prey and new territory and, as such, are at high risk from anthropogenic pressures (Farris et al., 2015). Human pressures include poaching, persecution, road/train accidents, disease transmission from domestic species and reduced available habitats (Woodroffe et al., 2007).

When small and/or isolated populations of a naturally dispersing species exist, the level of genetic divergence between each population increases due to increased inbreeding (Frankham, 1996). Reduced immigration and emigration between isolated habitats could rapidly lead to reduced species fitness as has been demonstrated in canids globally. In wild Scandinavian grey wolves (*Canis lupus*), pup survival through their first winter was reduced where inbreeding was increased (Liberg et al., 2005). Inbreeding in captive grey wolves negatively affected adult reproduction and juvenile weight as well as longevity of adult animals (Laikre & Ryman, 1991). Conversely, merging founding lineages of captive Mexican wolf (*Canis lupus baileyi*) increased fitness traits such as proportion of live births, litter size and pup survival (Fredrickson et al., 2007). In the African wild dog, Ethiopian wolf and Mexican wolf, the genetic variation of fitness-related genes in the major histocompatibility complex is low compared to that of other wild canids (Table 2.1; (Marsden et al., 2009). Such reductions in alleles are thought to be caused by genetic bottlenecks, which could lead to reduced individual fitness (Leigh et al., 2012; Marsden et al., 2009; Marsden et al., 2012).

Table 2.1. Comparison of the number of Major Histocompatibility Complex class II, DLA-DQA1 and DLA-DQB1 (dog leukocyte antigen-DQ α1 and β1 respectively) alleles found in different canid populations. (Adapted from Marsden et al., 2009).

Study Species	DLA-DQA1	DLA-DQB1	Reference
Domestic Dog: European purebred dogs *n* = >8000	18	47	Kennedy et al., (2001)
African wild dog: Eastern & Southern Africa *n* = 368	1	2	Marsden et al., (2009)
Grey wolf: Canada & Alaska *n* = 194	12	15	Kennedy et al., (2007)
Grey Wolf: Northern Europe *n* = 163	9	10	Seddon and Ellegren (2002)
Grey wolf: Total *n* = 407	18	21	Kennedy et al., (2001); Kennedy et al., (2007); Seddon and Ellegren (2002)
Mexican wolf: Captive American Population *n* < 7	5	3	Hedrick et al., (2000); Kennedy et al., (2001); Kennedy et al., (2007)
Ethiopian wolf: Bale Mountains Ethiopia *n* = 99	2	5	Kennedy et al., (2011)

Human conservation interventions such as maintenance of sustainable captive and wild populations aim to safeguard these species from extinction (Woodroffe et al., 1997). The risk of inbreeding can be overcome by the translocation of genetically valuable animals between fragmented populations (Armstrong & Seddon, 2008). However, only between 11% and 53% of bird and mammal translocations result in a self-sustaining population (Wolf et al., 1996; Fischer & Lindenmayer, 2000). A common problem limiting translocation success is animal dispersal away from the release site, which occurs in canids if they are unfamiliar with the area (Fritts et al., 1997). To reduce dispersal from release areas and facilitate pack cohesion prior to release in social canids, wild-caught animals are often temporarily held in captivity at the release site during the translocation process (Fritts et al., 1997). Stress has been recognised as a major constraint to the success of translocation in wild canids (Landa et al., 2017; Marneweck, et al., 2019). When translocated animals experience prolonged stress, a number of problems arise including reduced social cohesion, poor pair-bonding, reduced reproduction, increased rates of infanticide, immune suppression, appetite suppression, and energy mobilisation at the cost of energy storage (Vrekoussis et al., 2010; Sheriff et al., 2011; Van den Berghe et al., 2012). In this review, the consequences of translocation will be discussed in terms of stress and aggression, as well as current management options and emerging pheromone treatments to mitigate these effects. Moreover, the physiology of pheromone perception, species-specificity and the mechanism by which appeasing pheromones modulate stress and aggression will be reviewed.

2. Conservation management of wild canids

Metapopulation management, where spatially isolated groups of individual animals are translocated between fragmented habitats (Wells & Richmond, 1995), has been proposed as a useful method for recovery planning of threatened canids. This strategy has been recommended for the Ethiopian wolf, red wolf, grey wolf and African wild dog (Laikre & Ryman, 1991; Mills et al., 1998; Moehrenschlager & Somers, 2004; Simonis et al., 2018). The extent of activities in each of these programs may differ depending on goals, release techniques and environmental aspects (such as predator/prey abundance, size of existing populations, and size of release site; (Sillero-Zubiri et al., 2004). Both successful and unsuccessful reintroduction attempts have been previously documented in grey wolves, red wolves, Mexican wolves, African wild dogs and the swift fox (Moehrenschlager & Somers, 2004). Most successful reintroductions are supported by local landowners, and involve holding animals in captivity prior to release to acclimatise animals and promote breeding soon after release (Moehrenschlager & Somers, 2004). Many wild canids have been released and monitored in Europe and America but ongoing management of populations between fragmented habitats appears limited, in part due to limited funding and population stability (Moehrenschlager & Somers, 2004).

Currently the only wild canid actively managed as a metapopulation is the African wild dog, a highly endangered canid which in the last century has been limited to just 7% of its historical range (IUCN/SSC, 2009; Woodroffe et al., 1997). This reduction in available habitat has been accompanied by mass persecution and hunting which has reduced the wild population from an estimated 500,000 to approximately 6,600 adult animals (Woodroffe & Sillero-Zubiri, 2012). Given that Kruger National Park is the only suitable conservation area large enough to support a self-sustaining population in South Africa, a metapopulation approach has been adopted to manage other fragmented habitats (Mills et al., 1998; Davies-Mostert et al., 2009). This is achieved by managing a series of small, isolated sub-populations as a single population through the movement of individuals between suitable areas (Davies-Mostert et al., 2009). This approach involves the translocation and artificial pack formation of animals between habitats, thereby mimicking natural immigration and emigration patterns (Davies-Mostert et al., 2009). Today, managed wild populations of African wild dogs have shown greater pup and yearling survival and annual population growth than is recorded in established and unmanaged populations (Davies-Mostert et al., 2015). As one of a few wild canids that are actively managed, the African wild dog presents a unique opportunity to review the effect of such conservation intervention on behaviour and physiology.

2.1. Translocation of wild animals

Translocation is the deliberate and mediated movement of wild individuals from one part of their range to another (IUCN, 1998). This technique is increasingly used to augment populations that are in decline or to restore extirpated populations of animals (Dickens et al., 2010). Translocations aim to create self-sustaining populations, and the ability for the animal to survive through the establishment phase strongly affects the outcome (Armstrong & Seddon, 2008). Translocation objectives should include (i) animal survival post release; (ii) settlement in the release location; and (iii) reproduction of released animals in the area of translocation (Gosling & Sutherland, 2000; Letty et al., 2003; Teixeira et al., 2007).

Historically, there have been a number of attempts to translocate both captive-bred and wild-caught canids. In the United States, most attempts to supplement existing grey wolf populations with wild-caught animals throughout the 1970s were unsuccessful due to human persecution and other anthropogenic factors (Weise, 1975; Fritts et al., 1985). The red wolf was reintroduced into North Carolina through translocation from 1987 to 1995, resulting in dispersal over large areas and successful establishment of a thriving population (Phillips, 1995). In Canada, the swift fox was reintroduced using captive-bred and wild-caught animals, with mixed results (Carbyn et al., 1994). Initial African wild dog translocation attempts were unsuccessful, largely due to the release of captive-bred animals which lacked the necessary survival skills for the wild (Woodroffe & Ginsberg, 1998). More recently, attempts to release captive-bred with wild-caught individuals has resulted in greater success rates (Lindsey & Davies-Mostert, 2009; Woodroffe & Ginsberg, 1998).

2.2. Artificial pack formation

Artificial pack formation involves the temporary capture of dispersing animals and their subsequent joining with unfamiliar conspecifics of the opposite-sex in order to form a reproductive unit (Gusset et al., 2010). Such pack formations can be used to either supplement populations or re-establish a species within a habitat (Seddon et al., 2007). Artificial pack formation increases the genetic diversity of isolated populations, ensuring continued species fitness (Woodroffe & Ginsberg, 1999). Releasing canids as pre-formed packs increases translocation success, with animals more likely to reproduce and form territories within the release site, thus increasing survival post release (Shier, 2006). Temporarily holding grey or red wolves in family groups or pairs prior to release into novel environments results in greater pack stability and retention at the release site than for wolves released as individuals (Fritts et al., 1997; Bradley et al., 2005; McLellan & Rabon Jr, 2006). It is important to consider integration strategies when introducing unfamiliar animals to each other in order to facilitate the formation of new social groups prior to release (Gusset et al., 2006).

The technique of holding animals in temporary enclosures (boma) prior to release has proven useful in a range of carnivores, ensuring animals remain within the release site (Fischer & Lindenmayer, 2000; Hayward et al., 2007; Trinkel et al., 2008; Buk et al., 2018). In Norway, Artic fox (*Vulpes lagopus*) are released as litters at eight months of age, or as opposite-sex pairs from captive breeding programs following habituation in natural enclosures. This approach has resulted in successful re-establishment of populations in the Alpine region of Norway (Landa et al., 2017). It has been observed that African wild dogs released together with the aim of pack formation regularly disperse away from one another unless social cohesion occurs prior to release (Woodroffe et al., 1997). Young African wild dogs naturally disperse away from natal packs as single-sex groups during their first year of sexual maturity (McNutt, 1996; Davies-Mostert et al., 2015), and search for groups of unrelated animals of the opposite-sex with which to form a new pack (Marneweck, Becker, et al., 2019). Initial contact between unfamiliar dispersing groups is often marked by aggression due to lack of established dominance hierarchies (Creel et al., 1997a). These interactions will either result in pack formation or continued dispersal. Holding unfamiliar males and females in a boma allows them to test their compatibility with each other, resulting in either the formation of an established pack or pack annulment (McCreery, 2000). When opposite-sex groups successfully form packs separate male and female dominance hierarchies are formed. The alpha pair have almost exclusive mating rights and subordinates help raise young, guard territory and hunt (Creel et al., 1997a; Girman et al., 1997; Creel & Creel, 2002).

Artificial pack formation is often performed in conjunction with translocation, with the aim of mimicking their natural dispersal pattern, and forming socially cohesive packs that will eventually lead to functional breeding groups (Creel & Creel, 2002). However, one of the largest limiting factors to the success of artificial pack formation and translocation is stress, caused by captivity, and related aggressive behaviours which can result in injuries and mortalities (Marneweck, Marchal, et al., 2019; Crossey et al., 2020). During translocation, chronic stress results from disruption of hierarchical or familial bonds, temporary pack separation and periods of captivity involving human husbandry, causing social instability (Scheepers & Venzke, 1995; Johnston et al., 2007; Vlamings, 2011). Moreover, many social species often experience stress during the establishment and maintenance of social hierarchies due to increased dominance behaviours such as fighting and aggression (Creel et al., 1997a; Engelhard et al., 2000). The boma design, size of groups brought together, and the way in which animals are brought together should be designed to minimise the length of time it takes for cohesive packs to form, while also providing all the basic necessities to animals.

Boma construction can include either single or split compartments depending on financial and human resources available (Marneweck, et al., 2019). The type of boma (1- or 2-compartment) and length of time spent within varies greatly at different translocation sites, though management of animals is performed so as to facilitate rapid social cohesion (Marneweck, et al., 2019). The amount of time spent within the boma may depend on group size, with larger groups requiring more time to form a stable pack regardless of boma design (O'Riain et al., 2015; Marneweck, et al., 2019). Conversely, smaller groups brought together appear to socially integrate more rapidly in single compartment bomas (Marneweck, et al., 2019). Odour familiarity has been found to decrease aggression related to social integration (Marneweck, et al., 2019), and it is now standard practice to rub wild-caught African wild dogs against each other while immobilised (Marneweck, et al., 2019). Sex ratio can also impact social integration with a higher proportion of females to males resulting in more rapid pack formation (Marneweck, et al., 2019). When social integration occurs and a cohesive pack forms between opposite-sex animals, affiliative behaviours can be observed including resting in close proximity (McCreery, 2000; O'Riain et al., 2015) and reduced long distance calls (O'Riain et al., 2015). However, all forms of captivity result in higher levels of stress hormones in African wild dogs and this is increased in wild-caught animals compared to captive-bred individuals (Crossey et al., 2020).

3. Stress and aggression during conservation management

When a stressful object or event is perceived/experienced, it results in a stress response which causes physiological and behavioural changes (Dickens et al., 2010; Sheriff et al., 2011). The magnitude and length of these changes is controlled by the hypothalamic-pituitary-adrenal (H-P-A) axis (Sheriff et al., 2011). This pathway regulates the release of glucocorticoids such as cortisol and/or corticosterone (Kirschbaum & Hellhammer, 1994). The consequences of glucocorticoid release include increased energy mobilisation at the cost of energy storage, and the suppression of growth, reproduction, immunity and the inflammatory response (Sheriff et al., 2011). Episodes of stress and aggression can either be acute (lasting minutes to hours) or chronic (lasting days to months; Dhabhar, 2000). During acute stress these adaptations are beneficial with a diversion of physiological and behavioural processes towards immediate survival (Romero, 2004). However, prolonged activation of the H-P-A axis during chronic stress results in detrimental suppression of physiological processes and subsequent behavioural coping, such as displays of heightened aggression (Sapolsky, 1992; Sapolsky et al., 2000).

Increased aggression is often correlated with an increase in the androgen hormone testosterone and the hormones dehydroepianderosterone (DHEA) and androstenedione (Goymann et al., 2001). Testosterone is produced under the influence of luteinising hormone, the level of which is controlled by the hypothalamic-pituitary-gonadal (H-P-G) axis. Upregulation of H-P-G axis activity is associated with elevated testosterone levels which has been linked to both male and female aggression, especially during the breeding season in social species (Engelhard et al., 2000; Dloniak et al., 2006; Van den Berghe et al., 2019b). Prolonged elevated levels of testosterone and cortisol are directly correlated with behaviours such as increased aggression and reduced levels of parental care/bonding, which negatively impact both male and female reproductive success, as well as dysregulate immune function in a number of different species (Sundqvist et al., 1984; Torres-Calleja et al., 2001; O'Neal et al., 2008; Kalantaridou et al., 2010).

The conservation management of wildlife often requires the capture, handling and temporary housing of animals in captivity, all of which may induce stress (Dickens et al., 2010). For example, delayed release during the translocation of eastern bettongs resulted in increased faecal glucocorticoid metabolite concentrations (fGCM; Batson et al., 2016). It is thought that translocation in swift fox caused chronic stress, which negatively impacted their post-release survival (Waters, 2010). Moreover, in one study, stress was thought responsible for driving self-inflicted physical injury during translocation of grey wolves (Fritts et al., 1997). Despite this, there is limited monitoring of parameters of physiological stress described in the literature during conservation management of wild canids even though behavioural distress is commonly reported (Fritts et al., 1997; Waters, 2010; Landa et al., 2017).

Interestingly, captive African wild dogs show significantly higher stress-related fGCMs than their free-living counterparts (Van der Weyde et al., 2015). Similarly, permanent and temporary captivity result in similar fGCM concentrations; both of which are higher than in free-living African wild dogs; although this study relied on a single deafecated sample, and did not report the length of time animals had been kept in temporary captivity prior to sample collection (Crossey et al., 2020).

In the African wild dog, increased glucocorticoid concentrations in blood are directly correlated with increased aggression (Creel, 2005). During the breeding season male African wild dogs experience higher glucocorticoids concurrent with higher testosterone levels, with dominant males having higher levels of both hormones than subordinates. (Creel et al., 1997a; Monfort et al., 1997; Johnston et al., 2007; Newell-Fugate et al., 2012). The level of aggression displayed by dominant animals is directly related to attainment and maintenance of dominance hierarchies; with alpha animals having elevated glucocorticoids year-round compared to subordinate pack mates (Creel, 2001b; Van der Weyde et al., 2015). Furthermore, dominant animals are more aggressive and fight more than subordinates during periods of mating and, as such, the breeding season is a suboptimal time for translocation and artificial pack formation (Creel et al., 1997a). This in part is due to the observation that high levels of aggression after group integration can lead to mortalities during artificial pack formation (Potgieter et al., 2015).

3.1. Impact on reproduction

Translocation stress induced by suboptimal conditions during temporary captivity in Arctic fox was thought to cause a failure of females to enter oestrus during the first and second breeding season after release (Landa et al., 2017). In *ex situ*-bred red wolves, fGCM levels can directly influence the frequency or propensity to engage in breeding behaviour, with females observed to engage in copulatory ties having low baseline fGCM (Franklin et al., 2020). Interestingly, fGCM levels are higher in dominant grey, Iberian (*Canis lupus signatus*) and Ethiopian wolves as well as African wild dogs compared to subordinates, but this does not appear to reduce a dominant pair's reproductive potential (Kreeger et al., 1992; Barja et al., 2008; van Kesteren et al., 2012; Van den Berghe et al., 2019a). Furthermore, semen quality in

African wild dogs does not appear to differ between dominant and subdominant males (Van den Berghe et al., 2019a). Despite a lack of evidence to suggest that increased fGCM levels, as a result of stress, physiologically inhibit reproductive potential in canids, elevated fGCM levels could influence parental care and reproductive behaviours, such as mate-guarding, with aggressive behaviours leading to reduced litter success (Creel et al., 1997a; Kreeger et al., 1992; Barja et al., 2008; van Kesteren et al., 2012; Van den Berghe et al., 2019a). It is thought that prolonged confinement and artificial pack formation of canids may result in chronic stress to such an extent that reproductive success post-release could be compromised (Marneweck, et al., 2019). Captive African wild dogs have relatively high rates of pup mortality despite improved husbandry and veterinary care (Yordy & Mossotti, 2016). According to the North American Studbook, mortality of entire litters and individual pups was 52% and 53% respectively between 1993-2013 (Williamson,2013). In South African captive breeding programs, individual pup mortality is as high as 63% (Frantzen et al., 2001). Factors that may contribute to such high mortality rate include age of females at first breeding, the number of previous litters by the female and level of inbreeding between captive packs (Wayne et al., 1997; Yordy & Mossotti, 2016). Similarly, in the red wolf, primiparous and multiparous females have greater reproductive success and lower baseline fGCM than nulliparous females (Franklin et al., 2020). Furthermore, litter success is relatively low with only 20% of captive females producing litters in 2016 compared to 31% among *in situ* populations (Franklin et al., 2020). As mentioned previously, female African wild dogs held in permanent captivity experience significantly more stress than free-living counterparts (Van der Weyde et al., 2015; Crossey et al., 2020). This can be attributed to their limited ability to exhibit natural behaviours, which may destabilise the social structure and co-operation and cohesion within the pack, resulting in reduced reproductive success (Crossey et al., 2020).

Both acute and chronic stress can reduce levels of parental care in non-canid species. In non-human primates, stressors, including lack of social support, crowding and being the recipient of aggression, can increase the rate of infant abuse (Troisi & D'Amato, 1994; Maestripieri & Carroll, 1998a, 1998b; Saltzman & Abbott, 2009). Human mothers that experience depression engage more often in negative and disengaging behaviours towards their children and have lower rates of positive behaviours compared to non-depressed mothers (Lovejoy et al., 2000; Windham et al., 2004). Abusive parents often have an overly sensitive stress response to infant distress (McCanne & Hagstrom, 1996). In males, concurrent high levels of cortisol and testosterone are negatively associated with quality of caregiving (Bos et al., 2018). Thus, high levels of stress may also be associated with reduced parental care in wild canids, however, more research is needed to determine whether a correlation exists in any species of canid. Despite the apparent stability of African wild dog packs, abandonment of pups from first litters does occur, especially when pack hierarchies remain unstable (Potgieter et al., 2015), or when mothers have limited experience (Yordy & Mossotti, 2016). Monitoring of packs after release is often limited due to practical constraints and, as such, hierarchy instability and litter abandonment may be under-reported and may also contribute to pup mortalities in artificially formed packs.

3.2. Impact on immune function

Vulnerability to disease is increased by stress-related immunosuppression in animals (Ben-Eliyahu et al., 2000; Quan et al., 2001). Chronic stressors cause a decrease in almost all functional immune measures as a result of chronically high levels of cortisol (Sergestrom & Miller, 2004). This increases an individual's susceptibility to infection (Chao et al., 1990; Cohen et al., 1991b), delays wound healing (Marucha et al., 1998), and impairs the immune response to vaccination (Glaser et al., 1992; Kiecolt-Glaser et al., 1996; Stammen et al., 2018a). The degree to which cortisol suppresses the immune system may vary depending on the duration of stressors (Sergestrom & Miller, 2004). During acute stress, brief natural stressors elicit a short lived stress response and thus immune suppression is short-lived. When chronic stress is experienced, the inability to predict when a stressor will end, such as during translocation and captivity, leads to long term immune suppression (Sergestrom & Miller, 2004). In cases of chronic aggression, the immune system may become maladaptive (Takahashi et al., 2018). Prolonged elevation of androgens can compromise immune function (Wingfield & Ramenofsky, 1999) and is associated with delayed wound healing, dysregulation of cytokines at wound sites and heightened pro-inflammatory cytokines and other immune cells (Kiecolt-Glaser et al., 2005; Coccaro et al., 2014; Das et al., 2016; Takahashi et al., 2018).

Chronic aggression and stress leading to immune system dysregulation and suppression during translocation of canids could impact the ability for animals to cope with pathogen threats in the habitat they are released into. A number of pathogens are of concern globally to canids such as canine parvovirus, canine distemper virus, and rabies virus. Canine parvovirus (CPV) has been identified in grey wolves and coyotes in Yellowstone National Park though seroprevalence suggests that disease-induced mortality is low, even in young animals (Almberg et al., 2009). Canine parvovirus has limited risks for adult African wild dogs but can result in a 38-40% reduction in litter size and pup survival during outbreaks (Creel et al., 1997b; Fuller et al., 1992). Canine distemper virus (CDV) has caused high pack mortalities and population eradications of African wild dogs (Creel et al., 1997b; Goller et al., 2010; Flacke et al., 2013), though some adults have been found with antibodies to this disease (Creel et al., 1997b; Alexander et al., 2010). Similarly, CDV outbreaks in Ethiopian wolf populations typically cause mortality in sub-adults and juveniles (Gordon et al., 2015). In Yellowstone National Park, CDV outbreaks affect both grey wolf and coyote populations, particularly pups (Almberg et al., 2009; Almberg et al., 2011). Lastly, rabies virus outbreaks have caused high rates of pack mortality and population eradication in African wild dogs (Gasgoyne et al., 1993a; Kat et al., 1995; Hofmeyr et al., 2004). In Ethiopian wolves, rabies caused mortality of over 70% of packs and has previously been the cause of local population extinctions (Sillero-Zubiri et al., 1996; Haydon et al., 2002; Randall et al., 2004; Randall et al., 2006; Marino et al., 2013). The risk of infection, outbreak and mortality resulting from pathogens of concern could be exacerbated during translocations and artificial pack formations due to immune suppression arising from chronic stress.

It has previously been proposed that the stress of immobilisation, radio collaring and in particular, vaccination could compromised the immune system of African wild dogs; this erroneous belief stems from reports of mortalities and animal disappearances shortly after vaccination of African wild dogs (Burrows, 1992; Gascoyne et al., 1993; Burrows et al., 1994; Kat et al., 1995; Woodroffe, 2001). These disease outbreaks occurred prior to onset of vaccine immunity and unmanipulated packs also suffered pathogen-related mortality during these outbreaks (Woodroffe, 2001). More research is required to determine how stress impacts the immune response of wild canids during conservation interventions.

4. Management of stress and aggression

Given the serious consequences of chronic stress and aggression in canids during conservation activities, it is important to consider ways in which these can be alleviated. In domestic dogs, castration is a common tool to reduce aggressive behaviour (D'Onise et al., 2017). Gonadectomy of aggressive male and female dogs resulted in 61% and 53% gentler dogs respectively (Takeuchi et al., 2000). Permanent (gonadectomy) and reversible (GnRH vaccination) castration has been used in African wild dogs housed in European zoos with debatable effectiveness (Richard Barnes, Personal communication). However, the use of castration to ablate aggressive behaviour in endangered wildlife is counterintuitive when often the goal is to breed from as many of these genetically-valuable animals as possible. Other methods of behavioural control may be more nuanced and efficacious.

4.1 Pharmaceuticals

In domestic dogs, pharmaceutical treatments for anxiety include the administration of a range of different psychoactive drugs such as fluoxetine (a selective serotonin reuptake inhibitor; Dodman et al., 1996), tricyclic antidepressants, benzodiazepines (a nervous system depressant), and buspirone (an azapirone neuroleptic; Simpson & Papich, 2003). Of these drugs fluoxetine is the most commonly prescribed due to its proven efficacy for treating anxiety and aggression (Simpson & Papich, 2003). These medications are prescribed to reduce the behavioural implications of stress due to separation anxiety, phobia to thunderstorms, fear and hospitalisation (Ciribassi & Ballantyne, 2014; Gilbert-Gregory et al., 2016). However, these drugs can have undesirable side effects such as diarrhea, vomiting, sedation, hypotension, agitation, ataxia, and excitement (Simpson & Papich, 2003).

The use of pharmaceuticals to manage stress and aggression during wildlife management is controversial. The administration of midazolam and azaperone to wild-caught mule deer did not reduce physiological (heart rate, blood oxygen saturation, body temperature), hormonal (fGCM, serum cortisol) or behavioural (vocalisations, kicking) stress (Ortega et al., 2020). Furthermore, observations of hesitation, stumbling and falling after release from drug-induced lethargy of mildly tranquilized deer could compromise animal welfare (Ortega et al., 2020). Fluoxetine has been reportedly administered to Asiatic bears (*Ursus thiberanus*; Jeong et al., 2019), brown bears (*Ursus arctos*; Uchida et al., 1998; Yalcin & Aytug, 2007), polar bears (*Ursus maritimus*; Poulsen et al., 1996) and Bengal tigers (*Panthera tigris tigris*; Baker, 2002) housed in zoos to treat anxiety and pacing behaviours. Reports indicate that during drug administration pacing behaviours are reduced and amount of time spent resting is commonly increased; a possible sign of drug-induced lethargy (Poulsen et al., 1996; Yalcin & Aytug, 2007; Ortega et al., 2020). Administration of fluoxetine to control aggression and excessive mounting behaviours in castrated male red-necked wallabies (*Macropus rufogrisues*) resulted in mild sedation for several days after administration and was effective at reducing aggression only when administered daily for a number of months (Olds, 2017). European zoological institutions have used fluoxetine hydrochloride and sedative drugs during introduction and translocation of African wild dogs (Vlamings, 2011). Such treatments did not appear to reduce stress in most situations (Vlamings, 2011) and their sedative effects could be detrimental to social interactions between pack mates thereby destabilising established hierarchies. In domestic dogs, a positive relationship was found to exist between cortisol and DHEA pre- and post-fluoxetine treatment. However, aggressive dogs' serum cortisol, DHEA and serotonin remained higher than control animals post-treatment and dogs that had high serum serotonin concentrations pre-treatment showed poorer improvement in behaviours, cortisol, DHEA and serum serotonin than control dogs (Rosado et al., 2011). In rats, Fluoxetine has other side effects including low libido, and delayed or inhibited ejaculation (Hueletl-Soto et al., 2012). In summary, drug-induced side effects such as sedation, reduced reproductive potential and change in mounting behaviours could alter dominance hierarchies and social cohesion in wild canids during conservation interventions, making pharmaceutical options for stress management inadvisable.

4.2. Pheromones

Pheromones are naturally occurring chemicals that are released by an organism to modulate the behaviour and/or physiology of conspecifics (Karlson & Lüscher, 1959). They offer a novel natural alternative for the management of animal behaviours. Pheromones are able to regulate endocrine status, signal individual identity, and evoke sexual, nurturing and aggressive behaviours (Liberles, 2014; Wyatt, 2017). In mammals, pheromones are excreted from almost all glands and are detected by the olfactory system (Liberles, 2014). They are highly specific to chemoreceptors and a single olfactory glomerulus is activated by each individual chemical component of the pheromone, causing excitation of interneurons in a precise combination to initiate a particular behavioural response (Wyatt, 2017).

While the mechanism by which pheromones are perceived in mammals is not completely understood, it is thought that the main olfactory and vomeronasal systems, along with additional olfactory organs, are involved in pheromone detection (Tirindelli et al., 2009; Liberles, 2014). These systems possess a similar histological organisation, possessing primary sensory neurons that project axons to mitral cells (second-order neurons) contained in specific regions in the main olfactory bulb (MOB) or the accessory olfactory bulb (Tirindelli et al., 2009; Liberles, 2014). Primary sensory neurons of the main olfactory epithelium (MOE) are responsible for transduction of certain pheromones to the mitral cells of the MOB (Spehr et al., 2006; Wang et al., 2006; Liberles, 2014). In the MOB mitral cells project to multiple higher centres in the brain including the amygdala and the piriform cortex. The MOB is the primary target region of olfactory neurons (Tirindelli et al., 2009; Liberles, 2014). The laminar organisation of this bulb is comprised of a superficial nerve layer with axonal projections of chemosensory neurons. A first-order synaptic region exists between sensory neurons and mitral cells, represented by a glomerular layer. Soma of mitral/tufted and granule cells reside in the external and internal plexiform layer (Meisami & Bhatnagar, 1998; Tirindelli et al., 2009;Liberles, 2014). Within the MOB, glomeruli are anatomically separated and encapsulated by peri-glomerular cells (Tirindelli et al., 2009; Liberles, 2014). Axons of olfactory sensory neurons express a particular odorant receptor which converges on only two glomeruli in the MOB (Ressler et al., 1993; Vassar et al., 1993; Mombaerts et al., 1996; Tirindelli et al., 2009; Liberles, 2014). Mitral/tufted cells within the MOB bear only one apical dendrite which extends to certain brain regions, thus they transmit signals from glomeruli to pyramid neurons in the olfactory cortex, bypassing thalamic relay (Fig. 2.1; Meisami & Bhatnagar, 1998; Tirindelli et al., 2009; Liberles, 2014).

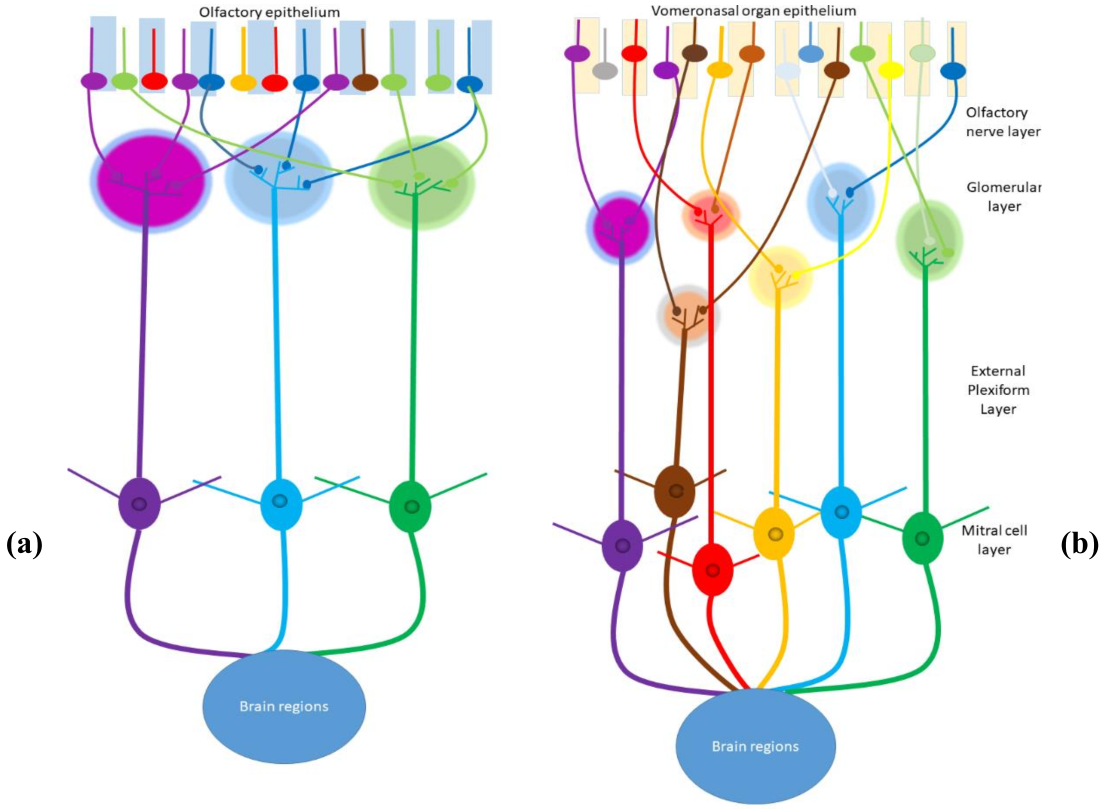


Figure 2.1. Schematic representation of sensory projections in the (A) main olfactory epithelium (MOE) and (B) vomeronasal organ (VNO). A: Within the MOE, olfactory sensory neurons in the olfactory epithelium expressing the same specific odorant receptor have axons that innervate to the same glomerulus (represented by the different colours: green, blue and purple), which in turn excite specific mitral cells to act on specific brain regions. B: within the VNO, sensory neurons expressing the same vomeronasal receptor innervate multiple small glomeruli, which excite mitral cells and thus specific brain regions. Each colour represents a population of vomeronasal sensory neurons, each of which expresses one different type of vomeronasal receptor. Adapted from (Ennis et al., 2007).

Unlike the separation between the MOE and the MOB, the vomeronasal epithelium is part of the vomeronasal organ (VNO) which is recessed with detection relying on pheromone molecules being dissolved in nasal mucus and being sucked into the lumen of the organ (Døving & Trotier, 1998; Tirindelli et al., 2009; Liberles, 2014). Access of stimuli to the lumen is modulated by vasodilation/constriction of blood vessels and sinuses lateral to the lumen

(Meredith & O'connell, 1979; Meredith et al., 1980; Meredith, 1994; Salazar et al., 2008), which is under hormonal control and can be actively modulated by some pheromones (Zancanaro et al., 1997). Vomeronasal sensory neurons are found in a pseudostratified epithelium in the basal and apical aspects of the vomeronasal organ (Liberles, 2014). Pheromones cause membrane depolarisation and increase the action potential firing rate of sensory neurons in the VNO (Taniguchi et al., 2000; Spehr et al., 2002), however, the signal transduction cascade is largely unknown. Signals from the VNO sensory neurons excite a given vomeronasal receptor and send axonal projections towards the anterior and posterior accessory olfactory bulb. Neurons that excite the same receptor have axons that merge into 10–30 glomeruli (Tirindelli et al., 2009). Differentially, inputs from neurons expressing different receptor types are received by one glomerulus (Tirindelli et al., 2009; Liberles, 2014). Mitral cells of the accessory olfactory bulb connect to the medial amygdala, posteromedial cortical amygdala, the accessory olfactory tract and the bed nucleus of the stria terminalis (Von Campenhausen & Mori, 2000; Tirindelli et al., 2009). Ultimately both the MOE and VNO pathway projections lead to specific areas of the amygdala and the hypothalamus to modify the behaviour and endocrinology of animals (Fig. 2.2).

The effect pheromones have on neuroendocrine status are mediated by the hypothalamus (Fleischer et al., 2007). Pheromones that act on the H-P-A axis generate action potentials along paraventricular neurons of the hypothalamus, and repeated exposure to stress causes increased expression of receptors that act to release corticotrophin releasing hormone (CRH; Tirindelli et al., 2009). Activation of the H-P-A axis by pheromones causes the synthesis and release of vasopressin and CRH, which act via the pituitary and adrenal glands to trigger a cortisol response in the presence of a stressor (Fig. 2.2; Tirindelli et al., 2009). Pheromones that activate the H-P-G axis activate gonadotropin releasing hormone (GnRH) neurons which lead to the stimulation of luteinising hormone and follicle stimulating hormone to stimulate the development and function of gonads and output of testosterone and other androgens that modulate aggression (Fig. 2.2; Engelhard et al., 2000; Dloniak et al., 2006; Plant & Zeleznik, 2014; Van den Berghe et al., 2019a). Pheromones can also downregulate the H-P-A and H-P-G axes by reducing paraventricular nucleus receptor expression and limiting anterior pituitary gland release of luteinising hormone and follicle stimulating hormone, thereby reducing stress and aggression (Pageat & Gaultier, 2003; Tirindelli et al., 2009). Pheromones which act on GnRH neurons are believed to control endocrine responses in the hypothalamus (Silverman, 1988; Meredith, 1998; Tirindelli et al., 2009). Studies using genetic approaches have concluded that the GnRH neurons in the hypothalamus are synaptically connected to thousands of neurons in over 50 diverse brain areas, including the main olfactory regions (Boehm et al., 2005; Yoon et al., 2005). This suggests that GnRH neurons may influence a large variety of brain functions and thus neuroendocrine status (Tirindelli et al., 2009).

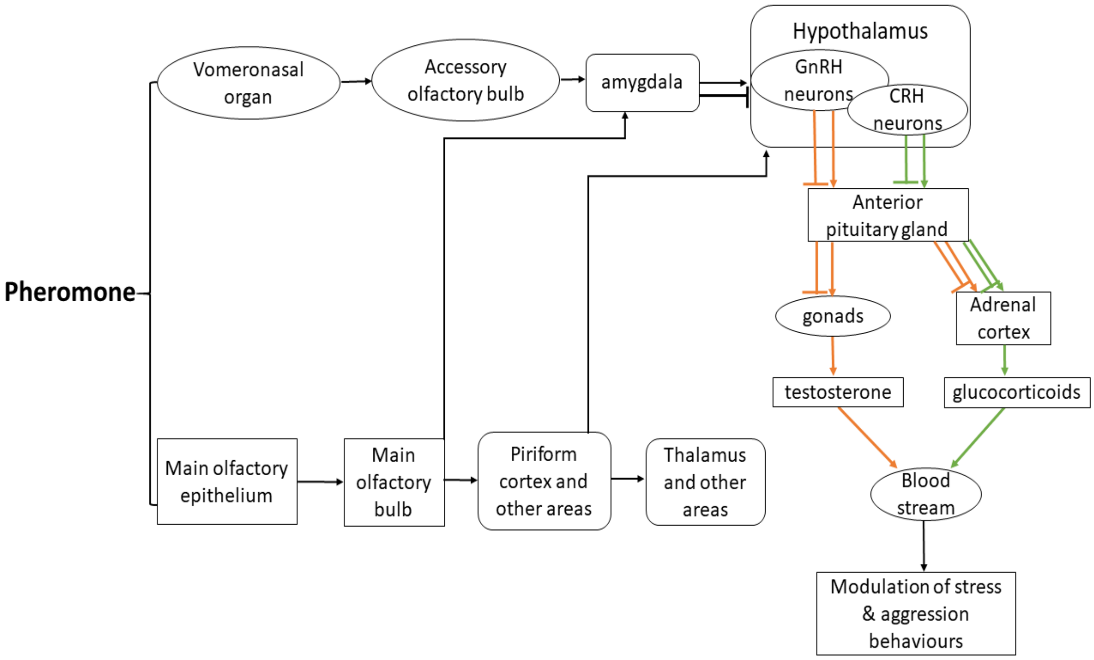


Figure 2.2. Pheromone perception pathways from the main olfactory epithelium and vomeronasal organ, through the main and accessory olfactory systems, and thalamic and hypothalamic higher brain regions, ultimately leading to a hormonal and/or behavioural response. Stress pathway upregulation (↓) or suppression (⊥); aggression pathway upregulation (↓) or suppression (⊥). Adapted from (Tirindelli et al., 2009).

Structurally, pheromones can be made up of small volatile molecules, steroid derivatives, peptides, or large protein-ligand complexes (Liberles, 2014). Pheromone-containing secretions, such as urine, sweat, saliva and tears, contain natural product blends which can be vastly complex (Liberles, 2014). Pheromones are chemically diverse although closely-related species often emit pheromones which are structurally similar (Weiss et al., 2015). Despite this, different ratios and components that constitute the same pheromone in different species contribute towards its species-specificity, preventing cross-reaction (Buchinger & Li, 2020). In many fish species, preference for water that previously held conspecifics compared to heterospecific odours provides strong evidence that conspecific odours rather than visual cues are used to mediate species recognition (Sorensen & Baker, 2015). In sea snakes, female lipids excreted from the skin create chemical cues that control male courtship behaviour, and the species-specific nature of such pheromones within these lipids prevents hybridisation by discouraging courtship behaviours between closely-related species (Shine et al., 2002). Social insects are able to distinguish between known and unknown conspecifics due to differences in pheromone and individual odour profiles (Wyatt, 2017). In mammals, chemical signals are thought to be unique to each species due to the high level of potential interference from odours of other species within the environment (Apps et al., 2015). Vomeronasal type-1 receptors (V1R) are partly responsible for pheromone detection in mammals and are responsible for mediating species-specific responses. The V1R in carnivores has between 50-90% similarity with domestic cats, cows and humans, which may help to explain how closely related species may distinguish pheromones of conspecifics and heterospecifics (Durairaj et al., 2019). Despite this, pheromones that have conserved chemical signatures across species can generate a response in the olfactory receptors or even processing in the central nervous system of closely related species (Apps et al., 2015). However, differences in genes of the vomeronasal organ and main olfactory epithelium have been found between species and subspecies, which may cause pheromones of closely-related species to be less effective (Apps et al., 2015; Grus & Zhang, 2008; Isogai et al., 2011; Niimura et al., 2014).

5. Pheromones for conservation

Research about pheromones of large carnivores is limited and to date has focused on territory-related pheromones in urine and faecal marks (Apps et al., 2012; Apps, 2013; Brahmachary & Poddar-Sarkar, 2015). Pheromones that mark territory could be used as spatial signalling compounds or 'bio-boundary markers' to prevent dispersal into agricultural land where large carnivores may face persecution (Apps et al., 2012). Targeted exposure of African wild dogs to species-specific scent marks from foreign individuals resulted in packs moving away from marks towards the centre of their defined home range (Jackson et al., 2012). While detection of volatile components of biological secretions has become more common (Asa & Valdespino, 1998; Apps et al., 2012; Apps, 2013; Brahmachary & Poddar-Sarkar, 2015), there is a lack of research on the behavioural and physiological changes triggered by these pheromone messages. Among the limited studies, most research has focused on the effect of domestic animal pheromones on behaviour and/or physiology in captive wildlife (Vlamings, 2011; Pageat et al., 2014; Van den Berghe et al., 2019b). Preliminary data suggest that pheromones could be useful to control or alleviate undesirable behaviours in wild canids.

5.1. Appeasing pheromones

Appeasing pheromones are chemical messages that are commonly released by lactating females, which act biologically to calm and reassure newborn animals (Pageat & Gaultier, 2003; Mills et al., 2006). Species-specific appeasing pheromones have been identified and isolated from a number of different domestic species (Pageat & Gaultier, 2003) and have been reported to reduce aggression and fighting in cats, dogs, pigs and horses (McGlone & Anderson, 2002; Falewee et al., 2006; Mills et al., 2006; Cozzi et al., 2010; DePorter et al., 2019). Furthermore, in pigs, this pheromone may increase feed intake as well as reduce fighting related lesions (McGlone & Anderson, 2002). In dogs, it reduces signs of stress such as whale eyes, licking, yawning, vocalisation and fearful postures (Mills et al., 2006).

The appeasing pheromone can be identified according to a conserved appeasing ratio of oleic, palmitic and linoleic acids. In addition to this core appeasing message, species-specific components begin with myristic acid and are composed of other fatty acids with variable ratios across species. A basic composition of oleic acid, palmitic acid, linoleic acid and derivatives thereof can have an appeasing effect in all mammals, however, preferred embodiments with enhancer pheromone composition for different species have been identified (Table 2.2; Pageat, 2002). These enhancer compositions are species-specific in mammals—they act to enhance or act synergistically with the core pheromone message and increase their behavioural and physiological effectiveness in a target species (Pageat, 2002).

Table 2.2. Comparison of different fatty acid ratios that constitute the appeasing pheromone identified for different mammals (Adapted from Pageat, 2002).

Component	Canine % (w/w)	Porcine % (w/w)	Goat % (w/w)	Bovine % (w/w)	Ovine % (w/w)	Equine % (w/w)
Oleic acid	21.5-27.8	24.7-36.8	20.1-22.3	24.9-28.6	32.8-38.8	35.2-40.3
Palmitic acid	20.8-24.9	15.5-26.8	22.3-26.8	19.2-23.1	21.6-25.9	22.8-26.7
Linoleic acid	20.5-25.4	29.5-40.6	20.2-22.5	20.5-24.3	21.2-25.7	22.1-27.1
Myristic acid	2.2-3.9	3.9-9.6	8.5-10.1	3.2-5.6	3.4-5.9	2.0-2.8
Lauric acid	0.4-1.8	2.8-8.7	11.4-14.8	1.9-4.2	2.6-4.4	2.3-3.7
Pentadecanoic acid	1.8-3.1					
Cholesterol	10.2-18.6					
Capric acid		0.5-3.5				
Squalene			9.5-11.2			
1-docosanol 2,2-dimethyl 1,3-dioxolane 4-methanol				18.4-22.8	7.4-9.7	4.4-6.7

*w/w = weight for weight

5.2. Dog appeasing pheromone

The commercially-available dog appeasing pheromone (ADAPTIL® Previously DAP; Ceva Sante Animale, Libourne, France) has been reported to reduce behaviours associated with fear and anxiety in puppies entering new environments (Taylor & Mills, 2007) and in adult dogs during transport, veterinary settings, thunderstorms and rescue shelters (Pageat & Gaultier, 2003; Gandia Estellés & Mills, 2006; Mills et al., 2006; Hermiston et al., 2018; Landsberg et al., 2015). Treatment with ADAPTIL® has been found to be as effective as pharmaceutical treatments for the reduction of stress-related behaviours that occur as a result of separation anxiety (Gaultier et al., 2005). This pheromone can be administered as an imbibed collar, diffuser or aerolite spray. A blinded, placebo-controlled study of the effect of ADAPTIL® on stress reduction in dogs subjected to a thunderstorm simulation found that while all dogs showed stress behaviours above baseline, the fear response between thunder noises was reduced in dogs with ADAPTIL® collars compared to placebo collars (Landsberg et al., 2015). Imbibed collars have been found to reduce anxiety-related aggression more effectively than diffusers, although duration of exposure may affect these results since collar studies often exposed animals to ADAPTIL® for 24 hours prior to behaviour analysis (Mills & Hargrave, 2004; Mills et al., 2006). By contrast, when dogs were only exposed to diffused ADAPTIL® in a veterinary setting 7 minutes prior to examination, no reduction in stress and aggression behaviours occurred, although their duration was reduced and the frequency of relaxed states increased (Mills et al., 2006). In a blinded placebo-controlled dog trial, ADAPTIL® released from diffusers for 2 days prior to parturition and for 3 weeks post-partum in maternity kennels increased the amount of time mothers nursed in a laying position and spent in close contact with their pups (Santos et al., 2020). Despite promising behavioural results, most studies rely on subjective observation, coupled with behavioural measures that can be less sensitive than physiological measures of stress (Koolhaas et al., 1999; Mills et al., 2006). While it is possible to infer emotional states from indirect measures, such as behaviour, any given state can be expressed by a variety of behaviours (Mills et al., 2006). Additionally, much of the literature on the use of ADAPTIL® lack baseline measures for individual animals (with some work relying entirely on owner surveys) or lack a blinded experimental design that would mitigate the introduction of observer bias (Gaultier et al., 2005; Gandia Estellés & Mills, 2006).

Limited reports have investigated the underlying physiological mechanisms affected by appeasing pheromones, but preliminary research suggests that, with the exception of one study (Vlamings, 2011), synthetic appeasing pheromones do not appear to affect the H-P-A axis to reduce stress—even if species-specific (Yonezawa et al., 2009; Siracusa et al., 2010; Van den Berghe et al., 2019b). These studies used a pheromone spray application method which, as stated previously, may not be as effective if not continuously released for extended periods of time. However, recent cross-species research suggests that the appeasing pheromone may work via the H-P-G axis at the level of testosterone, thereby reducing aggression and coping behaviours during stress (Van den Berghe et al., 2019b). Nevertheless, based on preliminary data by Vlamings (2011), it remains to be determined whether species-specific appeasing pheromones may be more effective in suppressing the H-P-A pathway. Thus collectively, species-specific appeasing pheromones could be a useful tool to modify both the stress and aggression pathways in wild canids during conservation interventions.

5.3. Application of dog appeasing pheromones to wild canids

Currently, the African wild dog is the only wild canid in which appeasing pheromones have been tested, and initial administration of dog appeasing pheromone in this species has shown some promise. Captive African wild dogs appear to perceive ADAPTIL® and exhibit a higher rate of favourable behavioural responses at the treated area such as resting, sniffing, urinating, licking mandibular, and panting with tongue out of mouth during perception studies (Vlamings, 2011). To further determine perception and behaviour modification due to ADAPTIL® treatment, three packs displaying aggression between animals were separated, treated with ADAPTIL® collars and spray and then reintroduced. On the day of reintroduction, all packs showed dominant and affiliative behaviours as expected, but two packs also exhibited severe and ritualised aggression. In Pack One, both dominant and affiliative behaviours persisted with no change in their frequency over the study period. In Pack Two, the frequency of dominant behaviours declined significantly over time without a change in the frequency of affiliative behaviours. In Pack Three which exhibited no severe aggressive behaviours until Day nine after reintroduction, the frequency of affiliative behaviour declined significantly over time (Vlamings, 2011). Unfortunately, limited sample-sizes, a lack of control groups, high variability between packs, and no initial baseline data make it difficult to conclude from this study whether ADAPTIL® was able to ameliorate aggression. In a third experiment, three female African wild dogs were separated from their respective packs, housed in isolation and treated with ADAPTIL® collars to investigate the effect of ADAPTIL® on physiological stress levels. In these females, mean fGCM concentrations were reduced by 0%, 68% and 82% respectively compared to pre-treatment levels, suggesting that ADAPTIL® may help to suppress the H-P-A axis (Vlamings, 2011).

A follow-up double-blinded placebo-controlled study found that, compared to controls, well-established zoo-based African wild dog packs treated with ADAPTIL® exhibited reduced contact dominance and active submission behaviours after separation, immobilisation and reintroduction events as part of routine health assessment in captivity (Van den Berghe et al., 2019b). Administration of a spot-on ADAPTIL® solution (as two 5ml aliquots between the shoulder blades and at the base of the tail) to male African wild dogs during this medical intervention did not significantly reduce fGCM concentrations after reintroduction (Van den Berghe et al., 2019b). However, compared to placebo-treated animals, ADAPTIL® treatment was found to suppress the faecal androgen metabolite surge which normally occurs after reintroduction in response to acute stress. This was accompanied by a significant shift from contact dominance to non-contact dominance behaviours (Van den Berghe et al., 2019b).

It is worth noting the difference not only in ADAPTIL® administration but also experimental design between these studies. Vlamings (2011) housed each of three females individually for the duration of one study to investigate the effect of ADAPTIL® on physiological stress, while in a separate experiment, the effect of ADAPTIL® on behaviour was investigated in unstable packs which had been previously separated due to high levels of aggression between individuals. By contrast, Van den Berghe et al., (2019b) measured both behaviour and physiology on the same groups of animals housed in established packs that showed low levels of aggression throughout the study. While both studies used a pre-treatment period to compare hormone levels before/after ADAPTIL® treatment in the same individuals, control animals were absent in the Vlamings (2011) study while double-blinded placebo-treated control packs were used by Van den Berghe et al., (2019b). Collectively, these studies suggest that cross-species ADAPTIL® may suppress the H-P-G axis to control testosterone surges and associated aggression in African wild dogs, but this pheromone appears to have a variably suppressive effect on the H-P-A axis, cortisol and stress. However, a stronger combined effect on both the H-P-A and H-P-G axes may be achieved if species-specific appeasing pheromones are isolated and administered to each canid species.

Appeasing pheromones could therefore be a useful tool to reduce agonistic interactions in African wild dogs and other wild canids during translocation and artificial formation of bonded pairs/packs among unfamiliar animals (Scheepers & Venzke, 1995; Johnston et al., 2007; Vlamings, 2011). Initial extraction and identification of appeasing pheromones has been derived from mammary gland secretions (Pageat, 2002; Pageat & Gaultier, 2003). However, they have subsequently also been found in other bodily secretions of the skin and in faeces - which can be collected with greater ease from wild canids (McGlone & Anderson, 2002; Aviles-Rosa et al., 2020). Gas chromatography coupled with mass spectrometry is used to quantify the different volatile substances and fatty acid compositions contained within secretions for the presence of the conserved appeasing message of oleic, palmitic and linoleic acid followed by the species-specific components (Pageat, 2002; Apps et al., 2012). Candidate pheromones can then be synthetically replicated using relatively cost-effective mass produced chemical components allowing for replication of species-specific pheromones that are readily available and feasibly deployed (Pageat, 2002). Bioassays that evaluate behavioural, hormonal, immunological or neurophysiological responses in a species can then be used to validate the effect of pheromones and their potential benefit (Apps, 2013). In wild canids we advise measuring behaviours (e.g. aggression, resting proximity and submission) and hormones (cortisol and testosterone) that are indicative of stress and aggression pre- and post- pheromone exposure. Their potential benefit to improve reproduction could also be measured through behavioural analysis, breeding outcomes and litter success post release. Furthermore, improvement in immune function could be investigated through safe immune challenge bioassays (Dugovich et al., 2017).

Metapopulation management has been proposed for a number of canids that are endangered or are limited to small spatially fragmented landscapes, including the red wolf, European grey wolf and Ethiopian wolf (Moehrenschlager & Somers, 2004; Laikre et al., 2016; Simonis et al., 2018). Appeasing pheromones could improve the efficacy and welfare of the conservation management of these species. Moreover, such pheromones could help mitigate aggression toward humans in problem populations of other wild canids such as Australian dingoes during breeding and whelping seasons (Appleby et al., 2018). They could also help improve captive breeding and reintroduction programs for species such as the Arctic fox in Norway (Landa et al., 2017). Given the species-specific nature of pheromones (Tirindelli et al., 2009) and the possibility of increased effectiveness when derived from and used upon the target species, it is possible that appeasing pheromones isolated from each species of canid may elicit a greater reduction in stress- and aggression-related hormones and behaviours than using cross-species dog appeasing pheromone. This would offer a natural, non-invasive tool to modify behaviour in a number of wild canids. This is particularly important to improve the success of translocation and pack formation in species such as endangered wolves, foxes and even dingoes that require metapopulation management in the face of habitat fragmentation, disease and human-wildlife conflict.

6. Conclusions

The management of wild canids is necessary to ensure the continued survival of many species due to habitat fragmentation and human persecution. This is most successfully achieved through metapopulation management, where artificially bonded packs/pairs of animals are held in acclimatisation pens for a period of time prior to release. However, temporary captivity leads to chronic stress and possible aggression, which may reduce fitness through suppressed immunity and/or reproduction, resulting in poor translocation outcomes. Current captive methods of castration and pharmaceutical sedation to manage stress and aggression are inadvisable and counterproductive for use in wild canids. Pheromones are naturally occurring chemical messages transmitted between conspecifics that act on the endocrine system to modulate behaviours. Most animals can differentiate these highly species-specific pheromones between conspecific and heterospecific individuals based on small molecular differences in the pheromone composition. Species-specific appeasing pheromones have been shown to reduce stress and aggression behaviours in domestic animals. Previous research on African wild dogs suggests that domestic dog appeasing pheromones can work cross-species to reduce aggression in established captive packs after stressful intervention through a reduction in both contact dominance and testosterone surges. Further research is required to investigate whether a domestic dog or a species-specific appeasing pheromone is able to directly reduce glucocorticoid levels and stress. This can be achieved through an integrative approach using bioassays for behavioural stress (ethogram) and cortisol (non-invasive faecal hormonal assays) in animals exposed to changing synthetic appeasing analogues. This tool has the potential to significantly increase the welfare of animals during conservation intervention. Reduced stress and aggression may lead to tangible translocation and artificial pack formation results. For example, managers should be able to observe reduced aggression between unfamiliar animals; accelerated and increased rates of successful pack bonding, thereby reducing the time spent in captivity prior to release; as well as an elevated immune function that could manifest in (i) reduced disease-induced mortality shortly after release and (ii) increased breeding success and first-litter pup survival.

Chapter 3: Non-Invasive measurement of immunoglobulin concentrations in the faeces of healthy canids

Riddell P, Paris MCJ, Joone C, Bertshinger H & Paris DBBP. Non-invasive measurement of immunoglobulin concentrations in the faeces of healthy domestic dogs (*Canis familiaris*), Australian dingoes (*Canis familiaris dingo*) and African wild dogs (*Lycaon pictus*). *Vaccine* (In prep).

COVID-19 Impact statement

In response to the COVID-19 restrictions implemented in Australia, a substantial amount of field-work based in South Africa and abroad was significantly delayed at the start of this PhD. These delays consequently impacted some of the previously established collaborations in country and impacted global supply chains and negatively impacted international shipping. Where it was possible, in situ personnel were requested to collect data resulting in significantly less control and increases in delays to obtaining important samples. As such the level of detail in the data collected in this chapter was less than originally intended for this aspect of the study.

Abstract

Globally, pathogens such as rabies, canine parvovirus and canine distemper virus (CDV) have caused population crashes and continue to limit growth of wild canid populations; especially those that persist in close proximity to humans where disease spill-over from domestic animals/dogs is high. Current vaccinations available for use in a range of canids appear to show limited success in curtailing the effects of disease. Furthermore, disease monitoring is often not feasible in wild canids due to the need to collect invasive blood samples. As such, this study aimed to (i) measure and compare baseline concentrations of immunoglobulins M (fIgM), G (fIgG) and a CDV-specific immunoglobulin G (fCDV-IgG) in non-invasive faecal samples from 3 different species of canid, (ii) Monitor immunoglobulin response to booster vaccinations in domestic dogs and Australian dingoes, and (iii) determine the stability of these antibodies post-defaecation in African wild dogs to mimic their collection in the field. A single faecal sample was collected from the ground immediately after defaecation from healthy domestic dogs (*Canis familiaris*, n=10) and Australian dingoes (*Canis familiaris dingo*, n=9), and within 48 hours from African wild dogs (*Lycaon pictus*, n=10) when keepers cleaned their enclosure. All samples were frozen shortly after collection. A saline extraction, containing 0.1M phosphate buffered saline, 0.5% Tween20, 0.05% sodium azide and protease inhibitor cocktail, was performed to isolate immunoglobulins from faecal samples. An enzyme-linked immunosorbent assay, previously validated for domestic dogs, and commercially available domestic dog antibodies were used to measure the fIgM and fIgG concentrations of all samples. Baseline rates of fIgM were not significantly different between the three species of canid but tended to be highest in domestic dogs. Comparatively, fIgG was significantly higher in domestic dogs compared to African wild dogs but not dingoes and may reflect differences in immune status/vaccination history, age or nutrition. fIgM and fIgG were stable at ambient temperatures (20-25°C) for up to 9 and 7 days respectively post-defaecation in African wild

dog faeces. In conclusion, commercially available domestic dog antibodies can be used in ELISAs to measure fIgM, fIgG and fCDV-IgG in both closely (dingoes) and distantly (African wild dogs) genetically related canids as long as faeces are collected within 7 days post-defaecation.

1. Introduction

Exposure to infectious pathogens threatens wild canid populations globally, especially where natural dispersal and genetic diversity are limited due to habitat fragmentation. Several pathogens, including canine distemper virus (CDV), canine parvovirus (CPV) and rabies virus, are known to be extremely virulent to canids causing significant morbidity and mortality within populations (Alexander et al., 2010). Epizootic and epidemic spread of these pathogens is a persistent and increasingly common threat to the conservation and management of wild populations (Pedersen et al., 2007). Outbreaks of rabies and CDV have previously caused local pack or broader population extinctions in African wild dogs (*Lycaon pictus*; Flacke et al., 2013; Woodroffe, 2022), Ethiopian wolves (*Canis simensis*; Sillero-Zubiri et al., 1996; Randall et al., 2004; Gordon et al., 2015), grey wolves (*Canis lupus*) and coyotes (*Canis latrans*; Almberg et al., 2011). Due to high levels of habitat fragmentation, many canids are restricted to small populations with low rates of immigration and emigration, which can lead to reduced genetic variability necessary to combat these virulent pathogens (Spielman et al., 2004). Furthermore, some pathogens are thought to limit population growth, resulting in small populations more vulnerable to extinction (Woodroffe, 1999).

Blood, saliva and faeces can be used to test immune function in mammals (Franz and Corthier, 1981; Kikkawa et al., 2003; Peters et al., 2004). Immunoglobulins, also known as antibodies, are made by the immune system in response to pathogens (Schroeder and Cavacini, 2010). Different types of antibodies are responsible for the physiological response to different immune invasions and, as such, it is important to know the role of each. There are five major immunoglobulin types: immunoglobulin G, A, M, E and D (IgG, IgA, IgM, IgE and IgD; Schroeder and Cavacini, 2010). IgGs are the most commonly produced antibody (75-80%) found in all body secretions/fluids/compartments (Ruiz-López et al., 2014). IgGs are important for immune responses to bacterial and viral infections and rise over the course of an infection to indicate pathogen exposure (Ruiz-López et al., 2014). IgA antibodies are secreted in mucus, tears, saliva and breastmilk. They protect mucosal surfaces that are exposed to foreign pathogens, providing protection against infections, allergens and foreign proteins (Zaine et al., 2011). IgMs are the largest antibodies, found in lymph fluid and blood. These antibodies are produced immediately in response to infection (Ruiz-López et al., 2014). IgEs are found in lungs, skin and mucous membranes and are involved in histamine release and hypersensitivity reactions. IgDs are found in low quantities in tissues lining the stomach and chest, but the way in which they work is still not well understood.

The immune system in mammals works on two levels, an innate and an adaptive immune response (Barnard et al., 1996; Baumgarth et al., 2005; Curno et al., 2009). The innate immune response is the body's first line of pathogen defence (Medzhitov and Janeway, 1998; Mogensen, 2009). Adaptive immunity produces a pathogen-specific, learned immune response. The activation of cells involved in adaptive immunity is much slower than the innate immune response and is triggered when pathogens persist for a longer duration or are encountered again after an initial exposure event (Maier and Watkins, 1998). IgM and IgG work together through the compliment system and are activated during the innate and adaptive immune response with IgM being upregulated first upon antigen exposure and IgG sequentially upregulated.

The efficacy of vaccinations can be measured through immunoglobulin production, which is mediated by the immune system and is upregulated in response to pathogen invasion (Schroeder and Cavacini, 2010). Immunoglobulin M (IgM) production occurs rapidly in response to novel foreign antigens/cells and can be a good indicator of innate immune responses to vaccination or pathogen exposure (Ruiz-López et al., 2014). Immunoglobulin G (IgG) is important for the development of adaptive, long-term, immunity and rises over the course of an infection (Ruiz-López et al., 2014). Vaccination with dead or attenuated pathogens or their immunoreactive antigens induces a similar response, in a safe manner, without infection. Blood is the most common sample used to measure changes in antibodies in response to vaccination. However, blood collection is complicated in wild species often requiring restraint and sedation, which may cause undue stress (Sheriff et al., 2011). The presence of secretory IgG, following vaccination, in the saliva, tears and bile of domestic dogs suggests that serum IgG is able to passively diffuse into these secretions (German et al., 1998). By contrast, mucosal secretions of IgM are actively produced and secreted from plasma cells of local glandular tissue (German et al., 1998). Faeces and urine are increasingly used to measure glucocorticoids and other hormone metabolites non-invasively, so could also be a source to non-invasively measure immunoglobulins. In faeces, total IgM and IgG concentrations appear stable at room temperature for up to 72h after defaecation and are influenced by breed in domestic dogs (*Canis familiaris*; Peters et al., 2004). The development of a domestic dog assay to measure immunoglobulin concentrations in faeces (Teramoto et al., 1984; Decaro et al., 2004; Peters et al., 2004), could be adapted and validated for use in other wild canids where collection of invasive blood samples is problematic.

Enzyme-linked immunosorbent assay (ELISA) is the most commonly used technique to quantify total concentrations of immunoglobulins. This technique can analyse blood serum and plasma as well as faecal samples (Peters et al., 2004). A number of different ELISA methods have been validated including capture, sandwich, dot and indirect ELISAs. Detection of an immune history of infection can be achieved by measuring antibody concentrations (Day, 2007b). Detection of positive IgM concentrations indicates current infection or recent exposure to a pathogen. Positive IgG concentrations indicate immunity to a disease due to prior pathogen exposure. Anti-canine distemper virus and anti-canine parvovirus IgG antibodies persist throughout life in domestic dogs after overcoming exposure to the disease (Williams and Barker, 2001). To date, ELISAs have only been used to measure IgG and IgM antibodies in the faeces of domestic dogs (Peters et al., 2004). However, this tool could offer an advantage to monitor immune function in wild canids by reducing the need for stress-inducing blood sampling. The wolf-like clade of the Canidae diverged around 6 million years ago and at this time the African wild dog evolved separately from the lineage that developed into the domestic dog (Lindblad-Toh et al., 2005). Comparatively, the Australian dingo (*Canis familiaris dingo*) became distinctly different from the domestic dog 8,000 to 11,000 years ago (Bergström et al., 2020; Zhang et al., 2020). As such, potential differences may occur in baseline immunoglobulins and responses to vaccinations. Thus, the aim of this study is to validate an ELISA for the measurement of total IgM, total IgG and CDV-IgG concentrations in the faeces of 3 members of the Canidae: the domestic dog, Australian dingo and African wild dog, and determine the stability of African wild dog samples at ambient temperatures post-defaecation.

2. Materials and methods

2.1. Animals

All samples were collected from healthy animals with no signs of diarrhoea or gastrointestinal upset on the days of collection. All samples were frozen within an hour of collection to reduce the action of bacterial enzyme degradation of hormones and immune fractions post-defecation (Washburn and Millspaugh, 2002).

Samples were collected from 10 adult domestic dogs receiving annual C3/4 booster vaccinations (Breed: Golden Retriever *n*=1, Rottweiler *n*=8, Kelpie cross *n*=1; Location: Townsville, Australia), nine Australian dingoes receiving annual C4 vaccinations (Location: Billabong Wildlife Sanctuary *n*=3, Wildlife Surrounds *n*=4, Townsville, Australia and privately owned *n*=2, Ingham, Australia) and 11 African wild dogs receiving no annual vaccinations (Location: Perth Zoo, Perth, Australia). This study was approved by the James Cook University Animal Ethics Committee (A2710).

Table 3.1. Demographics and vaccine treatment for each group of canids.

Parameter	Domestic dog (*Canis famililaris*)	Dingo (*Canis familiaris dingo*)	African wild dog (*Lycaon pictus*)
n	10	9	11
Age range	1 – 5y	3 – 12y	1 – 3 y
No. males (no. neutered)	4 (1)	3 (0)	–
No. females (no. neutered)	5 (1)	4 (0)	11 (0)
Annual vaccines	C3	C4	–

C3 (Protech, Boehringer Ingelheim, North Ryde, Australia), core vaccine 3 – protects against CPV, CDV and adenovirus type 1 & 2; C4 (Protech, Boehringer Ingelheim, North Ryde, Australia or Nobivac, MSD Animal Health, North Ryde, Australia), core vaccine 4 – protects against CPV, CDV, adenovirus type 1 & 2, parainfluenza virus; y, years

2.2. Vaccination challenge

Adult domestic dogs were administered 1 ml C3 vaccine (Protech, Boehringer Ingelheim, North Ryde, Australia; core vaccine 3 – protects against CPV, CDV and adenovirus type 1 & 2) subcutaneously between the shoulder blades as an annual booster. All dogs had received annual boosters since their initial vaccinations as puppies. Dingoes were immunised with either 1 ml live attenuated C4 vaccine administered subcutaneously between the shoulder blades (Billabong Wildlife Sanctuary n=3; Protech, Boehringer Ingelheim, North Ryde, Australia; core vaccine 4 – protects against CPV. CDV, adenovirus type 1 & 2, parainfluenza virus) or 1 ml live attenuated Nobivac C4 vaccine (Wildlife Surrounds n=4 and privately owned animals n=2; MSD Animal Health, North Ryde, Australia). All dingoes had received annual boosters since arrival at respective wildlife centres. African wild dogs housed at Perth Zoo were not immunised against CDV so these samples were only used to evaluate baseline measures of faecal immunoglobulins.

2.3. Faecal sample collection and processing

(i) Baseline immunoglobulin concentrations in domestic dogs, Australian dingoes and African wild dogs.

Baseline levels of immunoglobulins were determined for all 3 species of canid from faecal samples collected either on the day of booster vaccination (domestic dogs and dingoes) or as opportunistic once-off samples during enclosure cleaning which did not coincide with a vaccination event (African wild dogs). Faecal samples were collected from the ground by owners or wildlife keepers immediately (domestic dogs and dingoes) or up to 48 hours (African wild dog) after spontaneous defaecation. All samples were placed in individually labelled Ziplock bags and stored at -20°C until required for further processing. Where possible, the individual animal identity was documented for each sample (domestic dogs and dingoes). However, this was not possible for African wild dog samples because animals were group-housed and unidentified faecal samples could only be collected from members of the pack within 48 h of defaecation at the time of enclosure cleaning. Samples were subsequently transported on dry ice and remained frozen when delivered to the laboratory.

(ii) Immunoglobulin response to booster vaccination in domestic dogs and Australian dingoes.

To validate that the ELISA was sensitive to changing levels of immunoglobulins, faeces from domestic dogs and dingoes were collected once weekly from the day of vaccination until 8 weeks post-vaccination. Faecal collection from n=3 dingoes at Billabong Wildlife Sanctuary only commenced 1 week after vaccination. Samples were frozen at -20°C until use as described above.

(iii) Stability of African wild dog faecal immunoglobulins at ambient temperatures.

To test whether the ELISA would be a tool suitable for use on African wild dog samples collected under field conditions, we needed to determine the stability of faecal immunoglobulins subjected to ambient temperatures over time. Degradation of fIgM and fIgG in African wild dog samples were assessed by looking at changes in concentration over time prior to sample storage at -20°C. Faecal samples from n=3 female African wild dogs were collected within 48h of defaecation and frozen at -20 °C. In the laboratory, a single stool from each animal was subsequently thawed, homogenised then pre-cut into three 1 g aliquots per sampling timepoint and maintained at room temperature (20 – 25°C) for up to 7 days. Each day from Day 0 to 7 post-thaw, three aliquots of the stool from each animal was frozen at -20 °C then processed as describe below to measure fIgG and fIgM concentrations.

2.4. Faecal sample processing

A modified saline extraction method (Peters et al., 2004) was performed on faecal samples to extract IgM, IgG and CDV-IgG antibodies in supernatant. Briefly, 10 ml of extraction buffer (0.01M phosphate buffered saline pH 7.4, 0.5% Tween and 0.05% sodium azide; Sigma-Aldrich, Sydney, Australia) was added to 1 g wet weight faeces in a centrifuge tube. Samples were thoroughly homogenized using manual shaking and vortexing before centrifugation at 1,500 x g for 20 min. A 2 ml aliquot of supernatant was then transferred to a sterile Eppendorf tube containing 20 µl of protease inhibitor cocktail (Sigma-Aldrich, Sydney, Australia), mixed thoroughly by vortexing and re-centrifuged at 3,000 x g for 10 min. Thereafter, protease-inhibitor-treated supernatant containing IgM and IgG antibodies was frozen at -20°C in sterile Eppendorf tubes in 1 ml aliquots.

2.5. fIgM, fIgG and fCDV-IgG Enzyme-linked immunosorbent assays.

Total fIgM, total fIgG and fCDV-IgG concentrations in faecal samples were quantified using capture ELISAs. Assay specificity was assessed using purified canine IgG (Sapphire Bioscience, Redfern, Australia), and IgM (InVitro Technologies, Nobel Park North, Australia, Table 3.2). These antibodies were also used to determine levels of cross reactivity between immunoglobulin classes. Validation of assays was completed by measuring the inter- and intra-plate coefficients of variation by running eight repeats of a single sample of pooled domestic dog faecal supernatant and pooled African wild dog faecal supernatant on separate plates. Checkerboard titrations were performed to determine optimum primary, secondary and tertiary antibody/antigen dilutions (Table 3.2). ELISAs used to quantify total canine IgM and IgG were as described by Peters et al., (2004) and German et al., (1998) respectively with the following modifications. Polyvinyl microtiter plates (Starstedt; South Australia, Australia) were initially coated with 75 µl primary antibody diluted to optimal concentration (1:80 and 1:800 for IgM and IgG assays respectively) in 0.05 M carbonate bicarbonate buffer (pH 9.6; Sigma-Aldrich) and incubated overnight at 4 °C in the dark. Plates were then washed three times in 0.01 M PBS containing 0.05% Tween (PBST) before residual protein binding was blocked using 100 µl of 20% non-fat milk (IgM assay) or 2.5% polyvinylpyrrolidone (IgG assay) in PBST incubated at room temperature for 1 h. Sample, secondary and tertiary antibody incubations were carried out on the benchtop and sample and capture antibody incubations were carried out on a shaking plate. All plates were washed three times with PBST at the end of each sample/antibody incubation. ELISAs were developed with the addition of 75 µl 3,3',5,5'-Tetramethylbenzidine liquid substrate (Sigma-Aldrich, Sydney, Australia) per well incubated for 15 min at room temperature before the colourimetric reaction was stopped with 75 µl 0.1 N H_2SO_4 per well. Absorbance was read at 450 nm using a Polarstar Omega microtitre plate reader (BMG Labtech, Victoria, Australia).

Table 3.2. Reagents and their optimal dilutions used in ELISAs.

Immunoglobulin	Primary antibody/antigen	Secondary antibody	Tertiary antibody	Standard
IgM	Affinity-purified goat anti-canine IgM (Fc)[a] (1:80)	Affinity-purified rabbit anti-canine IgM (Fc)[b] (1:100,000)	Affinity-purified goat anti-rabbit IgG (Heavy + Light chains) – HRP conjugated[c] (1:3,200)	Purified canine IgM (1 mg/ml)[a]
IgG	Affinity-purified rabbit anti-canine IgG (Fc)[b] (1:800)	Affinity-purified rabbit anti-canine (Heavy + Light chains) – HRP conjugated[d] (1:10,000)	None	Purified canine IgG (1 mg/ml)[d]
CDV IgG	Native canine distemper virus antigen (Lederle strain)[d] (1:10)	Rabbit anti-canine IgG (Fc) polyconal antibody – HRP conjugated[d] (1:25,000)	None	Purified canine CDV IgG (1 mg/ml)[e]

[a] InVitro Technologies, Nobel Park North, Australia, [b] Sigma Aldrich, Merk, Darmstadt, Germany, [c] Stratech Scientific, Sydney, Australia, [d] Sapphire Bioscience, Redfern, Australia, [e] Jomar Life Research, Mulgrave, Australia

The ELISA protocol used to detect canine CDV-specific IgG was adapted from Teramoto et al., (1984) with the following modifications. Polyvinyl microtiter plates (Starstedt; South Australia, Australia) were initially coated with 100 ng CDV antigen per well (CDV-specific IgG assay) diluted 1:10 in 0.05 M carbonate bicarbonate buffer (pH 9.6; Sigma-Aldrich, Sydney, Australia) and incubated overnight at 4 °C in the dark. Plates were then washed and residual protein binding blocked with the addition of 2.5% polyvinylpyrrolidone (Sigma-Aldrich, Sydney, Australia) in PBST incubated at room temperature for 1 h. All incubations were carried out on the benchtop and sample and capture antibody incubations were carried out on a shaking plate. Plates were washed three times with PBST between antigen, sample and antibody incubations. ELISAs were developed with the addition of 75 µl of 3,3',5,5'-Tetramethylbenzidine liquid substrate (Sigma-Aldrich) per well incubated for 15 min at room temperature. The colourmetric reaction was stopped with the addition of 75 µl 0.1 N H_2SO_4 per well. Absorbance was read at 450 nm using a Polarstar Omega microtitre plate reader (BMG Labtech, Victoria, Australia).

For each plate, optimal dilutions for samples were determined by two-fold serial dilution ranging from 1:50 to 1:6,400 in PBST for all plates. After primary antibodies were blocked, 75 µl diluted sample was added to each well and incubated at room temperature for 1 h. Secondary and tertiary antibodies, at concentrations outlined in Table 3.2 for each plate, were subsequently added and incubated at room temperature for 1 h. All immunoglobulin concentrations in faecal supernatant were expressed as micrograms per millilitre. Additionally, faecal supernatant concentrations were normalised to the wet weight of faeces to account for the number of immunoglobulins per gram of faecal matter. The values obtained were expressed as micrograms of immunoglobulin per gram of wet weight faeces.

2.5. Statistics

Statistical analysis was performed using SPSS statistics premium v27 (IBM®SPSS®Statistics 27, SPSS inc., IBM, Armonk, New York, USA). Analysis of Variance (ANOVA) coupled with a post hoc Tukey's honestly significant difference test was used to compare (i) baseline fIgM, fIgG and fCDV-IgG concentrations between domestic dogs, dingoes and African wild dogs, (ii) fIgM, fIgG and fCDV-IgG concentrations over the 8 week post vaccination period for dingoes and domestic dog, and (iii) fIgM and fIgG degradation in African wild dog subsamples held at ambient temperatures over time. $P \leq 0.05$ was considered significant for all analyses.

3. Results

There was no difference in baseline or post-vaccination collection IgM, IgG or CDV-IgG concentrations in dingoes or domestic dogs between sexes. Due to limited recruitment of castrated animals in either species the effect of castration was not able to be determined.

3.1. ELISA validation

Optimal antibody concentrations for all primary, secondary and tertiary antibodies used were determined through checkerboard titration (see Table 3.2 of optimal concentrations). The intra- and inter-plate coefficients of variation were 1.3 and 7.3, 2.3 and 7.0, and 4.6 and 3.0 for the IgM, IgG and CDV-IgG ELISAs respectively. The specificity and cross-reactivity of each ELISA was determined using purified canine IgM and IgG (Fig. 3.1). Antisera in the fIgM ELISA assay was specific for IgM, showing no cross-reactivity against IgG or CDV-IgG purified standards (Fig. 3.1a). Similarly, both the fIgG and fCDV-IgG ELISA assays showed the strongest affinity for IgG and CDV-IgG respectively and showed no cross-reactivity to the IgM purified standard (Fig. 3.1b & c). However, as expected, antisera in the IgG ELISA cross-reacted with about ~40% affinity to the CDV-IgG purified standard, while antisera in the CDV-

IgG ELISA cross-reacted with about ~65% affinity to the IgG purified standard (Fig. 3.1b & c).

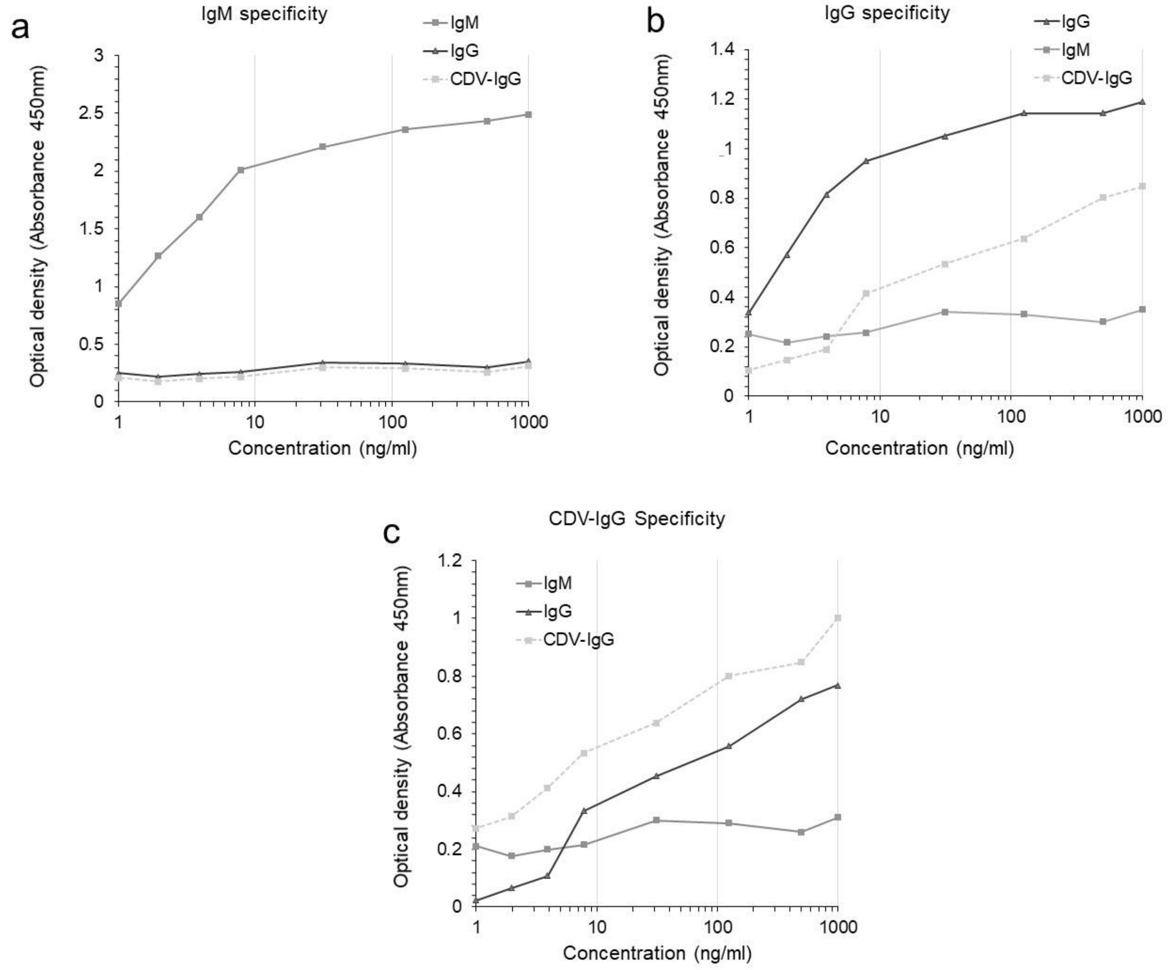


Figure 3.1. Mean (± SEM) baseline concentration of faecal (a) IgM, (b) IgG and (c) CDV-IgG in adult domestic dogs (*Canis familiaris*), Australian dingoes (*Canis familiaris dingo*) and African wild dogs (*Lycaon pictus*). Numbers in parenthesis indicate sample size. ^{a,b} values not sharing the same letter differ significantly in baseline immunoglobulin concentration between species. CDV, canine distemper virus.

3.2. Baseline immunoglobulin concentrations in domestic dogs, Australian dingoes and African wild dogs.

Baseline concentrations of fIgM were not significantly different between the three species of canid (F=0.814, df=3, *P*=0.456, Fig. 3.2a). In contrast, fIgG concentrations were highest in domestic dogs and lowest in African wild dogs (F=5.761, df=3, *P*=0.009), with dingoes showing no statistical difference to African wild dogs (F=5.761, df=3, *P*=0.407) or domestic dogs (F=5.761, df=3, *P*=0.253, Fig. 3.2b). Comparatively, baseline fCDV-IgG were only measured in the domestic dog and Australian dingo samples and were not significantly different between species (F=4.357, df=2, *P*=0.200; Fig. 3.2c).

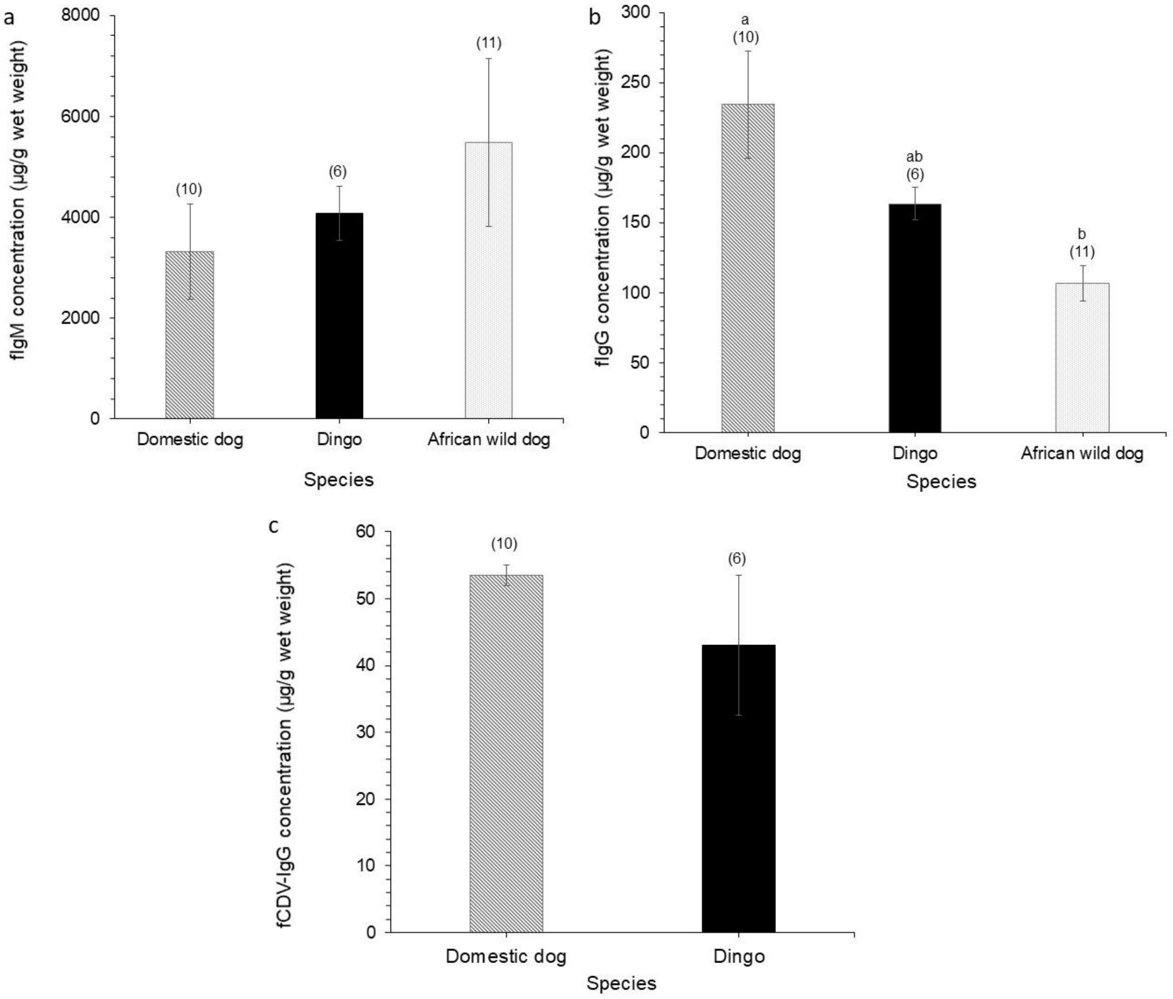


Figure 3.2. Mean (± SEM) baseline concentration of faecal (a) IgM, (b) IgG and (c) CDV-IgG in adult domestic dogs (*Canis familiaris*), Australian dingoes (*Canis familiaris dingo*) and African wild dogs (*Lycaon pictus*). Numbers in parenthesis indicate sample size. Different letters indicate significant differences in baseline immunoglobulin concentration between species. CDV, canine distemper virus. ^{a}, ^{b}, ^{ab} denote significance in the concentration of faecal immunoglobulin concentrations between species

3.3. Immunoglobulin response to booster vaccination in domestic dogs and Australian dingoes

There was no significant change in the concentration of IgM in the faeces of domestic dogs (F=0.516, df=10, *P*=0.842), but there was a declining trend in Australian dingoes (F=9.095, df=7, *P*=0.052) up to 8 weeks post-vaccination (Fig. 3.3a). Faecal IgG concentrations increased significantly from Week 5 to Week 7 onwards after vaccination in domestic dogs (F=1.981, df=10, *P*≤0.001) but not Australian dingoes despite a similar trend (F=0.717, df=7, *P*=0.290; Fig. 3.3b). Similarly, faecal CDV-IgG concentrations increased significantly from Week 6 to Week 8 post-vaccination in domestic dogs and from Week 4 to Week 6 onwards and rose further from week 5 to week 8 after vaccination in dingoes (F=9.151; df=10; *P*≤0.001 and F=9.151; df=7; *P*≤0.001 respectively; Fig. 3.3c).

3.4. Stability of African wild dog faecal immunoglobulins at ambient temperatures.

Total IgM concentrations detected in African wild dog faeces thawed and held at ambient temperature for 0 to 7 days (up to ~9 days after initial defaecation) were highly variable between animals but did not differ significantly over time (F=13.440 , df=3, P=0.207). In contrast, total IgG concentrations were more consistent between animals and stable until Day 5, then declined significantly from Day 6 onward after defaecation (Fig. 3.4; F=4.647, df=3, *P*≤0.001; ~8 days post defaecation).

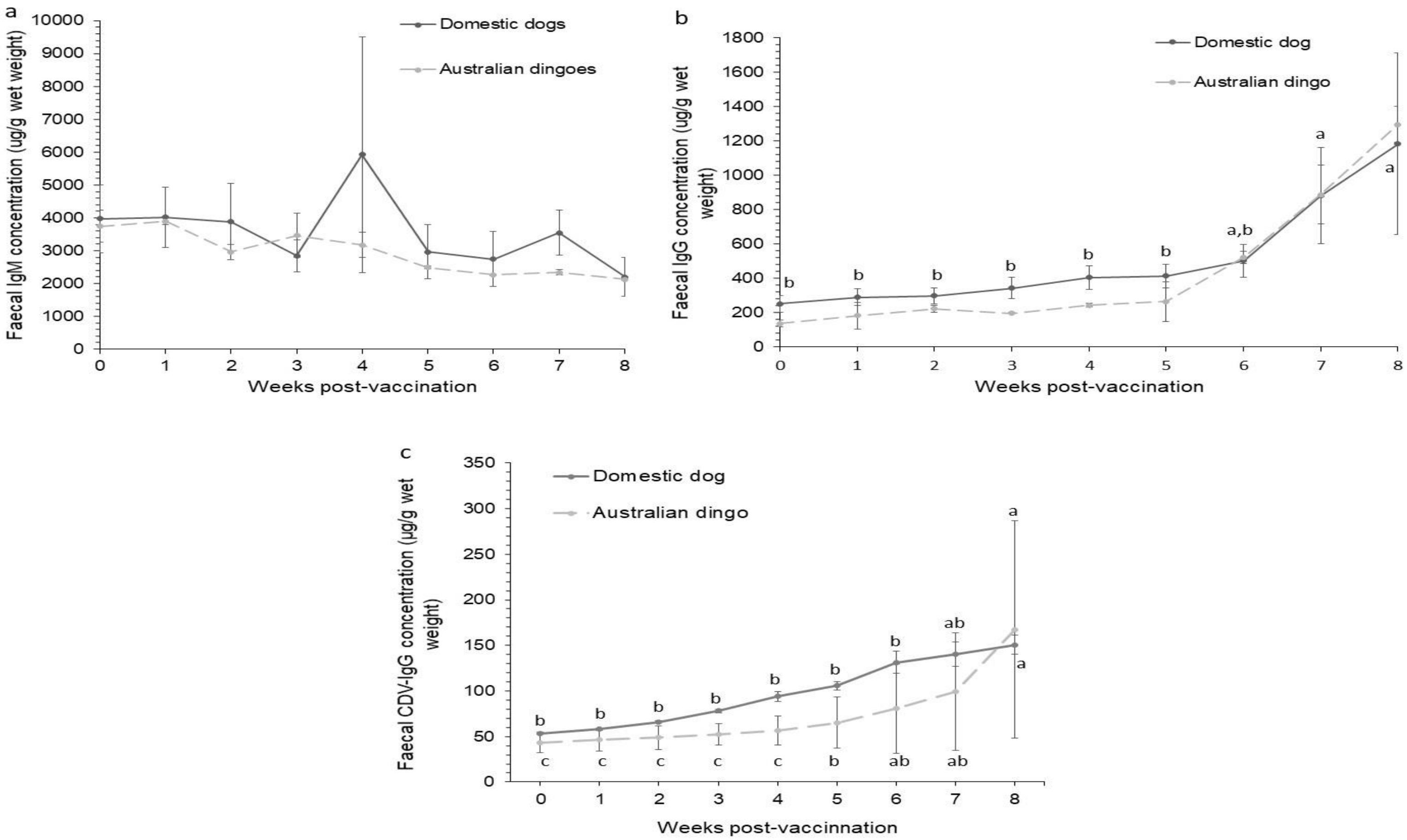


Figure 3.3. Mean (± SEM) concentration of faecal (a) IgM, (b) IgG and (c) CDV-IgG over time in adult domestic dogs (*Canis familiaris*; n=10) and Australian dingoes (*Canis familiaris dingo*, n=6 at Week 0, n=9 thereafter) after C3/C4 booster vaccination. ^{a,b,c} values not sharing the same letter differ significantly over time within a species. *P*≤0.05 is considered significant. C3, CPV, CDV and adenovirus type 1 & 2 vaccine; C4, CPV, CDV, adenovirus type 1 and 2, parainfluenza virus vaccine.

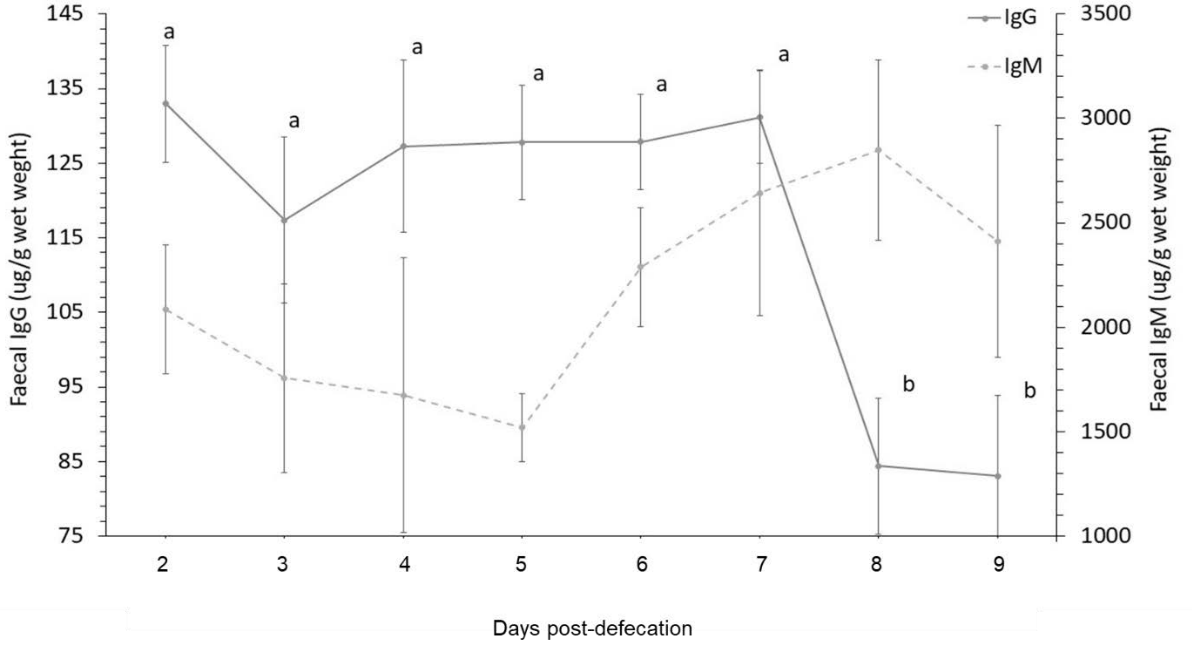


Figure 3.4. Mean (± SEM) concentration of IgM and IgG in the faeces of African wild dogs (*Lycaon pictus*) collected within 24 – 48 h after defaecation and held at ambient temperature (20 - 25°C) for up to a further 7 days. Values at each time point constitute technical triplicates obtained from the faeces of n=3 females (9 samples total). ^{a,b} values not sharing the same letter differ significantly over time; $P \leq 0.05$ is considered significant.

4. Discussion

In this study we successfully validated three domestic dog faecal ELISAs for the measurement of baseline levels of immunoglobulins (IgM, IgG and CDV-IgG) in dingoes and African wild dogs and their changes during vaccination in domestic dogs and dingoes or degradation in African wild dogs. While total IgM concentrations did not differ between canids, as expected, total IgG was significantly lower in unvaccinated African wild dogs. We also demonstrated that vaccination resulted in an earlier significant rise in CDV-IgG in dingoes (Week 4 to 6) than domestic dogs (Week 6 to 8), suggesting a more rapid adaptive response in this semi-wild canid. Additionally, under controlled ambient temperatures, immunoglobulins in the faeces of African wild dogs appear relatively stable for extended periods of time. Our results indicate that total IgM appears stable, but highly variable, at ambient temperature for up to 9 days post defaecation, while total IgG appears consistently stable up to 5 days post-defaecation. In summary, the faecal IgM, IgG and CDV-IgG ELISAs appear to be reliable to monitor immune function and vaccination response in both domestic and wild canids and appear robust and well-suited to the analysis of samples collected under field conditions.

Measurement of the antibodies of mammals can be useful to monitor changes in the immune system in response to pathogens and vaccinations but have been often developed for use in blood samples only (Tabor et al., 1987; Waner et al., 1998; Waner et al., 2003; Schroeder and Cavacini, 2010; Wang et al., 2010; Jeon et al., 2012). Measurement of IgM and IgG are useful indicators of pathogen exposure and development of long-term antibodies for protection against infections. ELISAs have been validated previously to measure IgM and IgG in body secretions other than blood, such as tears, saliva and faeces, in domestic dogs (Teramoto et al., 1984; German, Hall & Day, 1998; Peters et al., 2004). Our study shows that these assays can be adapted for use to detect antibodies in the faeces of wild canids both closely and distantly related to domestic dogs. These assays could provide a stress-free, non-invasive alternative to blood sampling for the monitoring of immune function. More specifically, measuring the peak of the predicted rise in IgM and IgG in response to initial and booster vaccinations could provide an indication of vaccine efficacy, while longitudinal measurement of their decline over time could provide an indication of the duration of immunity, which could help guide the timing of booster vaccinations in wild canids

IgM antibodies are produced in the body rapidly as a response to foreign pathogen detection, whereas IgG antibodies are produced more slowly and rise over the course of an infection but are able to confer long-term protection against future infections by the same pathogen (Ruiz-López et al., 2014). Secretions of bile from the gastrointestinal tract and liver contribute the immunoglobulins contained within faeces. Secretory IgM is actively transported across the epithelial barrier into the gastrointestinal system and accelerates the primary immune response (Mestecky and Russell, 1991). Comparatively, IgG is not actively transported into mucosal secretions and is most likely added to faeces through the contribution of bile (Peters et al., 2004). Thus, as expected, baseline fIgM concentrations did not differ between species of canid despite their different vaccination status, because all animals in this study were healthy and apparently free of infection at the time of these initial baseline collections. Moreover, baseline concentrations of fIgM and IgG of domestic dogs in this study were similar to those reported previously (Peters et al., 2004). However, fIgG concentrations were significantly higher in domestic dogs than African wild dogs, but not Australian dingoes, which we attribute to the fact that African wild dogs were the only species in the study with no recent history of vaccination. There is a negative relationship between IgG concentrations and length of time since vaccination suggesting that recently and regularly vaccinated animals have higher circulating IgG compared to unvaccinated or not-recently vaccinated animals (Babalola et al., 2016). Other factors are unlikely to explain this difference between domestic and African wild dogs because individual animal variation was low and studies have shown day-to-day, sex and diurnal variations that influence immunoglobulin concentrations in tears and saliva of domestic dogs, does not occur in faeces (German et al., 1998; Peters et al., 2004).

Vaccinations against rabies, CDV and CPV that are able to confer long-term protection to domestic dogs appear to offer limited long-term protection in African wild dogs (Connolly et al., 2013, 2015; Anderson and Smith, 2019; Woodroffe, 2021) and no studies to date have quantified the safety or efficacy of vaccinations in the Australian dingo. The detection of acquired immunity following vaccination currently requires the collection of multiple invasive blood samples over time and at least some form of temporary captivity, which may increase stress in wild individuals (Riddell et al., 2021). African wild dogs in permanent or temporary captivity experience stress beyond that of free-roaming counterparts (Crossey et al., 2020), which can be exacerbated during reintroduction of pack mates and cause contact dominance and in extreme cases fighting that could impact animal health (Potgieter et al., 2015; Van den Berghe et al., 2019b). While the impact of chronic stress on immune function is unclear in

African wild dogs, it is widely known in other mammals to suppress the immune system, which in turn can decrease vaccine efficacy and resistance to disease (Sergestrom and Miller, 2004; Vrekoussis et al., 2010; Sheriff et al., 2011; Potgieter et al., 2015; Yordy and Mossotti, 2016). Our study shows that it is possible to measure the immune response of canids to vaccination through non-invasive measurement of IgM, IgG and IgG specifically against CDV in faeces. Our results using faecal samples in dingoes and domestic dogs were similar to those reported using serum in domestic dogs and red wolves, where total and specific IgG antibodies were elevated 8-9 weeks after vaccination (Harrenstien et al., 1997; Waner et al., 2003). Providing further evidence of their potential for use to non-invasively measure immunity in African wild dogs and other canids. Moreover, these assays could help investigate whether stressful procedures such as capture, sedation, translocation, captivity, reintroduction or new pack formation suppresses immune function and vaccination efficacy in African wild dogs.

The use of non-invasive measures is increasingly important in animal research, especially as human intervention can cause unnecessary stress to animals. Faecal samples offer one of the least invasive methods of measuring physiological changes in animals and allow feedback-free sampling (Ganswindt et al., 2012). Assays to measure the physiological responses through hormones associated with stress and aggression in faecal samples have been validated previously (Van den Berghe et al., 2019b) and the longevity of these hormones assessed (Van den Berghe et al., 2019b; Crossey et al., 2020). Freezing of faeces post-collection halts the action of bacterial enzymes which might breakdown hormone or immune fractions post-defaecation and thus, samples collection immediately after defaecation is most desirable (Washburn and Millspaugh, 2002). However, under field conditions this is often not possible or freezing is delayed. As such it is important to validate how long immunoglobulins within faeces are stable at ambient temperatures. Our results indicate that total IgM and IgG are stable in African wild dog samples for up to 9 and 5 days post-defaecation respectively. Due to a need to ship samples to the laboratory degradation trials were performed using frozen thawed faeces (which may partly degrade immunoglobulins). Moreover, samples were also pre-cut into smaller segments, increasing surface area, leading to more rapid dehydration and potentially speeding up immunoglobulin degradation. As such, it is possible that the results of this study are an under estimation of the length of stability of faecal immunoglobulins in African wild dog samples.

In conclusion, we have validated a robust, field-applicable, non-invasive set of assays suitable to measure baseline immune function and its response during vaccination in two species of wild canids for the first time. We also demonstrated that immunoglobulins associated with the adaptive immune system are lower in unvaccinated individuals and rise more rapidly following booster vaccination in wild canids compared to their domestic counterparts. Moreover, degradation trials suggest that immunoglobulin concentrations can be reliably measured in African wild dog faeces at ambient temperatures for up to 7 days post-defaecation. These tools provide a valuable non-invasive alternative to blood sampling that can improve animal welfare during research and facilitate increased sampling frequency and longitudinal disease monitoring.

Chapter 4: African wild dog vocalisation changes in response to behaviours, contexts and housing

Riddell P, Malone K, Joone C, Paris MCJ & Paris DBBP. The range and daily rate of African wild dog (*Lycaon pictus*) vocalisations increase, syllables lengthen and fundamental frequency becomes lower during anti-social behaviours and contexts and within captivity. *Animal Behaviour* (In prep)

Abstract

Changes in vocalisation rates and acoustic parameters can be indicative of fluctuations in emotional states among canids and thus, could be good indicators of social cohesion within pack-living Canidae. The African wild dog is a socially complex canid that uses vocalisations, scent marking and physical behaviours to communicate between pack members. These are important to efficiently coordinate hunting, area defence, and communal rearing of pups and care of compromised pack-mates. As such, effective communication and social cohesion are crucial for survival. Despite being a highly vocally complex species, little is known about how African wild dogs use vocalisations and acoustic modulation to maintain social cohesion. This study aimed to determine whether changes in the rate and acoustic parameters of African wild dog vocalisations could be indicative of changes in emotional states and pack cohesion in different contexts. The vocalisations and behaviours of five African wild dog packs of different captive status (n=3 traditional zoo packs; n=1 open-range zoo pack and n=1 free-roaming pack) were recorded for three consecutive days during social contexts that included feeding, social interaction, and facing animal or human threats. Daily rates, syllable length and fundamental frequency of vocalisations emitted during anti-social, pro-social and feeding behaviours were recorded and compared between social contexts and housing types. We found that multi-purpose vocalisations, such as social and mob twitters, whimpers, huffs, sneezes, yelps and whines predominantly occurred during pro-social behaviours, while other multi-purpose vocalisations, such as spar twitters, begging squeals, whistle squeals, full moans, begging cries, social growls, rumbles, attack barks and attack twitters increased during anti-social behaviours. Interestingly, most vocalisations associated with negatively social behaviours had longer syllables and lower fundamental frequency than when emitted with socially positive behaviours. Moreover, barks, rumbles, growls and moans only occurred with negatively social behaviours and thus may be good indicators of negative emotional states. Social contexts in

which negatively social behaviours predominated (human and animal threats) had vocalisations with increased rates, longer syllables and lower fundamental frequencies compared to contexts in which positively social behaviours were more commonly experienced (social interaction). Additionally, open-range zoo packs used multi-purpose vocalisations with negative social behaviours more than traditional zoo and free-roaming packs (21, 16 and 13 vocalisation categories, respectively). Of those vocalisations, those which occurred with negatively social behaviours occurred more often in traditional zoos. Moreover, traditional zoo packs had more vocalisations with longer syllables and open-range zoo packs had lower frequency vocalisations. These results suggest that traditional and open-range zoo packs may experience more negative emotional states indicative of reduced social cohesion compared to free-roaming packs. This may be caused by a reduced ability to (i) display normal behaviours such as ranging and group feeding in traditional zoos, and (ii) disperse from natal groups (unless facilitated by managers) in open-range zoos. Our results suggest that African wild dog vocalisations and their acoustic parameters are sensitive to changes in emotional states elicited by different behaviours, contexts and housing. As such, they may make useful tool to measure the social cohesion of packs with further research.

1. Introduction

Vocalisations in canids and other wildlife vary based on situational demands, which can influence the subsequent actions of conspecifics (Faragó et al., 2010; Kershenbaum et al., 2016). In ground squirrels (*Spermophilus sp.*), the audible pitch of alarm calls changes and is used as an indicator for the urgency of response to threats (Seyfarth et al., 1980; Yin & McCowan, 2004; Wilson & Hare, 2006). Vervet monkeys (*Chlorocebus pygerythus*) use different alarm calls to direct different patterns of escape from threats (Seyfarth et al., 1980). For domestic dogs (*Canis familiaris*), vocal parameters of barks and growls become longer, harsher and of lower pitch when threats are perceived (Yin and McCowan, 2004; Farago et al., 2010). Howls emitted by wolves (*Canis lupus*), coyotes (*Canis latrans*) and dingoes (*Canis lupus dingo*) differ between packs in a range of parameters and are thought to be used as a way for separated pack members to recognise their pack from others in proximal locations (Hallberg, 2007; Deaux & Clarke, 2013). Moreover, the rate of African wild dog (*Lycaon pictus*) long distance contact calls decreases as social cohesion increases during artificial pack formation (Marneweck et al., 2019a). As such, changes in rate and acoustic parameters of vocalisations may be indicative of alterations in social cohesion of group living animals with low pitched, long syllable barks and growls probably being associated with threats or negative arousal states, while higher pitched, short syllable vocalisations likely to indicate positive arousal states (Marx et al., 2021). Additionally, playback and individual animal removal trials have shown that wolf and dingo howls also serve to identify specific individuals (Mazzini et al., 2013; Palacios et al., 2015; Deaux et al., 2016) and thus vocal communication may serve to reinforce social bonds, maintain territory and hierarchy, communicate identity, and locate separated pack members.

In livestock and other domestic animals, vocalisations are increasingly used as a tool to evaluate welfare by indicating both positive and negative impacts (Schön et al, 2004; Laruijs et al, 2021). Vocalisations can therefore provide clues as to the emotional state of animals, as a proxy to assess welfare. For example, pigs (*Sus domesticus*) exposed repeatedly to moderately aversive stimuli produced more vocalisations overall with higher rates of low frequency grunts compared to pigs exposed to positive stimuli (Laurijs et al., 2021). In domestic dogs, increased rates of howling, barking and whining may be associated with human-related separation anxiety (Pongracz et al., 2017). Hearing the whines of a familiar individual also increases alertness and stress-related behaviours in conspecifics and may prompt more affiliative behaviours towards that individual (Quervel-Chaumette et al., 2016). In addition to increased rates of vocalisation during periods of separation anxiety in domestic dogs, acoustic parameters also appear to increase in spectral noise (Marx et al., 2021). Wild canids show similar patterns, with separation of pack members causing stress in wolves, as evidenced by a significant increase in cortisol from baseline during separation, that resulted in increased rates of howling, which was further exacerbated by denying visual contact between individuals (Mazzini et al., 2013). Suggesting that long distance contact calling may be associated with the stress of separation of pack members in wild canids. As such, vocalisations would appear to be driven by the emotional and/or physical state of animals and measurement of changes in the rate and fundamental parameters of vocalisations may provide strong evidence to implicate specific drivers that change emotional states in pack living canids.

The African wild dog is a socially complex endangered carnivore, that lives at low population densities and occupies very large home ranges of up to 2,500 km^2 (Creel & Creel, 2002; Van den Berghe et al., 2012; Woodroffe & Sillero-Zubiri, 2012; Pomilia et al., 2015). Within African wild dog packs, animals work cooperatively to care for juvenile, old and sick or injured individuals, and to hunt and guard territory (Creel & Creel, 2002). Packs contain separate male and female dominance hierarchies, and generally only the alpha pair breed, requiring the cooperation of subordinate animals to help rear the litter (Creel & Creel, 2002;

Van den Berghe et al., 2012). Packs with less than five animals hunt less effectively and, as such, five is the minimum threshold for pack survival (Creel et al., 1995; Creel et al., 1997a; Girman et al., 1997; Creel & Creel, 2002). Social cohesion in African wild dog packs is of utmost importance to their survival due to their reliance on efficient group hunting, cooperative raising of pups and care for sick and injured individuals, as well as cooperative territory maintenance and defence. Thus, they must rely on physical behaviours and vocalisations to communicate effectively with one another in different contexts. Due to their limited and declining population of only 6600 animals and the fact that they remain endangered across Africa, zoo-based populations have been established as a safeguard against extinction (Woodroffe et al., 1997; Woodroffe & Sillero-Zubiri, 2020). Additionally, most free-roaming populations are found mainly within protected areas such as game reserves and National parks throughout Southern Africa and southern regions of Eastern Africa (Lindsey et al., 2004; Hayward et al., 2007). Currently, free-roaming African wild dog populations also exist in reduced numbers outside protected zones, but are threatened primarily by human activities such as snaring, poisoning and vehicle collisions, and diseases transmitted by domestic animals (Woodroffe et al., 2007; Prager et al., 2012; Nicholson et al., 2020).

Given that the amount of habitat available to African wild dogs has shrunk dramatically in recent decades, many remaining animals are part of management programmes, such as the metapopulation management programme in South Africa, that aim to protect existing populations, promote breeding success through artificial pack formation, and where possible, translocate and reintroduce packs into protected areas (Davies-Mostert et al., 2009). The success of these goals depends largely on a thorough understanding of the complex social nature of this species. Housing African wild dogs in confined spaces (permanently or temporarily) and introduction/reintroduction of individuals into packs are known to be stressors, which lead to increased stress and aggression related hormones along with anti-social behaviours (Marneweck, et al., 2019; Van den Berghe et al., 2019b). Furthermore, in zoos 23% of mortalities and 62% of morbidities have been attributed directly to aggressive behaviours (Cock et al., 2023). Thus, it is possible that high density living conditions and reduced ability to perform natural behaviours in captive packs could lead to decreased social cohesion exacerbating chances of problematic anti-social behaviours occurring. Current management often relies on the subjective observation of keepers/wildlife managers, which are often time poor and intrinsically biased by opinion. Acoustic monitoring could offer a sensitive, unbiased assessment of welfare in this species, but there is a need to first understand how vocalisations and their acoustic parameters are influenced by specific behaviours, contexts and whether confinement (in zoos or managed reserves) affects these.

The African wild dog has the largest vocal repertoire of all wild canids, consisting of 11 vocal classes and 18 subclasses (Matern, 1981; Robbins, 2000). Seven of these vocalisations are unique to this species (Robbins, 2000) but, despite their classification, very little is known about how vocalisations are used by African wild dogs. The only vocalisation that is well understood is the hoo-call, a long-distance vocalisation used to (i) locate separated individuals or (ii) mediate willingness of dispersing groups to form a new reproductive pack (Robbins, 2000; Marneweck et al., 2019). Some vocalisations may be context-specific, while others appear to be used across a range of contexts (Robbins, 2000). Changes in physical behaviours of pack members has been observed when specific vocalisations are expressed (Robbins, 2000; Potgeiter et al., 2015). Moreover, variations in vocalisations may be a sensitive indicator of social cohesion and welfare within packs during different contexts and within stressful captive settings. In this context, understanding whether any strong associations exist between their behaviour and, as yet under-studied vocal communication, could inform management decisions that lead to improved social cohesion, and ultimately resilience and persistence of packs when released into the wild. The current study aimed to better understand associations between specific vocalisations and specific behaviours in African wild dogs, and to determine whether the rate and acoustic parameters of these vocalisations differ according to context in which behaviours occur (feeding, social interaction, animal threat and human threat) and housing status (traditional zoo, non-traditional open-range zoo and free-roaming pack).

2. Materials and Methods

2.1. Animals

Behaviours and vocalisations were recorded from five African wild dog packs in South Africa, representing populations from free-roaming (TOOG pack, Waterberg, Limpopo), captive open-range (BOT pack, Bothongo Rhino and Lion Nature Reserve, Kromdraai, Krugersdorp) and traditional zoo-housed (JOB pack, Johannesburg Zoo, Johannesburg; AVDCC1 Monastery B and AVDCC2 Kennels packs, Ann Van Dyke Cheetah Centre, Brits) environments. All packs were observed during the denning seasons in 2022 (JOB) or 2023 (TOOG, BOT, AVDCC1 and AVDCC2). The TOOG packs was observed at two different den sites, firstly in June 2023 before a dispersal event ($\mathrm{TOOG_{BD}}$) and secondly in July 2023 after ($\mathrm{TOOG_{AD}}$) the dispersal of five two-year-old males from the natal pack after the pack moved from their initial den site. This data were analysed separately due to large differences in the rate of behaviours and vocalisations emitted by the pack after the dispersal of the five two-year-old males from the original pack. The composition, sex ratio and age of animals in each pack are summarised in Table 4.1.

Traditional zoo packs were fed every morning with either meaty bones, chicken carcasses or a mixture of these with dog kibble. The open-range zoo pack was fed sections of cow carcass every morning (Tuesday and Thursday) and midday (Wednesday, Saturday and Sunday) and fasted Friday and Monday. All zoo-housed animals had access to water ad libitum. The free-roaming pack was fed in the evening on each observation day with a whole or part of a carcass (Warthog, Zebra or Kudu) anchored to a tree by metal cable and were called up to the carcass using playback of a hoo-call to which they had been habituated.

Table 4.1. The demographics, enclosure type and mean (± SEM) duration of daily observations for each African wild dog (*Lycaon pictus*) pack from which vocalisations and behaviours were analysed.

Pack	Composition	Sex ratio	Age	Housing (enclosure size)	Daily observation time (min – max) h:m
$\mathrm{TOOG}_{\mathrm{BD}}$	3 adults	1F:2M	5+ y	Free-roaming	0:46 ± 0:04
	6 sub-adults	1F:5M	2 y		(0:37 – 0:53)
	6 yearlings	1F:5M	1 y		
$\mathrm{TOOG}_{\mathrm{AD}}$	3 adults	1F:2M	5+ y	Free-roaming	0:57 ± 0:50
	1 sub-adult	1F	2 y		(0:30 – 2:12)
	6 yearlings	1F:5M	1 y		
BOT	15 adults	5F:10M	3+ y	Open-range zoo	2:41 ± 0:29
				(6.0 ha)	(2:15 – 3:36)
JOB	2 adults	1F:1M	12+ y	Traditional zoo	1:01 ± 0:19
				(0.3 – 0.2 ha)	(0:55 – 1:28)
AVDCC1	2 adults	1F:1M	10 y	Traditional zoo	0:46 ± 0:01
	6 yearlings	1F:5M	1 y	(0.3 ha)	(0:45 – 0:48)
AVDCC2	5 adults	5M	3 y	Traditional zoo	0:48 ± 0:03
				(0.5 ha)	(0:44 – 0:55)

Min, minutes; max, maximum; h:m, hours:minutes; BD, before dispersal; AD, after dispersal; F, female; M, males; y, years; ha, hectares

2.2. Observations and analysis during different behavioural contexts

All behaviours were recorded using a Sony FDR-AX100E 4K digital video camera either from a stationary 4x4 vehicle or from public and zookeeper viewing areas over three days. The number of hours captured for each pack is listed in Table 4.1. Video data for each pack were analysed in BORIS Software (v. 7.13.8 2022-07-27, Olivier Friard – Marco Gamaba, University of Torino, Torino, Italy; Friard et al., 2016) for behaviours which occurred when pack members were vocalizing. Behaviours were classified using a modified ethogram from Van den Berghe et al., (2019b) to include feeding-specific behaviours and stereotypic behaviours (Table 4.2). Each specific behaviour was recorded and assigned to a behaviour category (Aggression, Dominance, Submission, Feeding, Play, or Affiliation; as shown in Table 4.2), behaviour categories were then assigned into one of four social contexts in which they occurred (Social interaction, Feeding, Animal threat, or Human threat) and captive status for the purpose of analysis (Table 4.2). Behaviours within the context of social interactions included all social interactions that occurred between pack members outside of feeding. Behaviours within the context of animal threats were behaviours directed towards an animal of a different species including animals in adjacent enclosures such as lions, or wild hyenas which intruded into the free-ranging packs territory. Behaviours within the context of human threats included all behaviours directed towards the keeper during feeding or cleaning of the enclosure for captive packs, or directed towards the observers within our vehicle for the free-ranging pack. Human and animal threats were deliberately separated due to the differences in the way African wild dogs respond to these threats. Animal threats pose a perceived risk to food security and life, while human threats pose more of a territorial threat as human keepers routinely entering enclosures for feeding and cleaning purposes. Feeding behaviour and feeding context differed in that only behaviours related to the act of feeding (the eating of or acquisition of food) were included in the feeding behaviour category, while both feeding behaviours and all other behaviours that occurred at or near a food source were included in the feeding context. In this study aggression, dominance and submission were categorised as anti-social due to the fact that during normal interactions, rates of these behaviours tend to be low but typically increase during periods of stress (Van den Berghe et al., 2019b). Behaviour categories were designated as anti-social (aggression, dominance and submission) or pro-social (play and affiliation, which increase positive social bonds between pack members). Feeding was not classified as either since feeding behaviours can be pro-social, anti-social or ambivalent in nature.

Table 4.2. African wild dog (*Lycaon pictus*) behaviour ethogram modified from Van den Berghe et al., (2019b).

Behaviour	***Description***
Aggressive behaviours	

Assault	A brusque approach at full speed, often followed by an aggressive physical contact.
Severe biting	The actor has closed jaws and teeth having a strong hold of any part of the actor's body e.g. legs, tail, throat or head. The bite is with full strength (uninhibited).
Chase away	The actor walks or runs in pursuit to elicit a flee response from the recipient.
Push Down	The actor presses down the recipient by an inhibited bite in the neck.
Embrace	The actor embraces the neck of the recipient from the front, the recipient can stand on four or two hind legs.
Snout bite	The actor seizes the snout of the recipient between its jaws from the side or from above and holds it gently for a short while.
Scruff bite	Scruff orientated inhibited bite.
Snap	The actor lunges with a bite movement towards the recipient, without making contact and often with an audible sound of the jaws, when they come together.
Ritualized approach	A restrained gallop in high posture towards the recipient, not resulting in physical contact.
Dominant behaviours	
Scruff orientated approach[b]	The actor approaches the scruff of the recipient without biting.
Stalk approach[a]	The actor slowly approaches the recipient with a prowling posture; that is with the head and neck in a straight line below the shoulder, the ears folded back, the tail relaxed or in a straight horizontal line and without losing eye-contact with the recipient.
Food approach	The actor approaches the recipient while looking at him in the context of food acquisition.
Intervention by approach stand or threat[a]	The actor stops an interaction between two interactants by approach, stand in between or threat towards one of the recipients respectively.
Fixating[a]	The actor looks straight at the recipient from a distance, motionless, in a high posture and with the ears forward.
Mark over urine or food	The actor secretes, with one (or both) feet lifted from the ground, a small amount of urine over a previous urine mark or food item on the ground.
Freezing[b]	The actor stands stiff with the head straight to the ground and the eyes fixated, either on the ground or on the recipient; the behaviour is shown mostly as a reaction to 'food approach'.
Inguino-genital inspection[b]	The actor initiates an inguinal contact and investigates the genitals of the recipient while the latter remains passive.
Point[a]	The actor directs, with an abrupt movement of his head or a short jump, towards the recipient.
Mount[b]	The actor places both its forepaws on the back of the recipient. It may do so from behind or from the side.
Stand over position[b]	The actor stands across a lying recipient.

Approach in high posture[a]	Moving towards the recipient in a high posture, while looking at him.
High posture snout[b]	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a high posture.
High posture face lick[b]	The actor licks the nose, lips and mandibular region of the recipient while being in a high posture.
Submissive behaviours	
Escape/flight[c]	The actor runs away from the recipient, often seen during conflicts.
Retreat[c]	The actor moves away from the recipient in a low position after having been approached by him. This also includes a retreat in the context of food acquisition.
Shrink back[c]	The actor jumps back from the recipient, after being approached by him.
Avoid[c]	The actor stands aside for the recipient, after being approached by him.
Active submission[d]	A behavioural complex in which the actor actively seeks contact with a recipient by approaching him in a crouched manner with curved back and bent legs, while the tale is curled down, often wagging, and while the ears are folded back. From this position, the actor tries to contact the recipient by licking its nose.
Passive submission[c]	The actor pushes himself down in front of the recipient.
Head turning[c]	The actor turns his head and avoid eye contact with the recipient, exposing the neck region towards the recipient.
Low posture standing[c,d]	Stand in a low position, with the ears pulled back.
Approach in low posture[c,d]	The actor moves towards the recipient in a low posture while looking at him.
Low posture snout contact[c,d]	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a low posture.
Present body[c]	The actor rolls on his side in front of the recipient or rolls towards him, awaiting his inspection.
Food solicit	The actor approaches or walks in parallel with the recipient while begging for food and trying to reach for his mouth corners. There is some resemblance with ’greeting’, which is an affiliative behaviour, but the context is different and the behaviour is not likely to be reciprocated.
Low posture face lick[c,d]	The actor licks the nose, lips and mandibular region of the recipient while being in a low posture.
Affiliative behaviours	
Close contact	The actor stands or lies within one body length from the recipient. The recipient may be standing, sitting or lying.
Approach in neutral posture	The actor moves towards the recipient in a neutral posture while looking at him.
Neutral posture snout	The actor brings his nose close to or pushes it towards the nostrils of the recipient while being in a neutral posture.
Neutral posture face lick	The actor licks the nose, lips and mandibular region of the recipient while being in a neutral posture.

Pass under head	The actor passes from a lateral side close under the head of the recipient, usually in a somewhat crouching manner; often a short nose-chin contact with the recipient is evident.
Head under	The actor pushes with his head towards the ventro-lateral side of the recipient, occasionally lifting the recipient's back quarters from the ground with his head.
Fur sniff/licking	Self-explanatory
Paw/head on	The actor places a paw or its head on the back of the recipient.
Grin	Only clear facial expression shown by AWDs. The mouth corners remain retracted and the mouth may be slightly open so that the teeth become visible. The behaviour occurs mostly in combination with 'giggle' and has a friendly nature
Greeting	The actor stands or walks in parallel with the recipient, tries to contact his muzzle, and performs a complex of behaviours including food-solicit or inspection behaviour, the facial expression 'grin' and the vocalisation 'giggle'.
Parallel walk or run	Two animals walking side by side in the same direction.
Regurgitation	Expulsion of undigested food from the mouth, pharynx, or oesophagus.
Rub on	The actor establishes intensive latero-lateral contact with the recipient. This may occur while both animals are in motion.
Greet without contact	The actor looks up at the approaching individual/s and vocalises before going back to what it was doing previously. No physical contact is involved.
Play behaviours	
Play solicit	The actor initiates a play interaction with the recipient by solicit behaviour such as nose pushing or tugging the recipients fur with an inhibited bite.
Fur bite	The actor tugs the recipient fur by an inhibited bite.
Play fighting	Playful non-competitive fighting in which attacker and defender exchanges roles and no winner or loser emerges. Interactions rarely include behaviours that can inflict injury.
Play chase	The actor follows in a fast pursuit the recipient, who tries to escape by abruptly changing the direction. The roles of follow and escape may change.
Play wrestle	This behaviour involves all play situations, in which the actor shows inhibited bite movements towards the recipient, while he keeps the recipient in constant eye contact.
Play sniff	Two or more pack members are engaged in extensive sniffing at an object or some particle on the ground, while their heads are in direct contact.
Social play	All other forms of play.

Feeding behaviours	
Food steal	One individual takes the food from another
Eating alone	Individual is consuming food
Eating in group	More than one animal eating from the same food source
Approach carcass	Individual approaches a carcass seeking access to food
Move off carcass	Individual moves away from carcass or food after eating without prompt from other animals
Fight over food	Two or more animals hold the same piece of food and tug away from each other
Leave feeding site	An individual leaves the feeding site to go elsewhere

[a] Behaviours included in non-contact dominant behaviour
[b] Behaviours included in contact dominant behaviour
[c] Behaviours included in passive submission behaviour
[d] Behaviours included in active submission behaviour

Audio recordings were converted to WAV files using Audacity® (v 3.1.2, The Audacity Team, Limassol, Cyprus). Vocalisations were then identified by spectral analysis using Wildlife Acoustics Kaleidoscope Basic® software (v 5.4.6, Wildlife Acoustics Inc., Massachusetts, USA), then classified according to Robbins (2000) into either social twitters, spar twitters, mob twitters, attack twitter, begging cries, yelps, whistle squeals, begging squeals, whimpers, whines, full moans, buzz moans, rumbles, social growls, alarm growls, alarm barks, attack barks, clear barks, howl barks, threat barks, yelp barks, hoo-calls, sneezes and huffs (see Appendix 6 for spectrograms). All vocalisation types except hoo-calls, yelp barks and howl barks were present in recordings. The rate, syllable length, and fundamental frequency (hereon referred to as F_0) were recorded for each vocalisation within each pack.

2.3. Statistical analysis

Data were analysed using RStudio (v 3.6.2; R Studio Team, 2023) for Windows. Mean ± standard error of the mean (SEM) was calculated for daily rate, syllable length and fundamental frequency of vocalisations within each behaviour category. Data were grouped by social context and enclosure type. Tests for normality were performed for each type of vocalisation using histograms and a Shapiro-Wilk test. $P \leq 0.05$ was considered significant for all analyses.

Total daily rate of vocalisations within behaviour categories and their syllable length and fundamental frequency were analysed using a generalised linear mixed model (GLMM) using the tidyverse (Wickham et al., 2019), lme4 (Bates et al., 2015), emmeans (Russell et al., 2024) and MASS (Ripley, 2002) packages; the dependent variables were set as the daily rate, syllable length or fundamental frequency of each vocalisation, the independent variable as the behaviour category, random effects of pack and day and duration of observation were included, and observation time were included as offset terms. GLMMs were fitted by maximum likelihood with Laplace approximation. Parsimonious models were generated using a two-step procedure, where the Akaike's information criterion was used to determine mean random-effects structure based on the full model containing all candidate fixed effects (pack, observation day, observation time, pack size, context, housing, behaviour categories, sex ratio), and then removed statistically insignificant fixed-effect terms (Zuur et al., 2009). Variables were then pruned using a backward stepwise selection based on likelihood ratio tests (Zuur et al., 2009). All GLMM were considered significant when $P \leq 0.05$.

A GLMM was performed to determine significant differences in daily rate, syllable length and fundamental frequency of vocalisations between behaviour categories within each context (feeding, social interaction, animal threat and human threat). The dependent variable was set as the daily rate, syllable length or fundamental frequency of each vocalisation, the independent variable as the behaviour category, and random effects of pack and day of observation were included. Another GLMM was used to determine significant differences in daily rate, syllable length and fundamental frequency of vocalisations between contexts within each behaviour category. The dependent variable set as the daily rate, syllable length or fundamental frequency of each vocalisation within behaviour category, the independent variable as the social context, and random effects of pack and day and duration of observation were included.

A GLMM was performed to determine significant differences in daily rate, syllable length and fundamental frequency of vocalisations between behaviour categories within each enclosure type (traditional zoo, open-range zoo, free-roaming). The dependent variable set as daily rate, syllable length or fundamental frequency of vocalisations within enclosure type, the independent variable as the behaviour categories, and random effects of pack and day of observation were included. A GLMM was also used to determine significant differences in daily rate, syllable length and fundamental frequency of vocalisations between enclosure types within each behaviour category. The dependent variable was set as the daily rate, syllable length or fundamental frequency of each vocalisation within behaviour category, the independent variable as the enclosure types, and random effects of pack and day and duration of observation were included.

3. Results

3.1. Association between vocalisations and behaviours

The results of the GLMM investigating the associations between vocalisations and behaviour categories are illustrated in Figure 4.1, with further detail, including specific P-values, found in Supplementary Table 4.1 (Appendix 1).

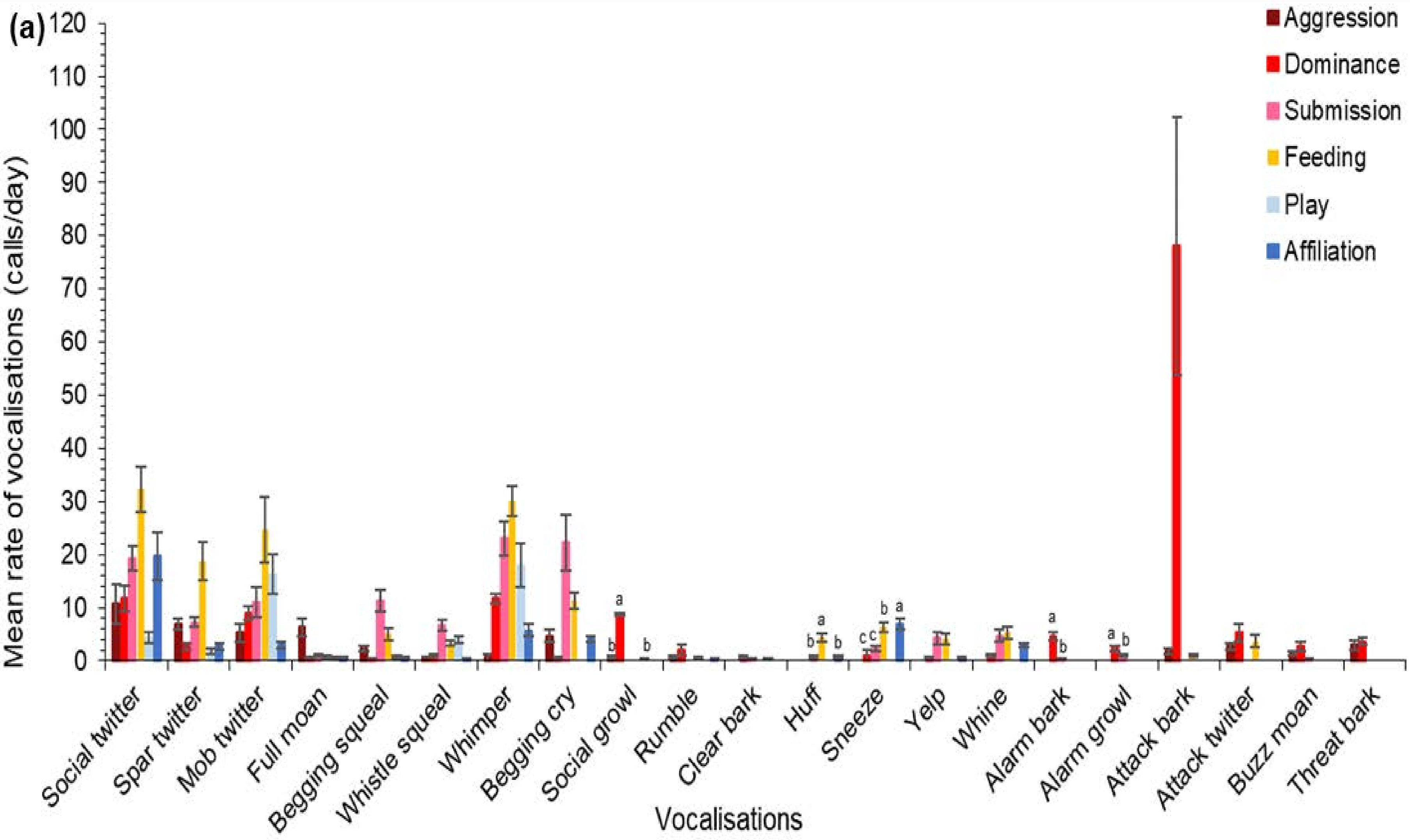
Anti-social dominant behaviours (dominance, 20 different vocalisations) tended to be accompanied by a wider range of vocalisations than other anti-social behaviours (submission, 16; aggression, 14 vocalisations), feeding (15 vocalisations) and pro-social behaviours (affiliation, 13; play, 9 vocalisations). Additionally, buzz moans, alarm growls, threat barks and alarm barks were only associated with anti-social behaviours, while attack twitters and attack barks were only associated with both anti-social and feeding behaviours.

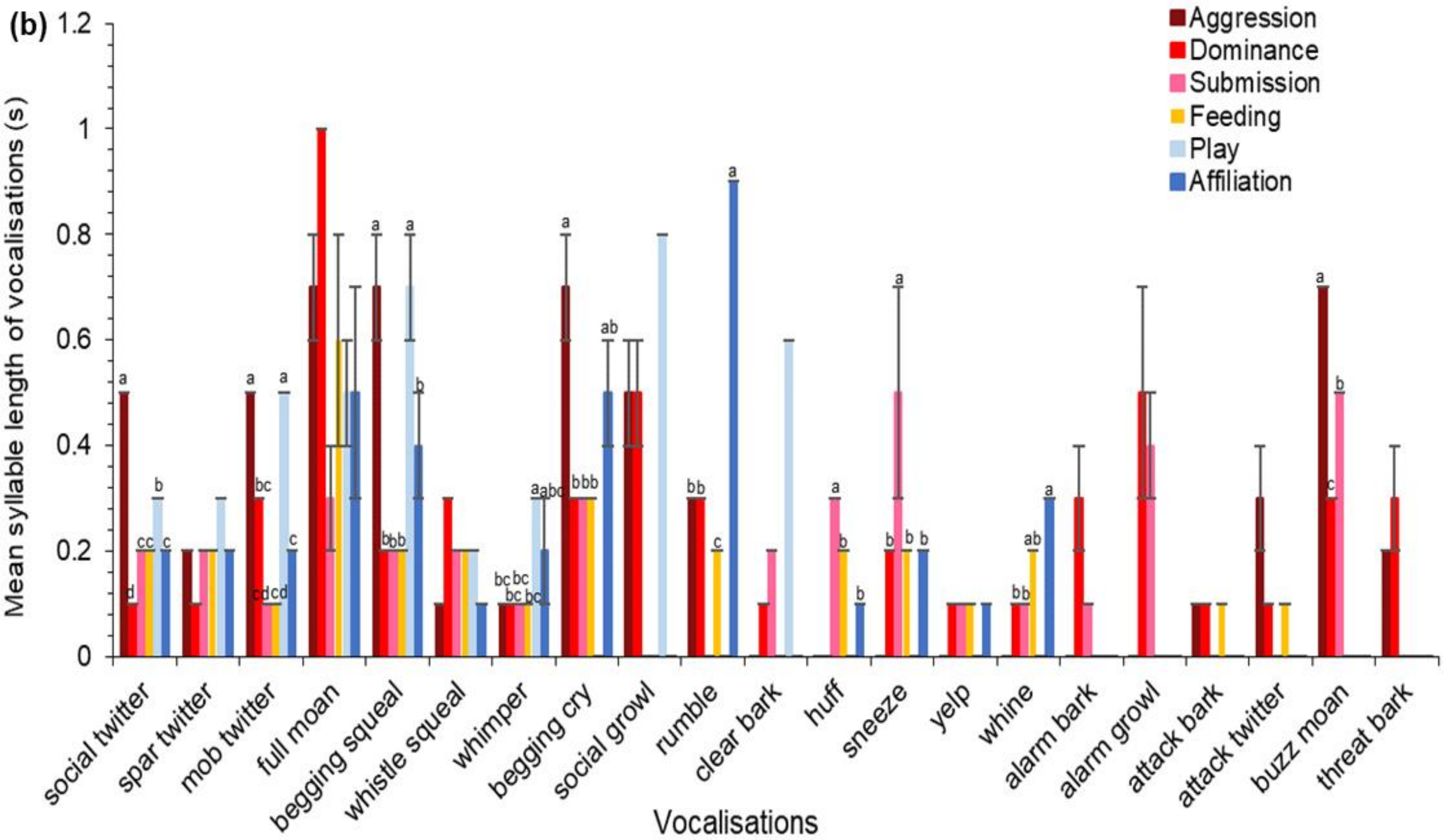
All vocalisations occurred during more than one category of behaviour. However, three vocalisations were each only associated with two anti-social behaviours, with alarm growls and alarm barks linked to dominant and submissive behaviours while threat barks were linked to dominant and aggressive behaviours (Fig. 4.1; Appendix 1). Alarm growls and alarm barks occurred at significantly higher daily rates and had higher F_0 during dominant compared to submissive behaviours (Fig. 4.1a-c; Appendix 1).

Six vocalisations were each associated with only three categories of behaviour, with buzz moans linked to anti-social behaviours only, while attack twitters and attack barks were linked to anti-social and feeding behaviours; clear barks and social growl to anti-social and play behaviours; and huffs to submission, feeding and affiliative behaviours (Fig. 4.1a-c; Appendix 1). Only the mean daily rate of social growls and huffs were significantly higher during dominance or feeding respectively compared to other behaviours (Fig. 4.1a; Appendix 1). Despite no significant difference being detected, attack barks appeared to occur at least 46-fold more often during dominance than other behaviours. The syllable length of buzz moans and huffs were significantly longer during aggression and submission respectively compared to other behaviours (Fig. 4.1b; Appendix 1). The F_0 of huffs, social growls and clear barks were significantly lower during feeding and play, aggression and dominance, and submission respectively compared to other behaviours in which they occurred (Fig. 4.1c; Appendix 1).

Sneezes, rumbles, whines, yelps, begging cries, whistle squeals, begging squeals, mob twitters, full moans, spar twitters, whimpers and social twitters tended to be less specific, being associated with four to six categories of behaviour, and all were emitted during anti-social, pro-social and feeding behaviours (Fig. 4.1a; Appendix 1). Furthermore, none of these vocalisations differed significantly in rate between behaviour categories (Fig. 4.1a; Appendix 1). However, the syllable length of sneezes, social twitters, begging cries, begging squeals, whines, rumbles and mob twitters were significantly longer during submission, aggression, aggression and affiliation, aggression and play, feeding and affiliation, affiliation, or aggression and play respectively when compared to other behaviour categories (Fig. 4.1b; Appendix 1).

The F_0 of social twitters, whines and yelps, and rumbles were significantly lower during aggression, dominance, or aggression and dominance compared to other behaviours respectively (Fig. 4.1c; Appendix 1).


(a)
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Mean rate of vocalisations (calls/day)
0
10
20
30
40
50
60
70
80
90
100
110
120
a
b
b
b
a
b
c
c
b
a
a
b
a
b
Social twitter
Spar twitter
Mob twitter
Full moan
Begging squeal
Whistle squeal
Whimper
Begging cry
Social growl
Rumble
Clear bark
Huff
Sneeze
Yelp
Whine
Alarm bark
Alarm growl
Attack bark
Attack twitter
Buzz moan
Threat bark
Vocalisations


(b)
Mean syllable length of vocalisations (s)
0
0.2
0.4
0.6
0.8
1
1.2
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Vocalisations
social twitter
spar twitter
mob twitter
full moan
begging squeal
whistle squeal
whimper
begging cry
social growl
rumble
clear bark
huff
sneeze
yelp
whine
alarm bark
alarm growl
attack bark
attack twitter
buzz moan
threat bark
a
d
cc
b
c
a
bc
cdcdcd
a
c
a
bbbb
a
b
bc bc
bc bc bc
a abc
a
bbbb
ab
bb
c
a
a
b
b
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a
b
b
bb
ab
a
a
c
b

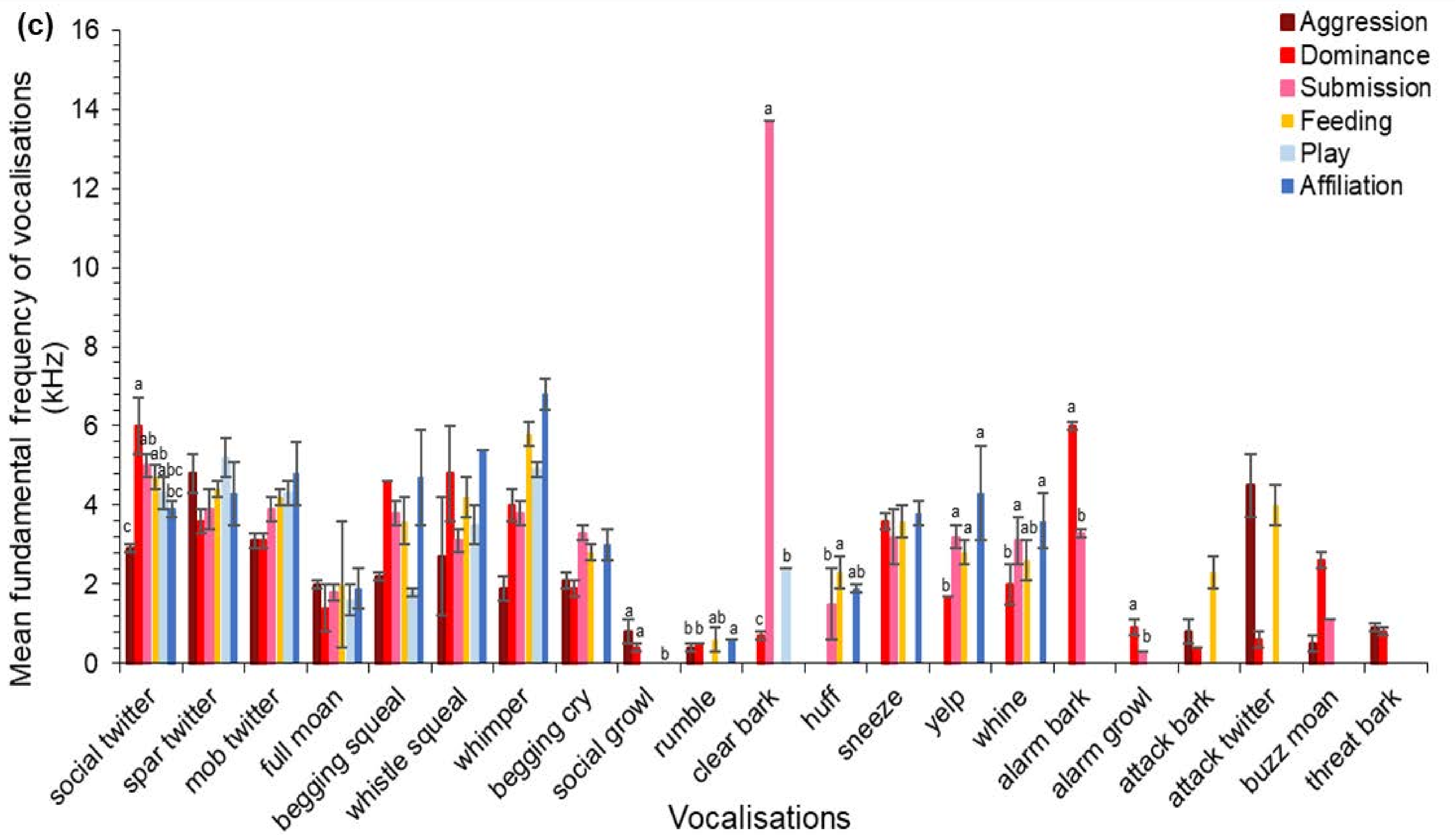


Figure 4.1. Bar graphs of the mean (a) daily rate, (b) syllable length, (c) fundamental frequency (F_0) of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*). ^{a,b,c} denotes a significant difference between behaviour categories within a vocalisation type.

3.2. Effect of context on association between vocalisations and behaviours.

The results of the GLMM investigating the associations between vocalisations and behaviour categories within and between contexts are illustrated in Figure 4.2 – 4.4, with further detail, including specific P-values, found in Supplementary Table 4.2 (Appendix 2).

The feeding context was associated with 17 different vocalisations, which were emitted during a range of anti-social, pro-social and feeding behaviours (Fig. 4.2; Appendix 2). Within the context of feeding buzz moans, alarm growls, threat barks and clear barks did not occur during any of the observed behaviours (Fig. 4.2t, q, u & k; Appendix 2). Attack twitters, social growls and alarm barks were only associated with one behaviour category (feeding, play and submission respectively; Fig. 4.2s, i & p; Appendix 2). Begging squeals, rumbles and attack barks were associated with only two behaviour categories, but their rates and acoustic parameters did not differ significantly different behaviour categories (Fig. 4.2-4e, j & r; Appendix 2). All other vocalisations occurred in three or more behaviour categories (Fig. 4.2-4; Appendix 2). The rate of whimpers and huffs were significantly increased during feeding compared to other behaviours; and the rate of whines was increased during submission compared to other behaviours in which they occurred (Fig. 4.2g & l; Appendix 2). The syllable length of huffs, whimpers and full moans were significantly longer during submission and feeding, affiliation, or dominance compared to other behaviours respectively (Fig. 4.3l, g &d; Appendix 2). The F_0 of social twitters, whimpers and spar twitters were significantly lower during affiliation, dominance, submission and feeding, or dominance and affiliation compared to other behaviours respectively (Fig. 4.4a, g & b; Appendix 2).

The social interaction context was associated with 14 different vocalisations, which were emitted during a range of anti-social and pro-social, but not feeding behaviours (Fig. 4.2-4; Appendix 2). Within the context of social interactions, attack twitters, social growls, alarm growls, attack barks, threat barks, alarm barks and huffs did not occur during any of the observed behaviours (Fig. 4.2s, I, q, r, u, p & l; Appendix 2). Rumbles and clear barks were only associated with one behaviour category (affiliation and play respectively; Fig. 4.2j & k; Appendix 2). Buzz moans, yelps, whines, full moans and sneezes occurred in two behaviour categories and all other vocalisations occurred in three or more behaviour categories (Fig. 4.2t, n, o, d & m; Appendix 2). Vocalisation rate did not differ between behaviour categories for any vocalisation (Fig. 4.2; Appendix 2). The syllable length of mob twitters, begging squeals, social twitters, and begging cries were significantly longer during play, submission and play, or affiliation respectively when compared to other behaviours (Fig. 4.3c, e, a & h; Appendix 2). The F_0 of social twitters, whimpers and mob twitters were significantly lower during dominance, play and affiliation, dominance, submission and play, or dominance and submission respectively compared to other behaviours (Fig. 4.4a, g & c; Appendix 2).

The context of animal threat was associated with 14 different vocalisations, which were emitted during a range of anti-social behaviours only (Fig. 4.2-4; Appendix 2). Within the context of animal threats, alarm growls, whistle squeals, whines, rumbles, attack barks, clear barks and huffs did not occur during any of the observed behaviours (Fig. 4.2q, f, o, j, r, k & l; Appendix 2). Attack twitters, buzz moans, begging squeals, mob twitters, full moans, spar twitters, begging cries and social twitters were only associated with aggression (Fig. 4.2s, c, d, b, h & a; Appendix 2). Social growls, yelps, threat barks, alarm barks and sneezes were only associated with dominance (Fig. 4.2i, n, u, p & m; Appendix 2). Whimpers were only associated with anti-social behaviours (aggression, dominance and submission) and its syllable length was significantly longer during submission compared to dominance and aggression (Fig. 4.2g; Appendix 2).

The context of human threat was associated with 16 different vocalisations, which were emitted during a range of anti-social and pro-social behaviours (Fig. 4.2-4; Appendix 2).

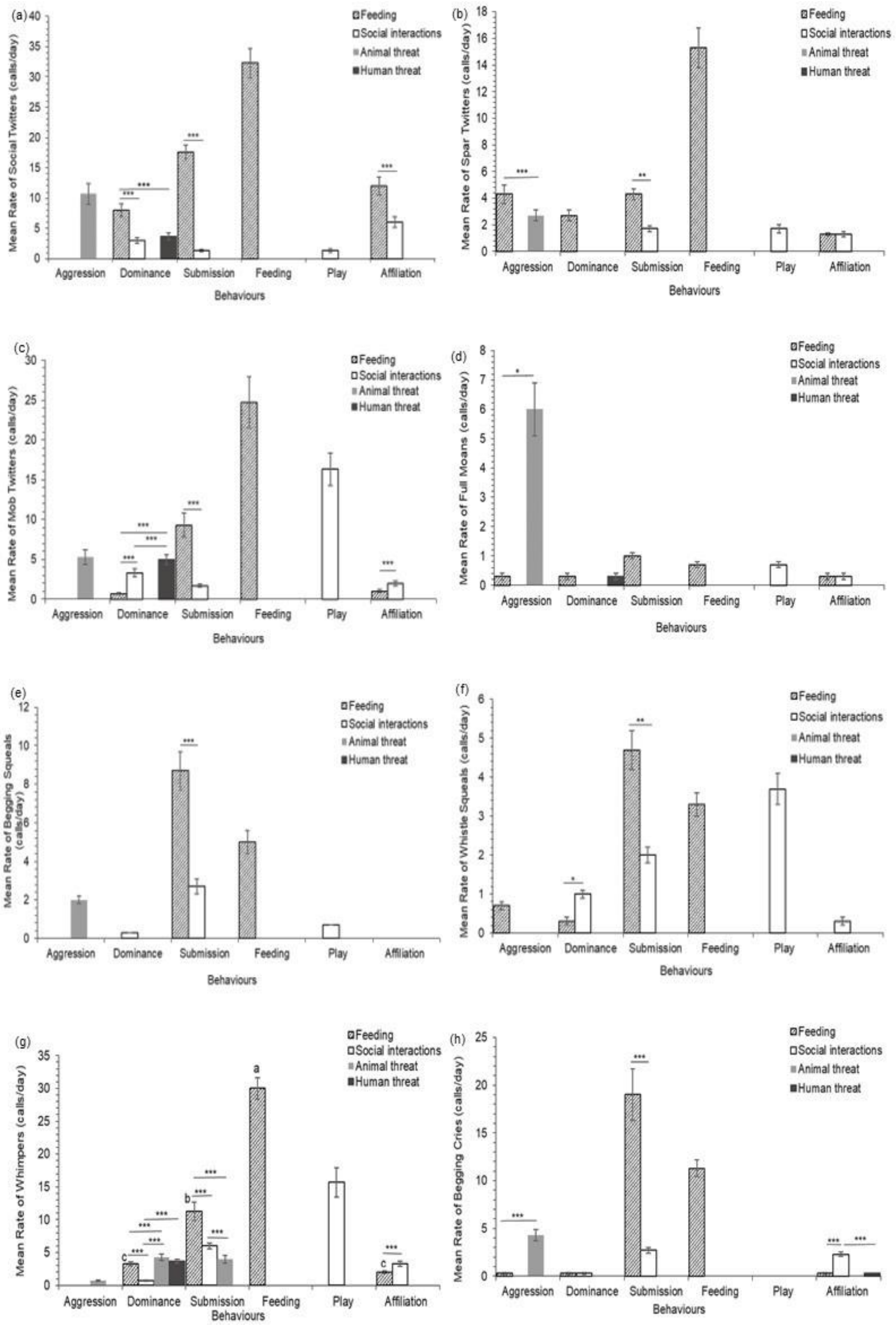
Within the context of human threats, buzz moans, whistle squeals, begging squeals, spar twitters and huffs did not occur during any of the observed behaviours (Fig. 4.2t, f, e, b & l; Appendix 2). Attack twitters, yelps, mob twitters, full moans, attack barks, alarm barks, clear barks, whimpers and social twitters were only associated with dominance (Fig. 4.2-4s, n, c, d, r, p, k, g & a; Appendix 2). Begging cries were only associated with affiliation (Fig. 4.2-4h; Appendix 2). Social growls, alarm growls, whines, rumbles, threat barks and sneezes were associated with two behaviour categories (Fig. 4.2-4i, q, o, j, u & m; Appendix 2). Vocalisation rate and syllable length did not differ between behaviour categories for any vocalisation (Fig. 4.2 and 4.3; Appendix 2). However, the F_0 of social twitters was significantly lower during affiliation than dominance (Fig. 4.4a; Appendix 2).

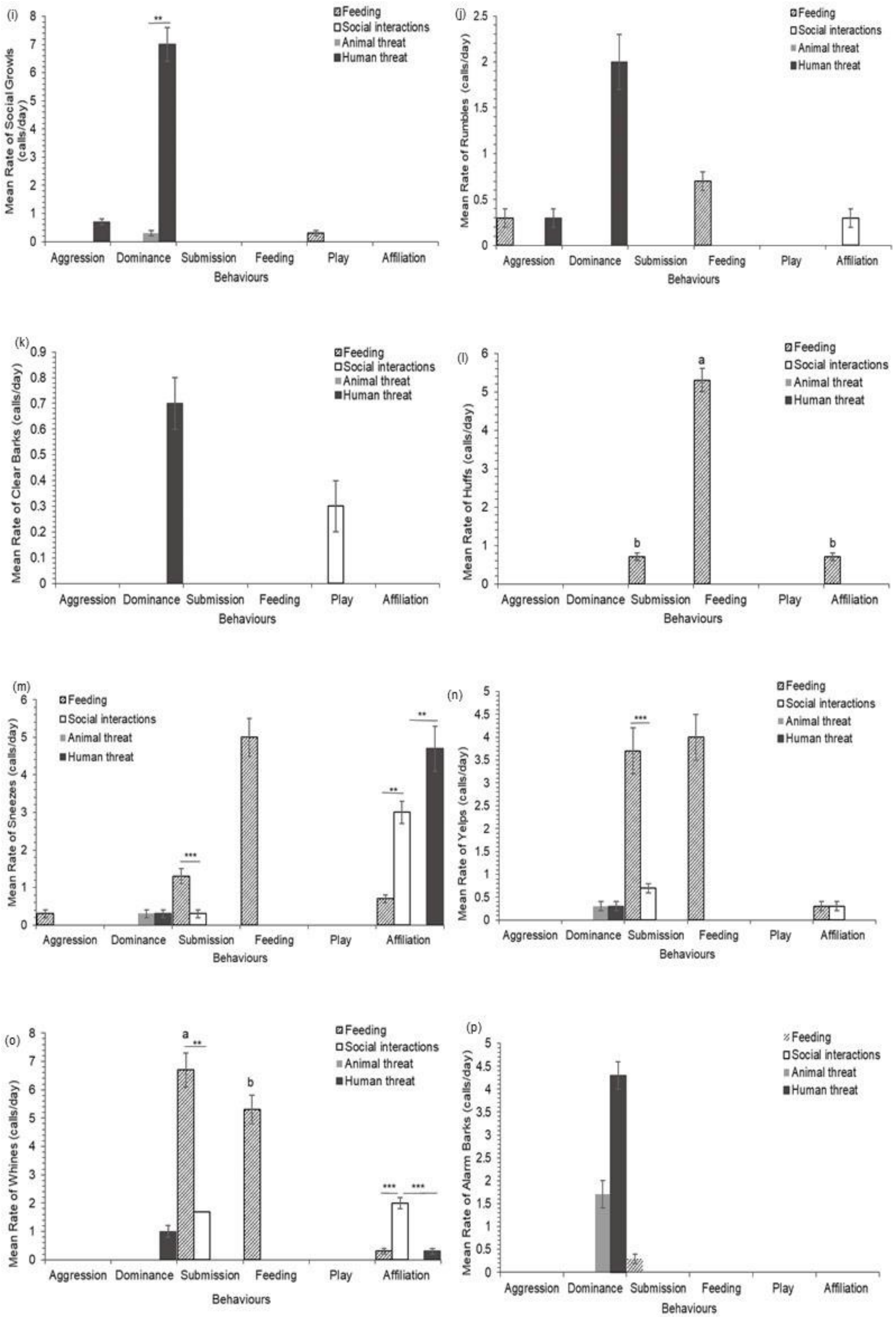
When compared between contexts during aggressive behaviours, the mean daily rate of begging cries, full moans and threat barks occurred significantly more often during threat context compared to the other contexts (Fig. 4.2h, d & u. Appendix 2). Additionally, spar twitters occurred significantly more often during feeding than animal threats but had longer syllables and lower F_0 during animal threats than feeding (Fig. 4.3b and 4.4b, Appendix 2).

During dominance behaviours, the mean daily rate of mob twitters, attack barks and threat barks occurred significantly more often during human threats, while whimpers were more frequent during both animal and human threats, and whistle squeals and social twitters were increased during social interaction and feeding than other contexts in which they occurred, respectively (Fig. 4.2c, r, u, g & a, Appendix 2). Moreover, threat barks and alarm barks had significantly longer syllables during animal threats, while whimpers were longer during both animal threats and social interaction, and mob twitters were longer during social interaction than other contexts in which they occurred (Fig. 4.3u, p, g & c, Appendix 2). Lastly, whimpers and social twitters had significantly lower F_0 during human threats, while alarm barks had lower F_0 during animal threats compared to other contexts in which they occurred (Fig. 4.4g, a & p, Appendix 2).

During submission, the mean daily rate of yelps, whistle squeals, begging squeals, whines, mob twitters, spar twitters, begging cries, whimpers, social twitters and sneezes were all significantly higher during the context of feeding (Fig. 4.2n, f, e, o, c, b, h, g, a & m, Appendix 2). Moreover, the syllable length of whimpers, begging cries and social twitters were significantly longer during animal threat, feeding and social interaction respectively (Fig. 4.3g, h & a, Appendix 2). Lastly, the F_0 of social twitters were significantly lower during feeding, while spar twitters and mob twitters were both lower during social interaction (Fig. 4.4a, b & c, Appendix 2).

During affiliation, the mean daily rate of begging cries, whimpers, whines and mob twitters were significantly higher during social interaction, while sneezes were higher during social interaction and human threats, and social twitters were higher during feeding (Fig. 4.2h, g, o, c, m & a, Appendix 2). Mob twitters were significantly longer and had a lower F_0 during feeding (Fig. 4.3c & 4.4c, Appendix 2). Finally, social twitters had a lower F_0 during human threats (Fig. 4.4a, Appendix 2).


(a)
Mean Rate of Social Twitters (calls/day)
0 5 10 15 20 25 30 35 40
Feeding
Social interactions
Animal threat
Human threat
***
Aggression Dominance Submission Feeding Play Affiliation
Behaviours
(b)
Mean Rate of Spar Twitters (calls/day)
0 2 4 6 8 10 12 14 16 18
***
**
(c)
Mean Rate of Mob Twitters (calls/day)
0 5 10 15 20 25 30
(d)
Mean Rate of Full Moans (calls/day)
0 1 2 3 4 5 6 7 8
*
(e)
Mean Rate of Begging Squeals (calls/day)
0 2 4 6 8 10 12
(f)
Mean Rate of Whistle Squeals (calls/day)
0 1 2 3 4 5 6
(g)
Mean Rate of Whimpers (calls/day)
0 5 10 15 20 25 30 35
a
b
c
(h)
Mean Rate of Begging Cries (calls/day)
0 5 10 15 20 25


(i)
Mean Rate of Social Growls (calls/day)
Feeding
Social interactions
Animal threat
Human threat
**
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(j)
Mean Rate of Rumbles (calls/day)
Feeding
Social interactions
Animal threat
Human threat
Behaviours
(k)
Mean Rate of Clear Barks (calls/day)
Feeding
Social interactions
Animal threat
Human threat
Behaviours
(l)
Mean Rate of Huffs (calls/day)
Feeding
Social interactions
Animal threat
Human threat
a
b
b
Behaviours
(m)
Mean Rate of Sneezes (calls/day)
Feeding
Social interactions
Animal threat
Human threat
***
**
**
Behaviours
(n)
Mean Rate of Yelps (calls/day)
Feeding
Social interactions
Animal threat
Human threat
***
Behaviours
(o)
Mean Rate of Whines (calls/day)
Feeding
Social interactions
Animal threat
Human threat
a
**
b
***
***
Behaviours
(p)
Mean Rate of Alarm Barks (calls/day)
Feeding
Social interactions
Animal threat
Human threat
Behaviours

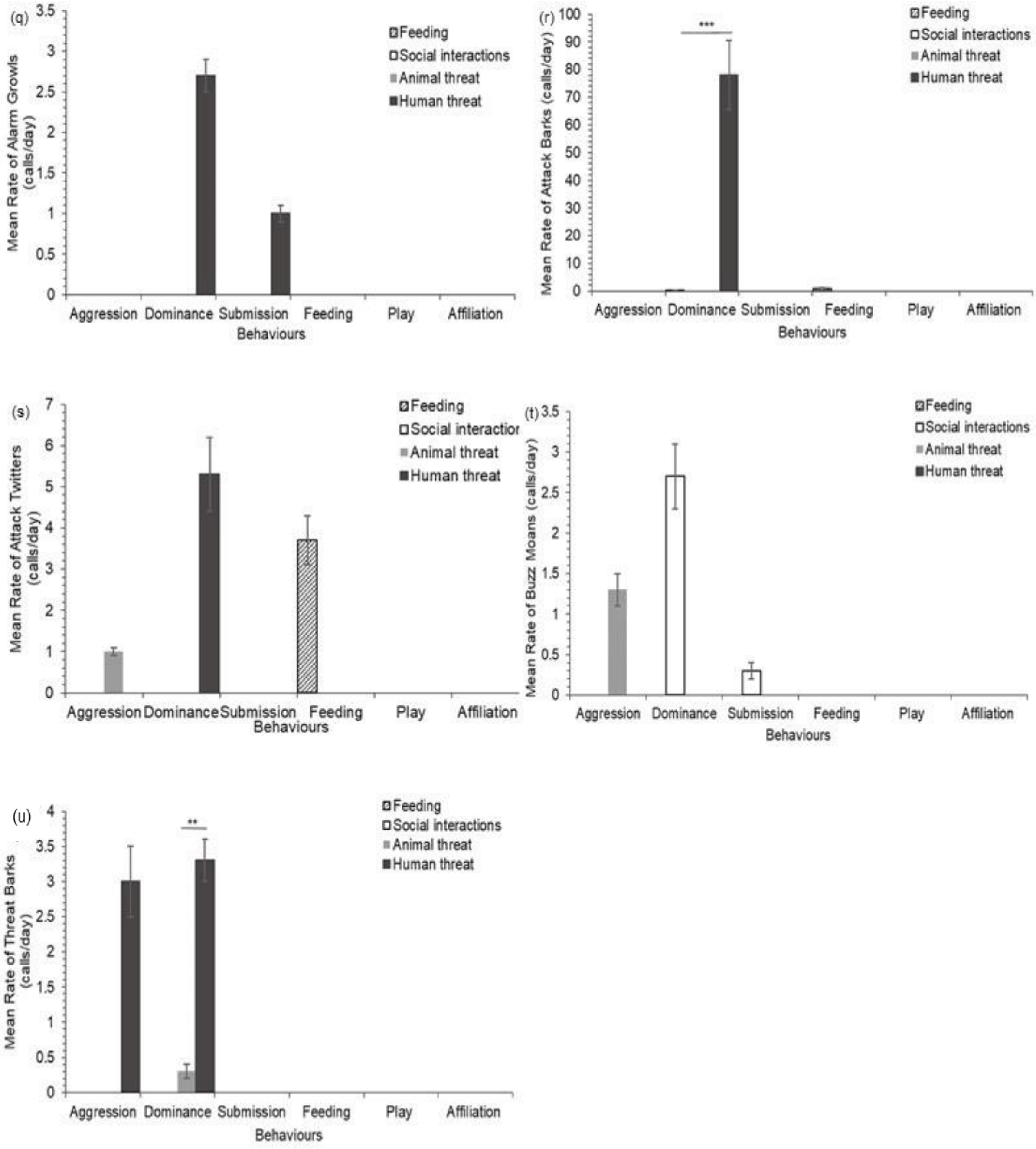
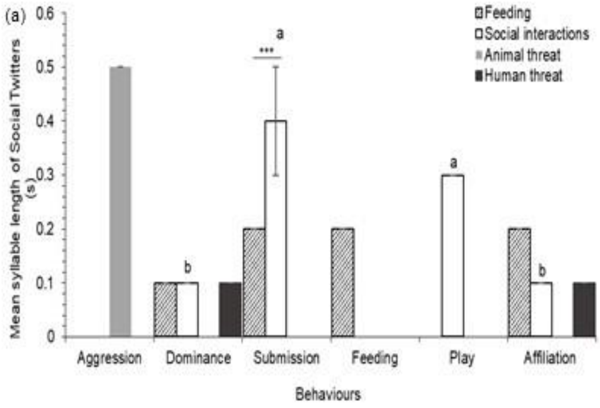
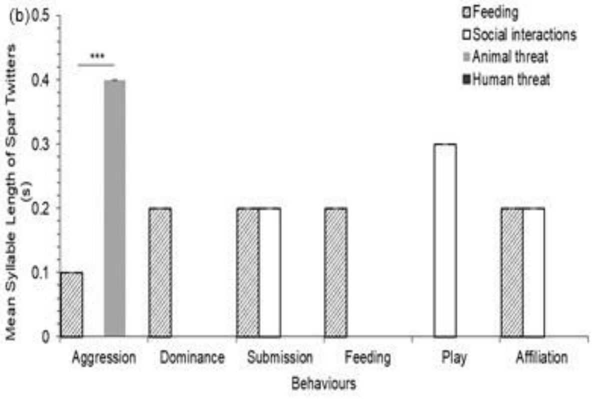
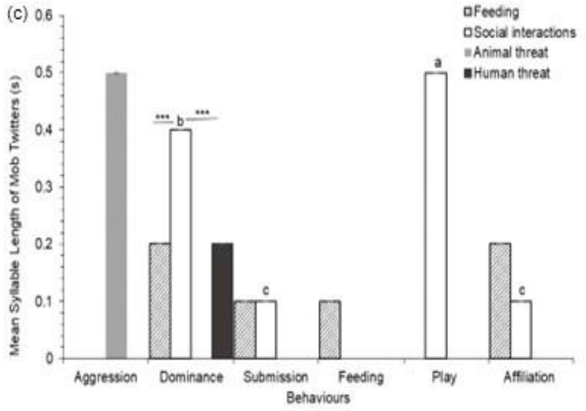
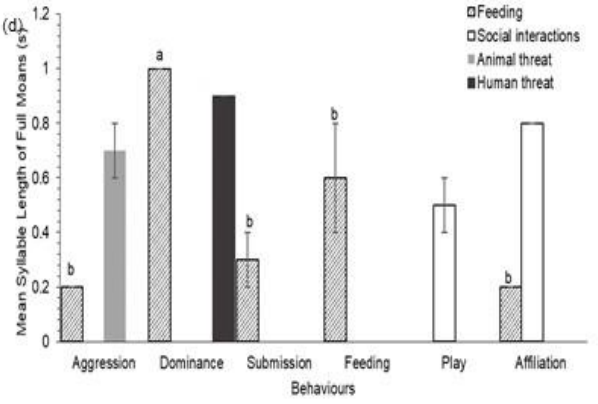


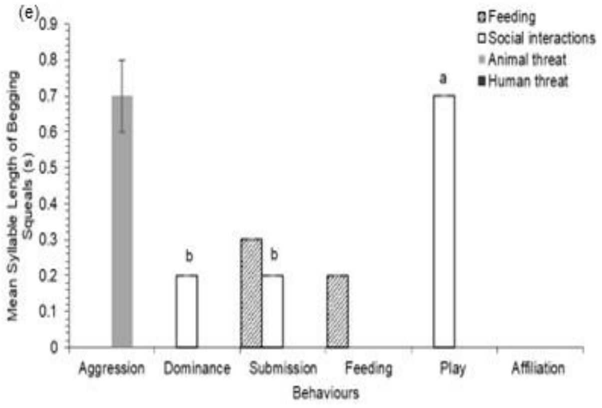
Figure 4.2. Bar graphs of the mean daily rate of vocalisations associated with different categories of behaviour (aggression, dominance, submission, feeding, play and affiliation) in African wild dogs (Lycaon pictus) within the context of feeding, social interaction, animal threat and human threat. ^{a,b,c} denotes a significant difference between behaviour categories within a behaviour context for a particular vocalisation type. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ denote significance between behaviour contexts.

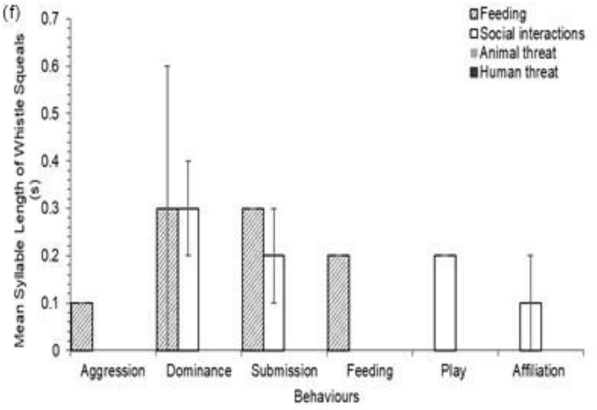

(a)
Mean syllable length of Social Twitters (s)
0.6
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
***
a
a
b
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

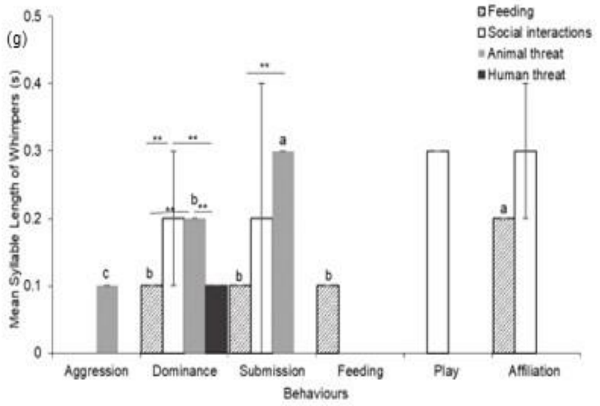

(b)
Mean Syllable Length of Spar Twitters (s)
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

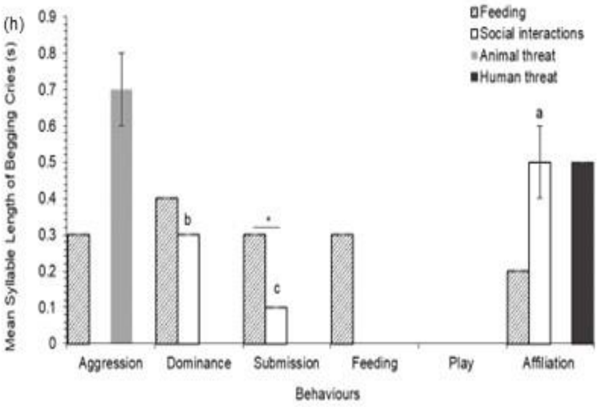

(c)
Mean Syllable Length of Mob Twitters (s)
0.6
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
***
b
***
a
c
c
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

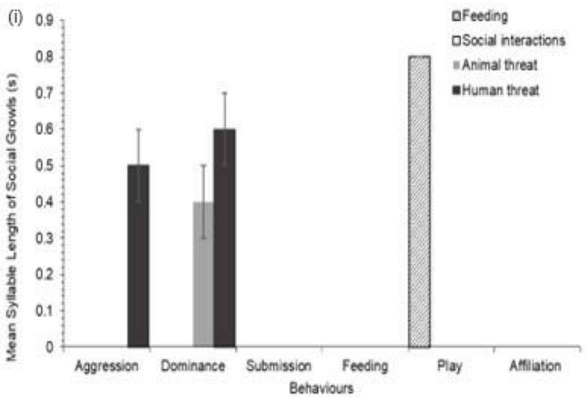

(d)
Mean Syllable Length of Full Moans (s)
1.2
1
0.8
0.6
0.4
0.2
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
a
b
b
b
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

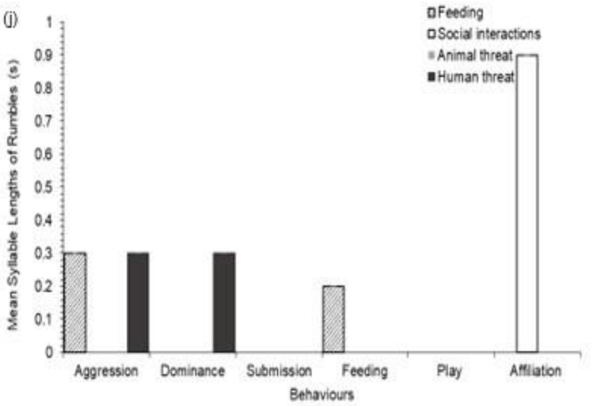

(e)
Mean Syllable Length of Begging Squeals (s)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
a
b
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

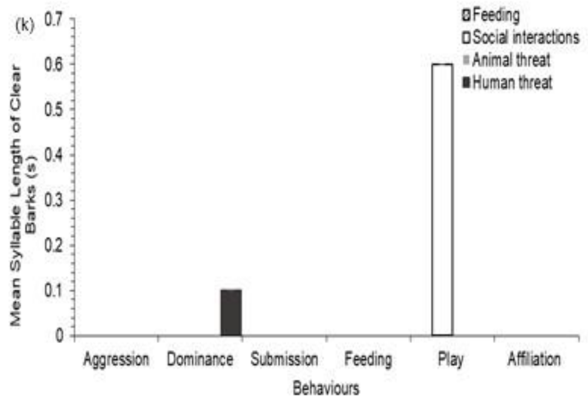

(f)
Mean Syllable Length of Whistle Squeals (s)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

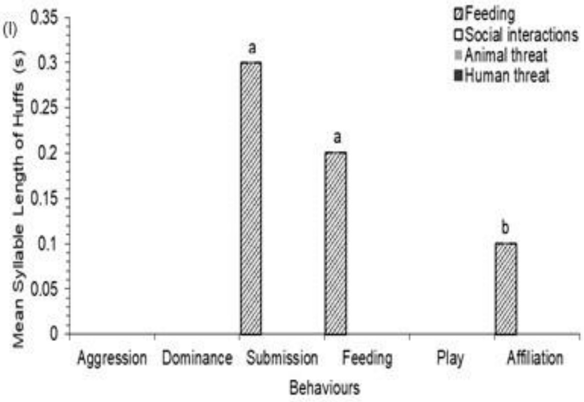

(g)
Mean Syllable Length of Whimpers (s)
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
**
**
**
**
**
c
b
b
b
b
a
a
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

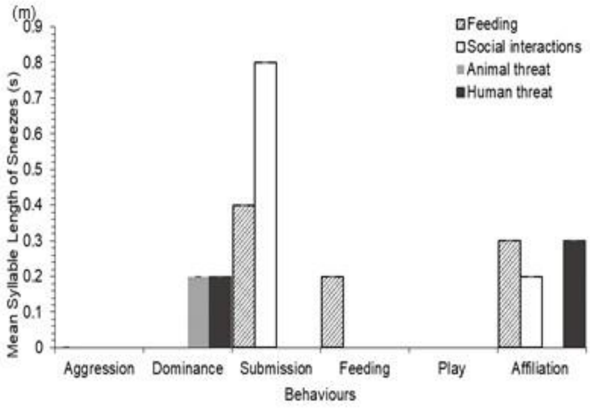

(h)
Mean Syllable Length of Begging Cries (s)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
▨Feeding
☐Social interactions
Animal threat
■Human threat
b
*
c
a
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

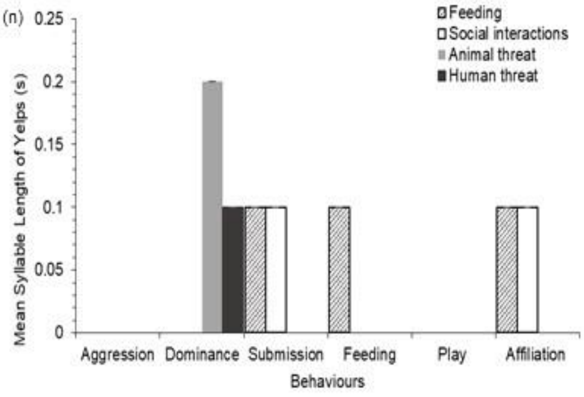

(i)
Mean Syllable Length of Social Growls (s)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat

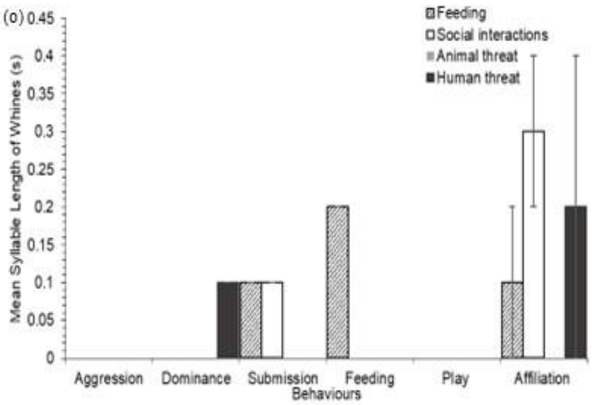

(j)
Mean Syllable Lengths of Rumbles (s)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat

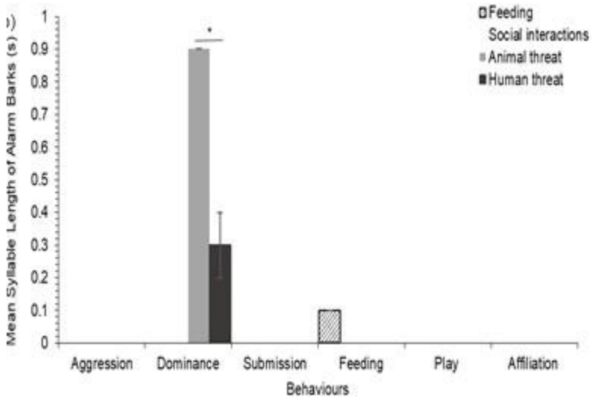

(k)
Mean Syllable Length of Clear Barks (s)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(l)
Mean Syllable Length of Huffs (s)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
a
a
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(m)
Mean Syllable Length of Sneezes (s)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(n)
Mean Syllable Length of Yelps (s)
0.25
0.2
0.15
0.1
0.05
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(o)
Mean Syllable Length of Whines (s)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


)
Mean Syllable Length of Alarm Barks (s)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat

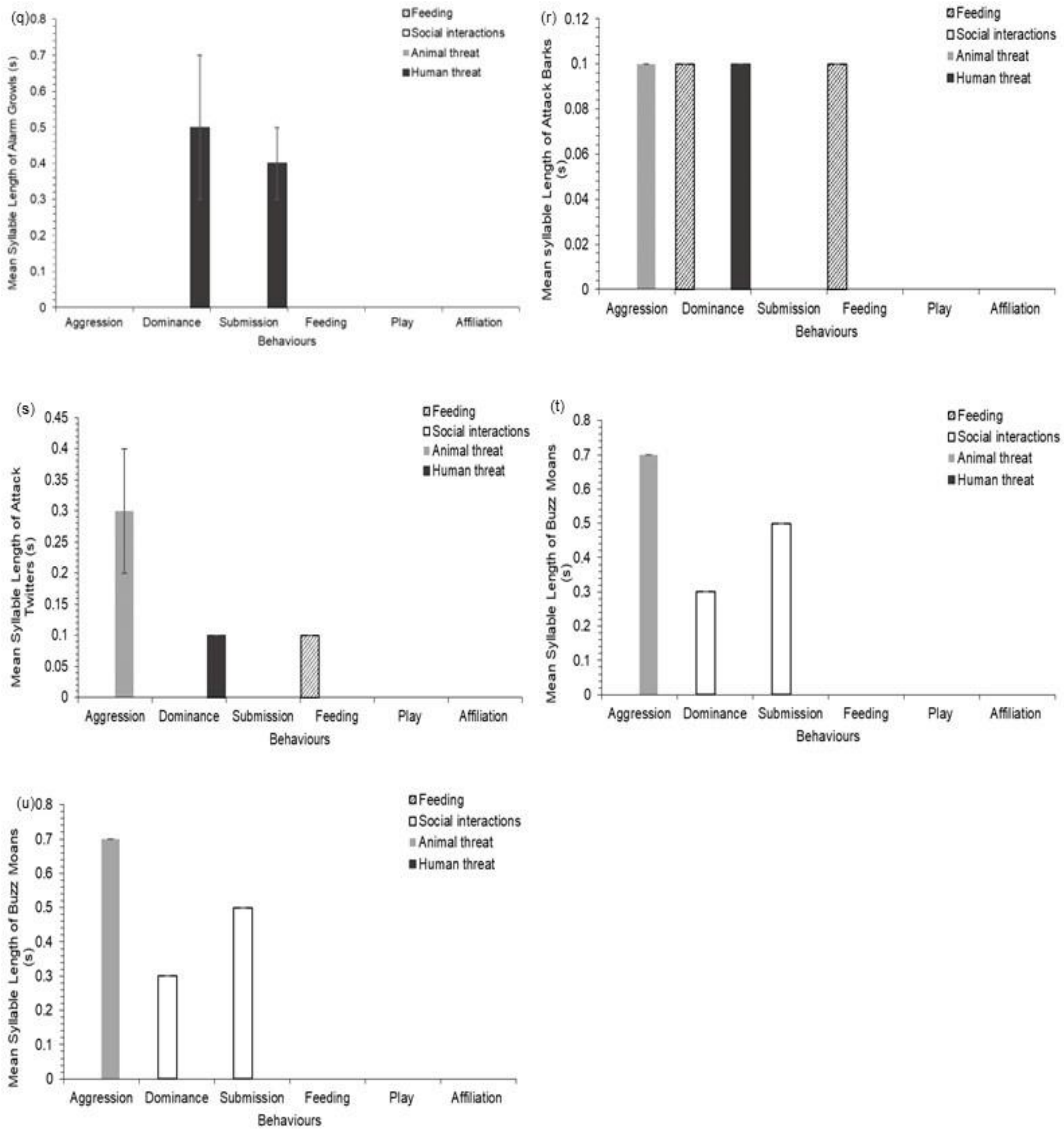
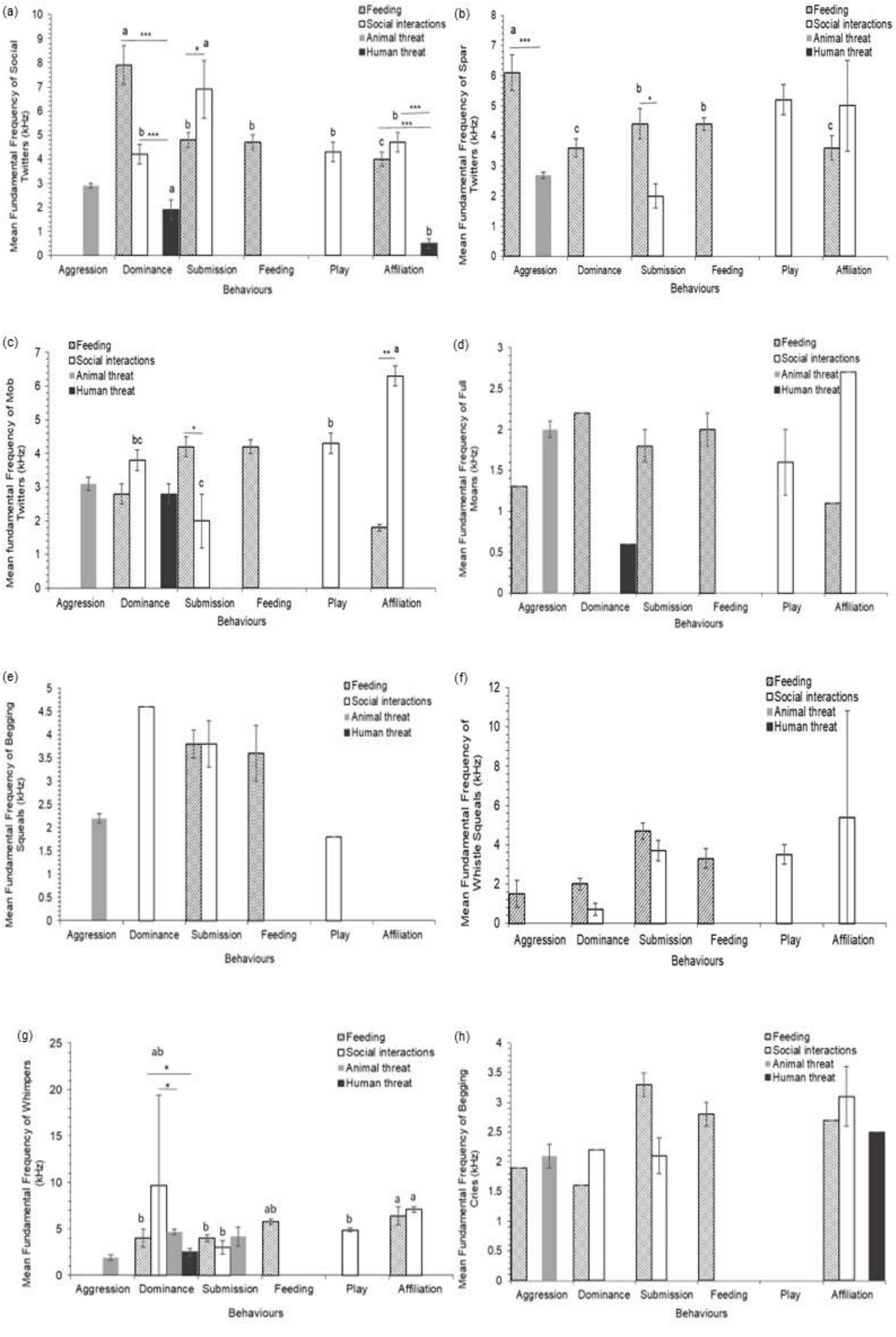
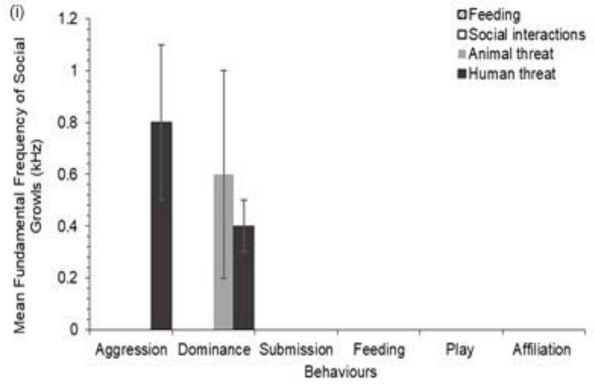
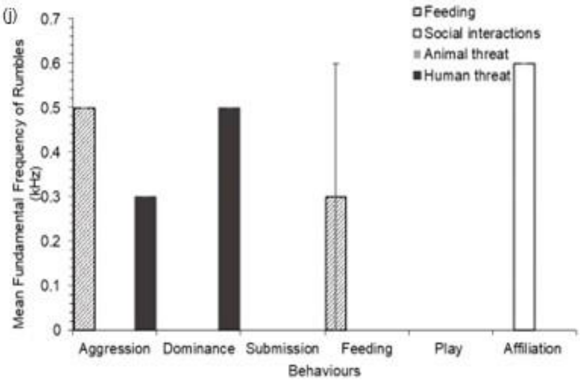
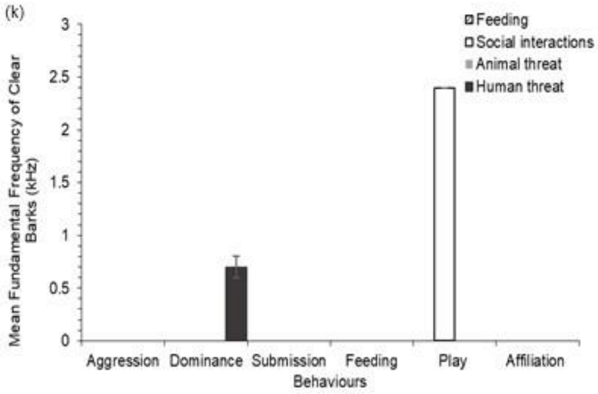


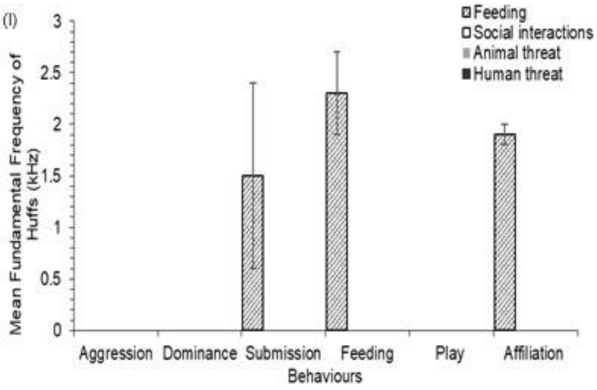
Figure 4.3. Bar graphs of the mean syllable length of vocalisations associated with different categories of behaviour (aggression, dominance, submission, feeding, play and affiliation) in African wild dogs (*Lycaon pictus*) within the context of feeding, social interaction, animal threat and human threat. ^{a,b,c} denotes a significant difference between behaviour categories within a context for a particular vocalisation type. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ denote significance between behaviour contexts.

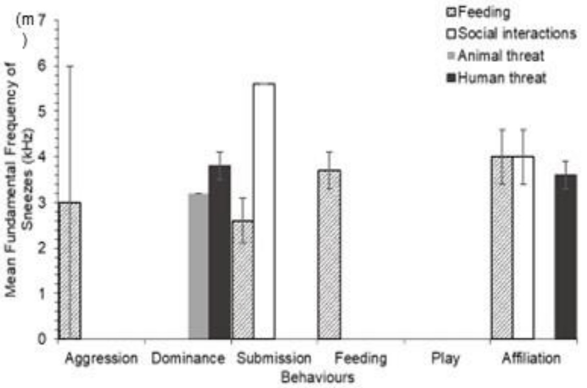

(a)
Mean Fundamental Frequency of Social Twitters (kHz)
(b)
Mean Fundamental Frequency of Spar Twitters (kHz)
(c)
Mean fundamental Frequency of Mob Twitters (kHz)
(d)
Mean Fundamental Frequency of Full Moans (kHz)
(e)
Mean Fundamental Frequency of Begging Squeals (kHz)
(f)
Mean Fundamental Frequency of Whistle Squeals (kHz)
(g)
Mean Fundamental Frequency of Whimpers (kHz)
(h)
Mean Fundamental Frequency of Begging Cries (kHz)
Feeding
Social interactions
Animal threat
Human threat
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
a
b
c
ab
bc
*
**
***

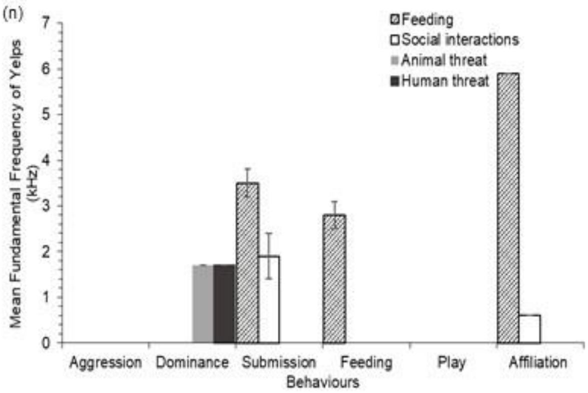

(i)
Mean Fundamental Frequency of Social Growls (kHz)
1.2
1
0.8
0.6
0.4
0.2
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat

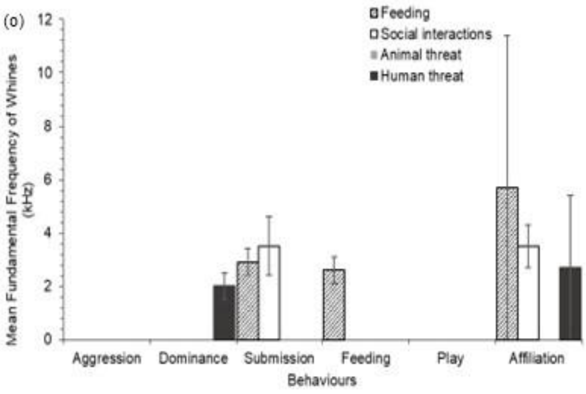

(j)
Mean Fundamental Frequency of Rumbles (kHz)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat

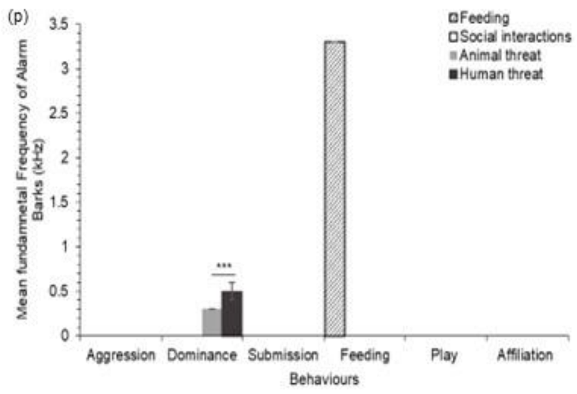

(k)
Mean Fundamental Frequency of Clear Barks (kHz)
3
2.5
2
1.5
1
0.5
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(l)
Mean Fundamental Frequency of Huffs (kHz)
3
2.5
2
1.5
1
0.5
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(m)
Mean Fundamental Frequency of Sneezes (kHz)
7
6
5
4
3
2
1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(n)
Mean Fundamental Frequency of Yelps (kHz)
7
6
5
4
3
2
1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(o)
Mean Fundamental Frequency of Whines (kHz)
12
10
8
6
4
2
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat


(p)
Mean fundamental Frequency of Alarm Barks (kHz)
3.5
3
2.5
2
1.5
1
0.5
0
***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Feeding
Social interactions
Animal threat
Human threat

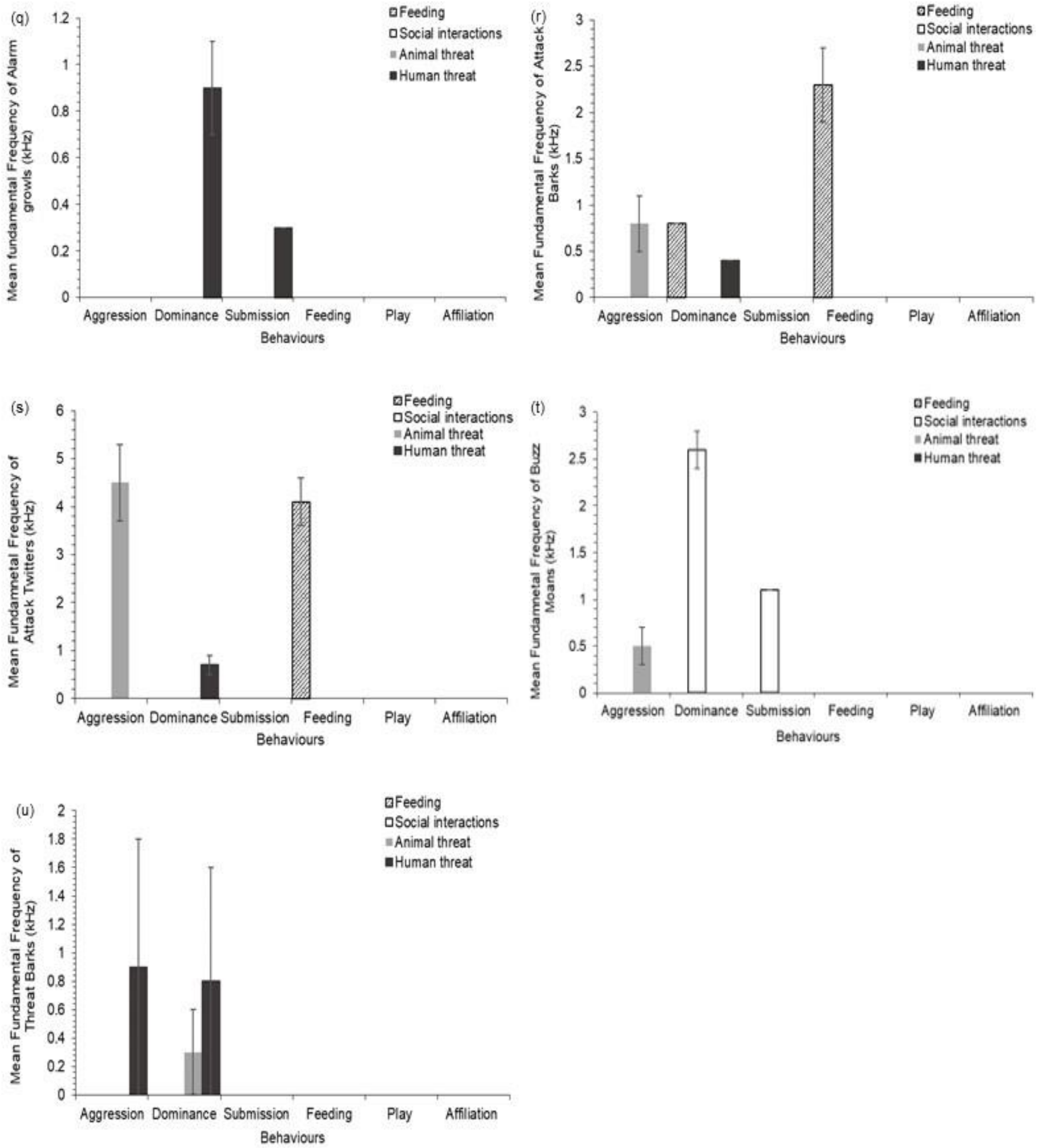


Figure 4.4. Bar graphs of the mean fundamental frequency of vocalisations associated with different categories of behaviour (aggression, dominance, submission, feeding, play and affiliation) in African wild dogs (*Lycaon pictus*) within the context of feeding, social interaction, animal threat and human threat. ^{a,b,c} denotes a significant difference between behaviour categories within a context for a particular vocalisation type. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ denote significance between behaviour contexts.

3.3. Effect of enclosure type on association between vocalisations and behaviours.

The results of the GLMM investigating the associations between vocalisations and behaviour categories within and between enclosure types are illustrated in Figure 4.5 – 4.7, with further detail, including specific P-values, found in supplementary Table 4.3 (Appendix 3).

Within traditional zoo packs, 19 vocalisations were recorded, with attack twitters and buzz moans not occurring during any of the observed behaviours (Fig. 4.5; Appendix 3). Full moans were only associated with aggression; yelps, attack barks and alarm barks only associated with dominance; whistle squeals and clear barks only associated with play; and huffs only associated with feeding behaviours (Fig. 4.5d, n, r, p, f, k & l; Appendix 3). Social growls, rumbles and threat barks were associated with dominance and aggression, with social growls occurring significantly more often during dominance than aggression (Fig. 4.5i, j & u; Appendix 3). Alarm growls were associated with dominance and submission; begging squeals and social twitters with aggression and play; and whines with submission and affiliation (Fig. 4.5q, h, a & o; Appendix 3). Moreover, the syllable length of threat barks and social twitters were both significantly aggression, while mob twitters during aggression and play, and finally whimpers were longer during submission, feeding and play (Fig. 4.6u, a, c & g; Appendix 3). Lastly, the F_0 of social twitters, begging cries and spar twitters were significantly lower during aggression, while mob twitters were lower during aggression and dominance, and whimpers were lower during dominance, submission and play (Fig. 4.7a, h, b, c & g; Appendix 3).

Within open-range zoo packs, all 21 vocalisations were recorded. Buzz moans were only associated with aggression, and alarm growls and threat barks only with dominance (Fig. 4.5t & q; Appendix 3). Social growls occurred significantly more often during aggression than play; alarm barks and clear barks were associated with both dominance and submission; huffs with submission and feeding; and rumbles with feeding and affiliation (Fig. 4.5i, p, k, l & j;

Appendix 3). The daily rate of spar twitters was significantly higher during feeding than submission then aggression (Fig. 4.5b; Appendix 3). Moreover, the syllable length of begging squeals and attack twitters were significantly longer during aggression, while whimpers and whines were longer during affiliation (Fig. 4.6e, s & g; Appendix 3). The F_0 of yelps and attack twitters were significantly lower during dominance, with attack barks lower during dominance and aggression, and whistle squeals and social twitters lower during submission and affiliation respectively (Fig. 4.7n, s, f & a; Appendix 3).

Within free-roaming packs, 16 vocalisations were recorded, with attack barks, threat barks, alarm barks and clear barks not occurring during any of the observed behaviours (Fig. 4.5r, u, p & k; Appendix 3). Attack twitters were only associated with aggression, social growls with dominance, alarm growls with submission, rumbles with feeding, and yelps with affiliative behaviours (Fig. 4.5s, q, j & n; Appendix 3). Buzz moans, whines and huffs were associated with two behaviour categories (Fig. 4.5 - 4.7t, o & l; Appendix 3). Buzz moans were associated with dominance and submission; and whines and huffs with feeding and affiliation (Fig. 4.5 - 4.7t, o & l; Appendix 3). Both huffs and whimpers were the only vocalisations that occurred significantly more often during feeding than any other behaviours (Fig. 4.5l & g; Appendix 3). Moreover, the syllable length of spar twitters was significantly longer during dominance, feeding and affiliation, while whimpers were longer during play and affiliation (Fig. 4.6b & g; Appendix 3). Lastly, the F_0 of begging squeals were significantly lower during aggression, whimpers were lower during dominance and submission, spar twitters were lower during dominance, submission and affiliation, and social twitters were lower during affiliation (Fig. 4.7e, g, b & a; Appendix 3).

When compared between enclosure types, during aggressive behaviours, traditional zoo packs had the largest and free-roaming packs the lowest range of vocalisations. The mean daily rate of begging cries was significantly higher in traditional zoos, while spar twitters were higher in traditional zoos and free-roaming packs (Fig. 4.5h & b; Appendix 3). Moreover, the syllable length of both vocalisations was significantly longer in traditional zoos (Fig. 4.6h & b; Appendix 3). Lastly, the F_0 of spar twitters were significantly lower in traditional and open-range packs, while begging squeals were lower in open-range and free-roaming packs (Fig. 4.7b & e; Appendix 3).

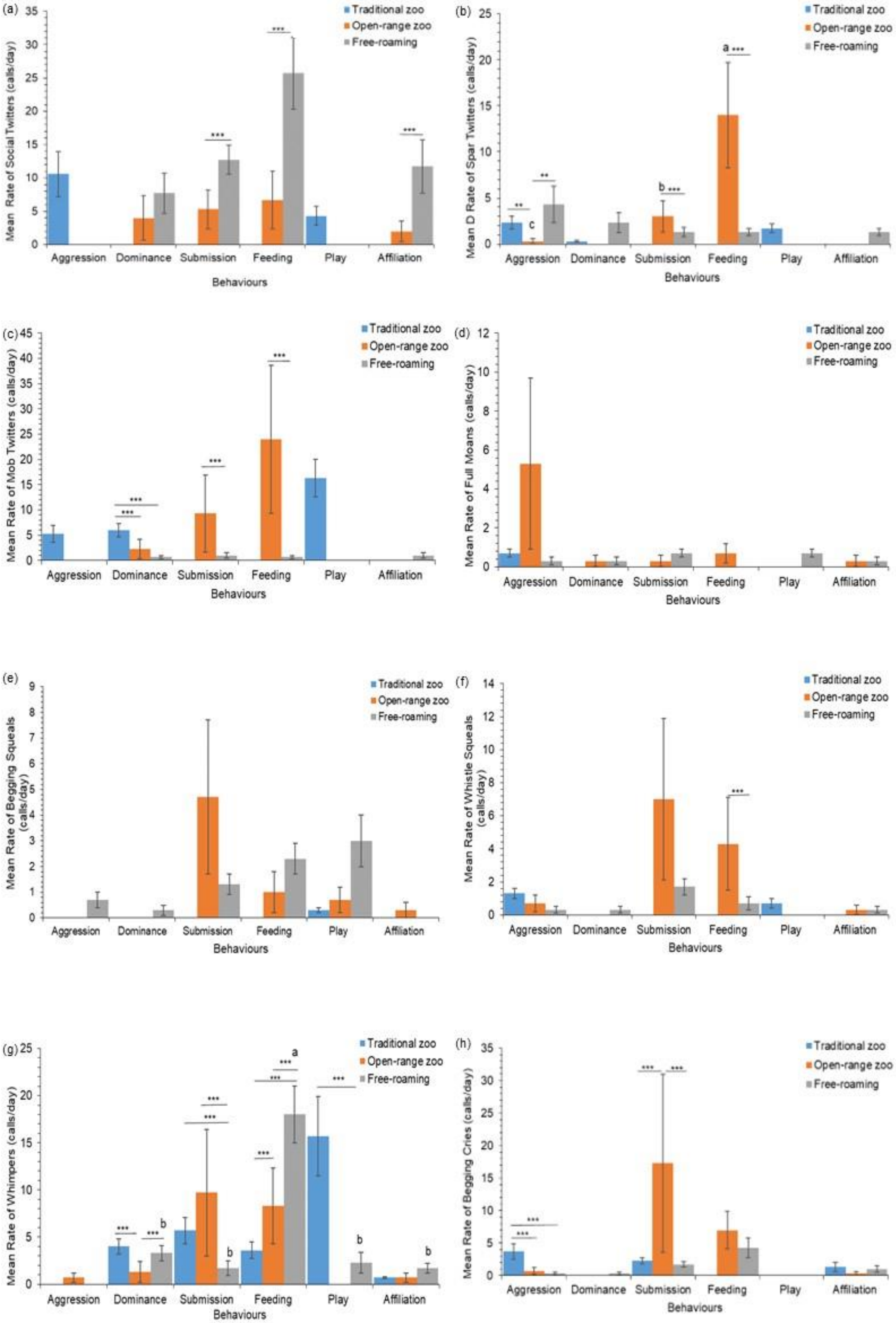
During dominant behaviours, open-range zoos had the largest and free-roaming packs the lowest range of vocalisations. The mean daily rate of mob twitters and social growls were significantly higher in traditional zoos, while whimpers were higher in both traditional zoos and free-roaming packs (Fig. 4.5c, i & g; Appendix 3). Moreover, the syllable length of whimpers and social growls, and social twitters were significantly longer in traditional zoos and free-roaming packs respectively (Fig. 4.6g & i; Appendix 3). Lastly, the F_0 of social twitters and whimpers were significantly lower in open-range zoos, while alarm growls were lower in traditional zoos and free-roaming packs (Fig. 4.6a, g & q; Appendix 2).

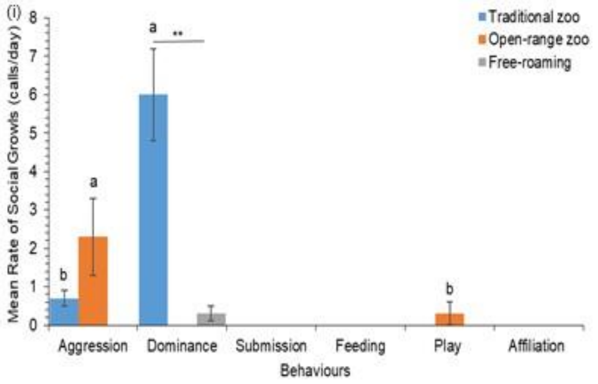
During submissive behaviours, open-range zoo and free-roaming packs had the largest and traditional zoos the lowest range of vocalisations. The mean daily rate of begging cries, spar twitters and mob twitters were significantly higher in open-range zoos, while whimpers were higher in both open-range and traditional zoos, and social twitters were higher in free-roaming packs (Fig. 4.5h, b, c, g & a; Appendix 3). Moreover, the syllable length of whimpers, social twitters and begging squeals, and spar twitters were significantly longer in traditional zoos, free-roaming packs, or open-range zoos respectively (Fig. 4.6g, a, e & b; Appendix 3). Lastly, the F_0 of whistle squeals and alarm growls were significantly lower in open-range zoos and free-roaming packs respectively (Fig. 4.7f & q; Appendix 3).

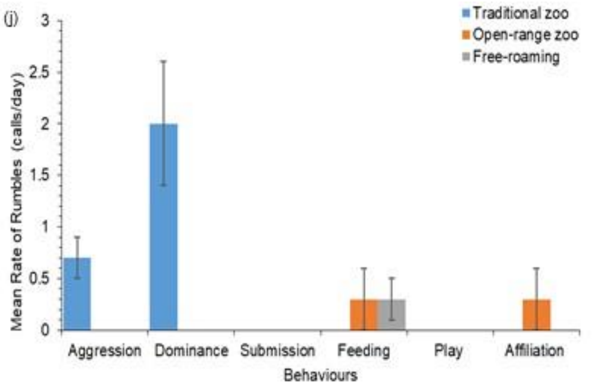
During feeding behaviours, open-range zoo and free-roaming packs had the largest and traditional zoos the lowest range of vocalisations. The mean daily rate of mob twitters and spar twitters were significantly higher in open-range zoos, while social twitters, whimpers and whines were higher in free-roaming packs (Fig. 4.5c, b, a, g & o; Appendix 3). Moreover, the syllable length of whimpers was significantly longer in traditional zoos, while social twitters and begging squeals were significantly longer in free-roaming packs (Fig. 4.6g, a & e; Appendix 3). Lastly, the F_0 of whimpers, begging squeals, spar twitters, and huffs were significantly lower in open-range zoos (Fig. 4.7g, e, c & l; Appendix 3).

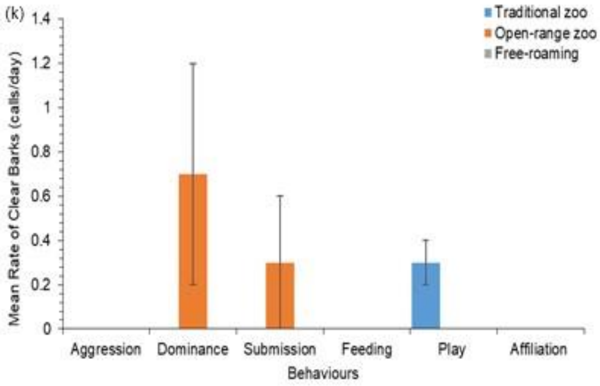
During play behaviours, traditional zoos had the largest, while open-range zoos and free-roaming packs the lowest range of vocalisations. The mean daily rate of whimpers and whistle squeals were significantly higher in traditional zoos and free-roaming packs respectively (Fig. 4.5g & f; Appendix 3). Moreover, the syllable length and F_0 did not differ significantly for any vocalisation between the different enclosure types (Fig. 4.5a-u; Appendix 3).

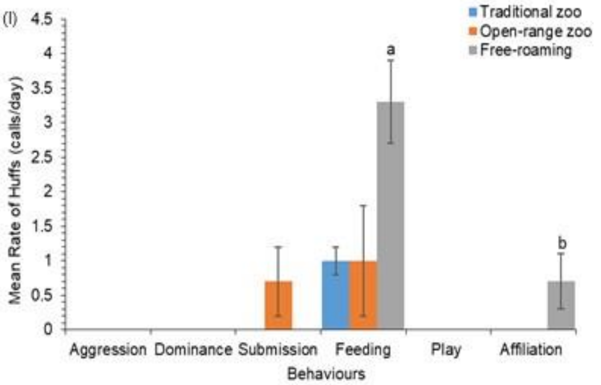
During affiliative behaviours, free-roaming packs tended to have slightly higher range of vocalisations than captive zoo packs. The mean daily rate of full moans and social twitters were significantly higher in open-range zoos and free-roaming packs respectively (Fig. 4.5d & a; Appendix 3). Moreover, the syllable length of begging cries was significantly longer in traditional zoos, whimpers were longer in open-range zoos, and sneezes were longer in both traditional and open-range zoos (Fig. 4.6h, g & m; Appendix 3). Lastly, only the F_0 of social twitters was significantly lower in open-range zoos (Fig. 4.7a; Appendix 3).

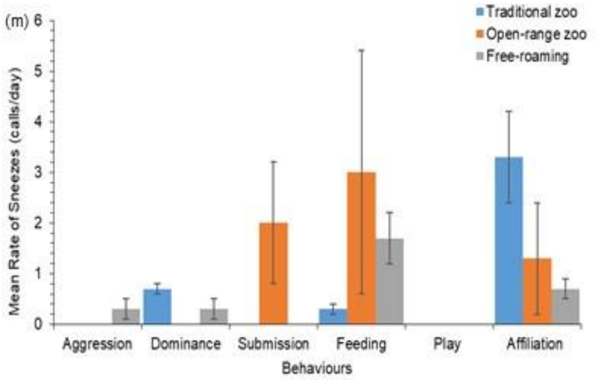

(a)
Mean Rate of Social Twitters (calls/day)
0
5
10
15
20
25
30
35
Traditional zoo
Open-range zoo
Free-roaming
***
***
***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(b)
Mean D Rate of Spar Twitters (calls/day)
0
5
10
15
20
25
**
**
c
b ***
a ***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(c)
Mean Rate of Mob Twitters (calls/day)
0
5
10
15
20
25
30
35
40
45
***
***
***
***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(d)
Mean Rate of Full Moans (calls/day)
0
2
4
6
8
10
12
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(e)
Mean Rate of Begging Squeals (calls/day)
0
1
2
3
4
5
6
7
8
9
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(f)
Mean Rate of Whistle Squeals (calls/day)
0
2
4
6
8
10
12
14
***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(g)
Mean Rate of Whimpers (calls/day)
0
5
10
15
20
25
***
***
b
***
***
b
***
***
***
a
***
b
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(h)
Mean Rate of Begging Cries (calls/day)
0
5
10
15
20
25
30
35
***
***
***
***
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours

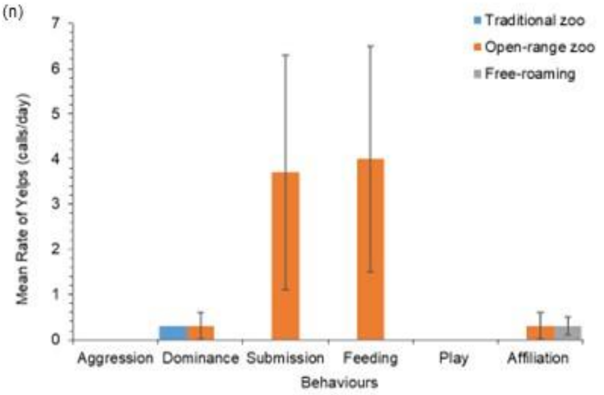

(i)
Mean Rate of Social Growls (calls/day)
0
1
2
3
4
5
6
7
8
a
**
a
b
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming

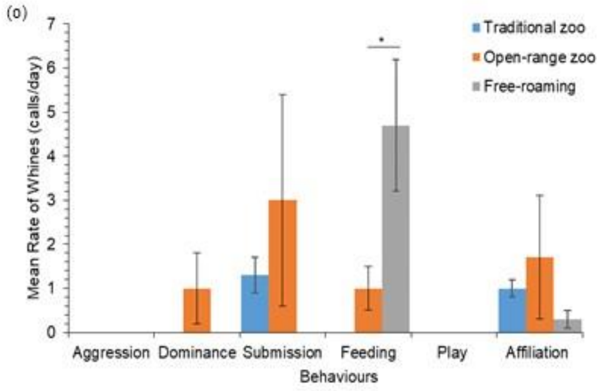

(j)
Mean Rate of Rumbles (calls/day)
0
0.5
1
1.5
2
2.5
3
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming

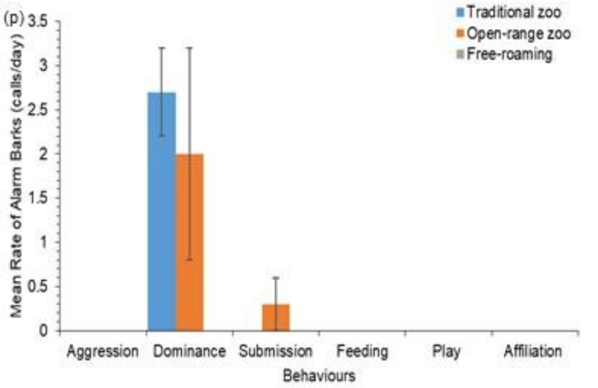

(k)
Mean Rate of Clear Barks (calls/day)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming


(l)
Mean Rate of Huffs (calls/day)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
a
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming


(m)
Mean Rate of Sneezes (calls/day)
0
1
2
3
4
5
6
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming


(n)
Mean Rate of Yelps (calls/day)
0
1
2
3
4
5
6
7
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming


(o)
Mean Rate of Whines (calls/day)
0
1
2
3
4
5
6
7
*
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming


(p)
Mean Rate of Alarm Barks (calls/day)
0
0.5
1
1.5
2
2.5
3
3.5
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming

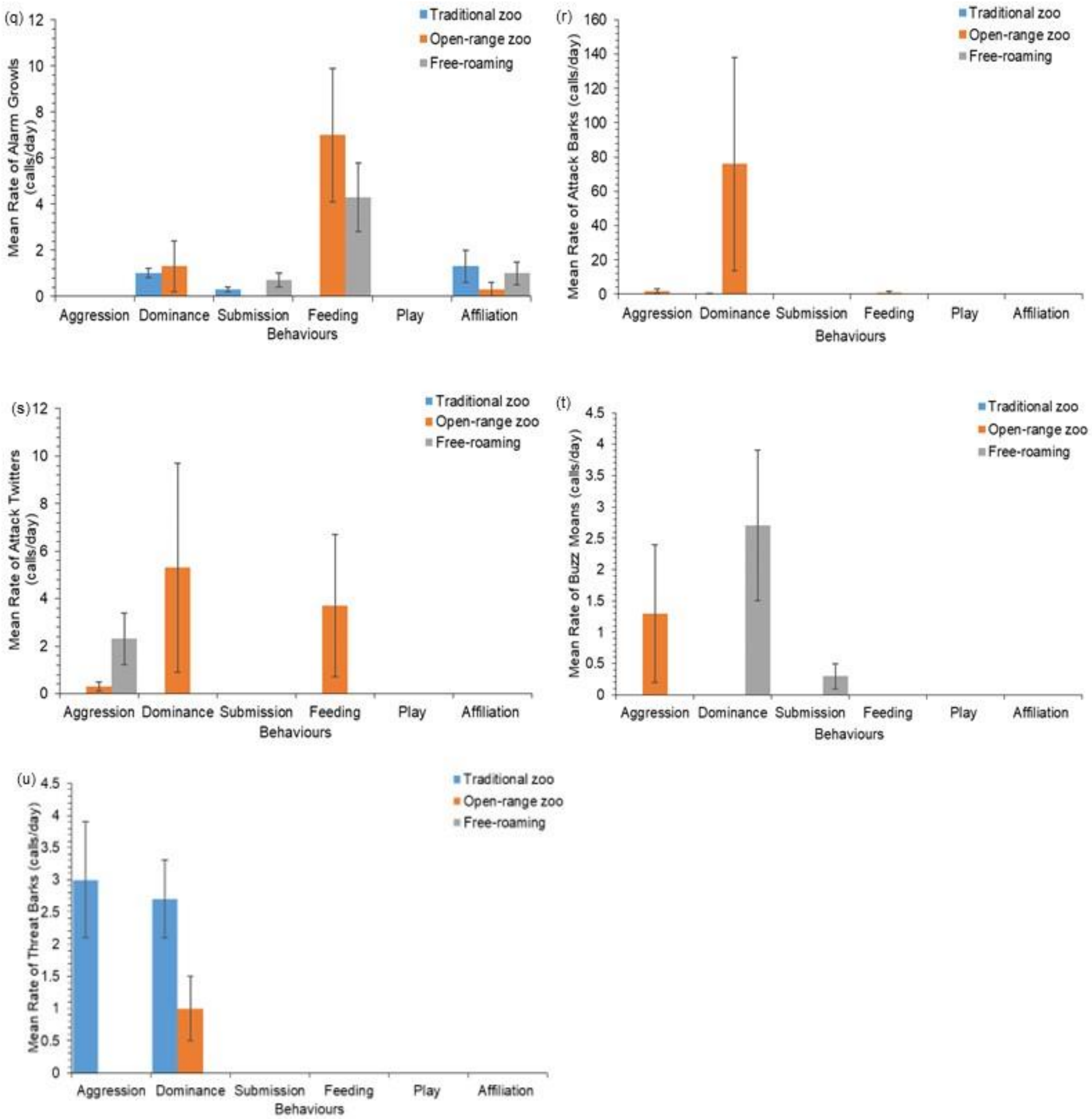
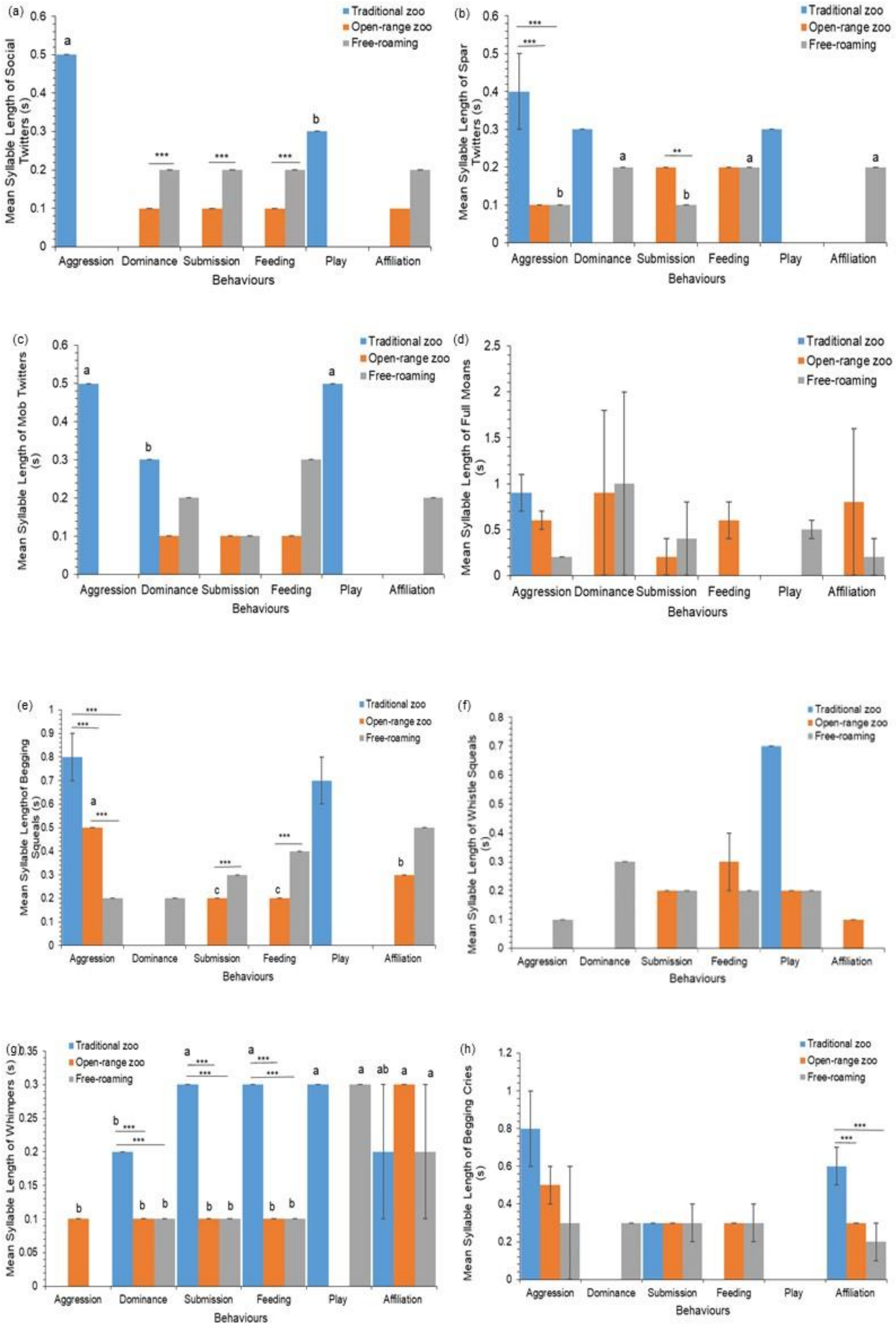
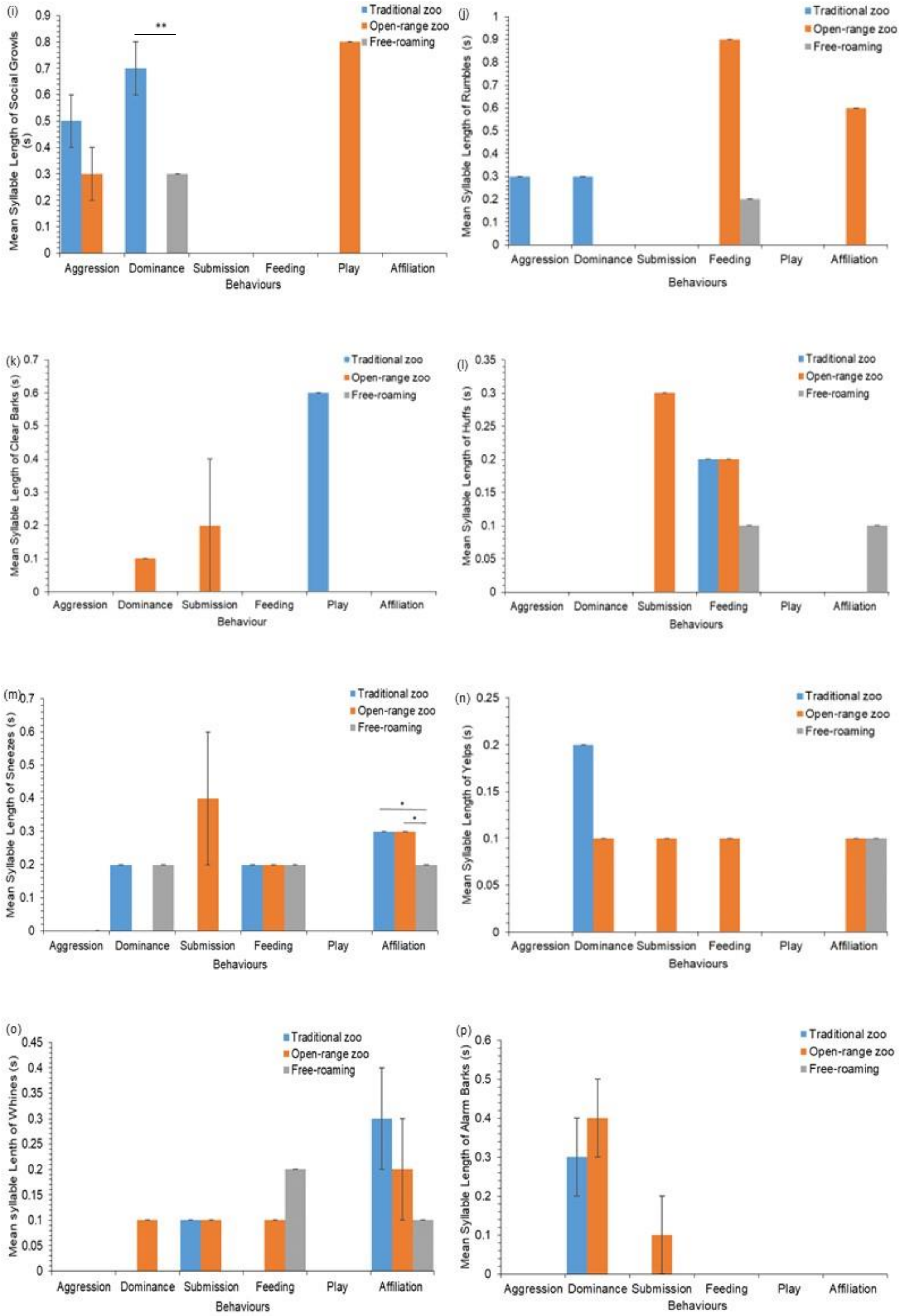


Figure 4.5. Bar graphs of the mean daily rate of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*) of different captive status in traditional zoo, open-range zoo, and free-roaming packs. ^{a,b,c} denotes a significant difference between behaviour categories within an enclosure type for a particular vocalisation type. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ denote significance between behaviour contexts.


(a)
Mean Syllable Length of Social Twitters (s)
Behaviours
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Traditional zoo
Open-range zoo
Free-roaming
(b)
Mean Syllable Length of Spar Twitters (s)
(c)
Mean Syllable Length of Mob Twitters (s)
(d)
Mean Syllable Length of Full Moans (s)
(e)
Mean Syllable Lengthof Begging Squeals (s)
(f)
Mean Syllable Length of Whistle Squeals (s)
(g)
Mean Syllable Length of Whimpers (s)
(h)
Mean Syllable Length of Begging Cries (s)
a
b
ab
c
***
**


(i)
Mean Syllable Length of Social Growls (s)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(j)
Mean Syllable Length of Rumbles (s)
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(k)
Mean Syllable Length of Clear Barks (s)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviour
Traditional zoo
Open-range zoo
Free-roaming
(l)
Mean Syllable Length of Huffs (s)
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(m)
Mean Syllable Length of Sneezes (s)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(n)
Mean Syllable Length of Yelps (s)
0.25
0.2
0.15
0.1
0.05
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(o)
Mean syllable Lenth of Whines (s)
0.45
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(p)
Mean Syllable Length of Alarm Barks (s)
0.6
0.5
0.4
0.3
0.2
0.1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming

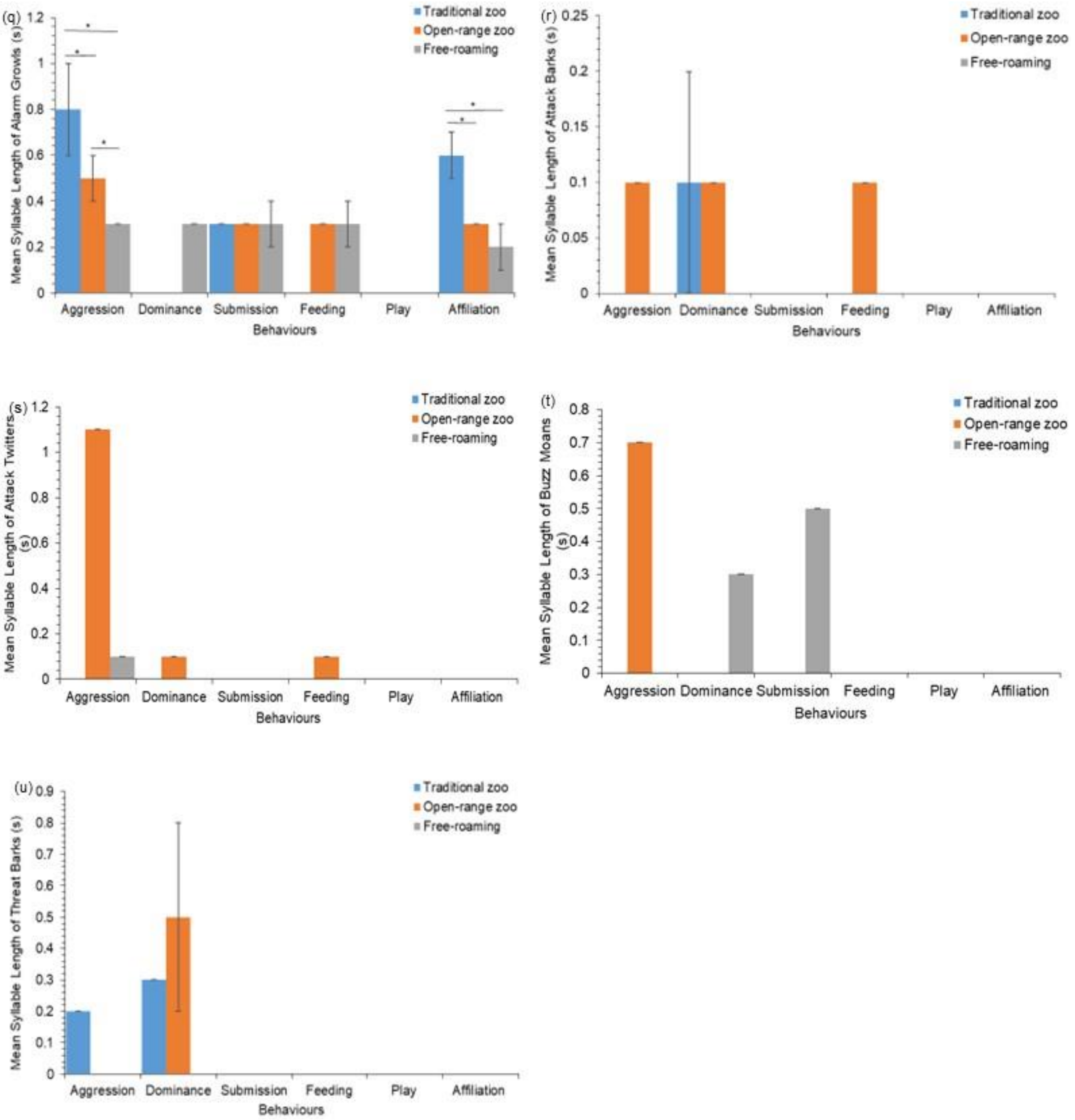
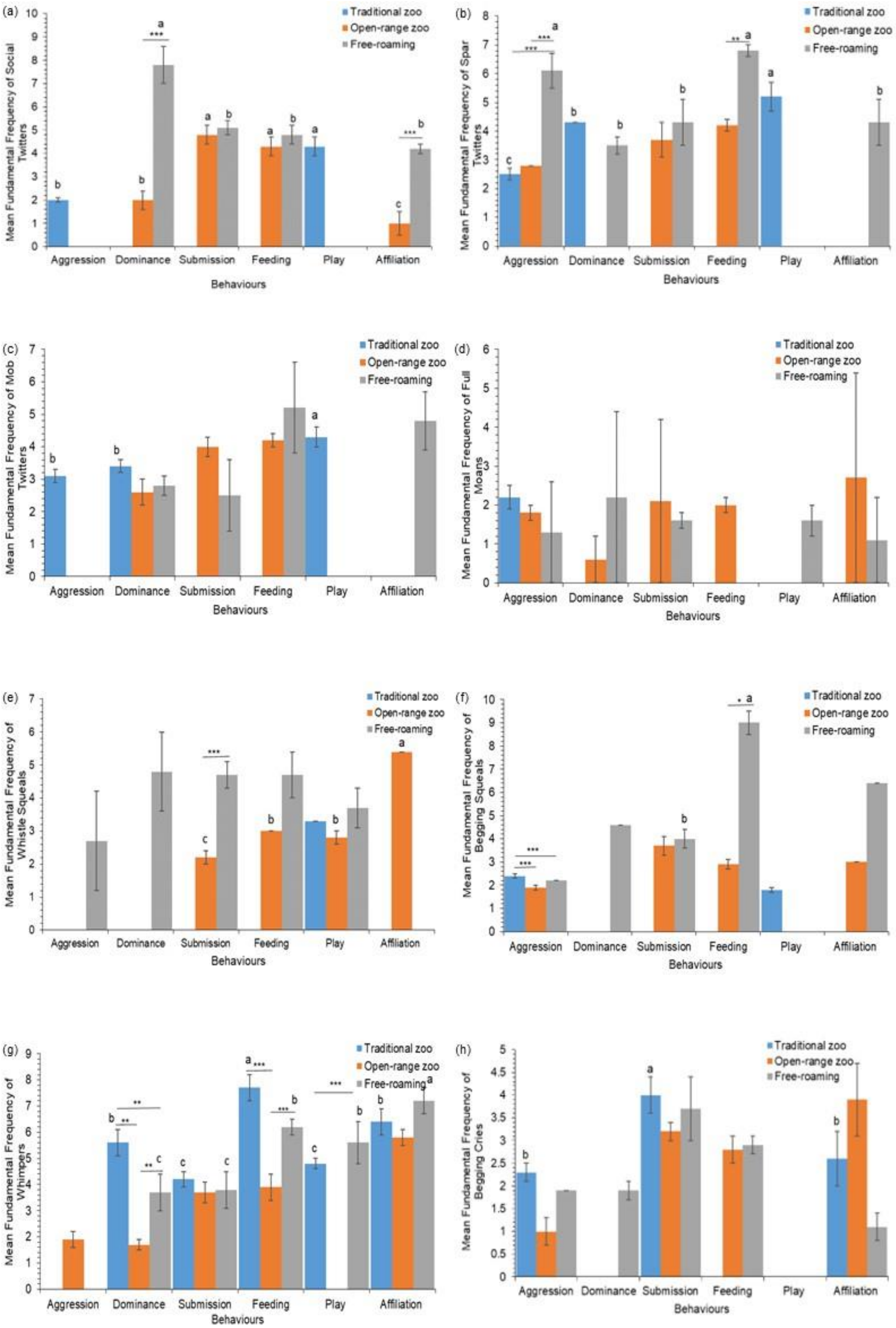
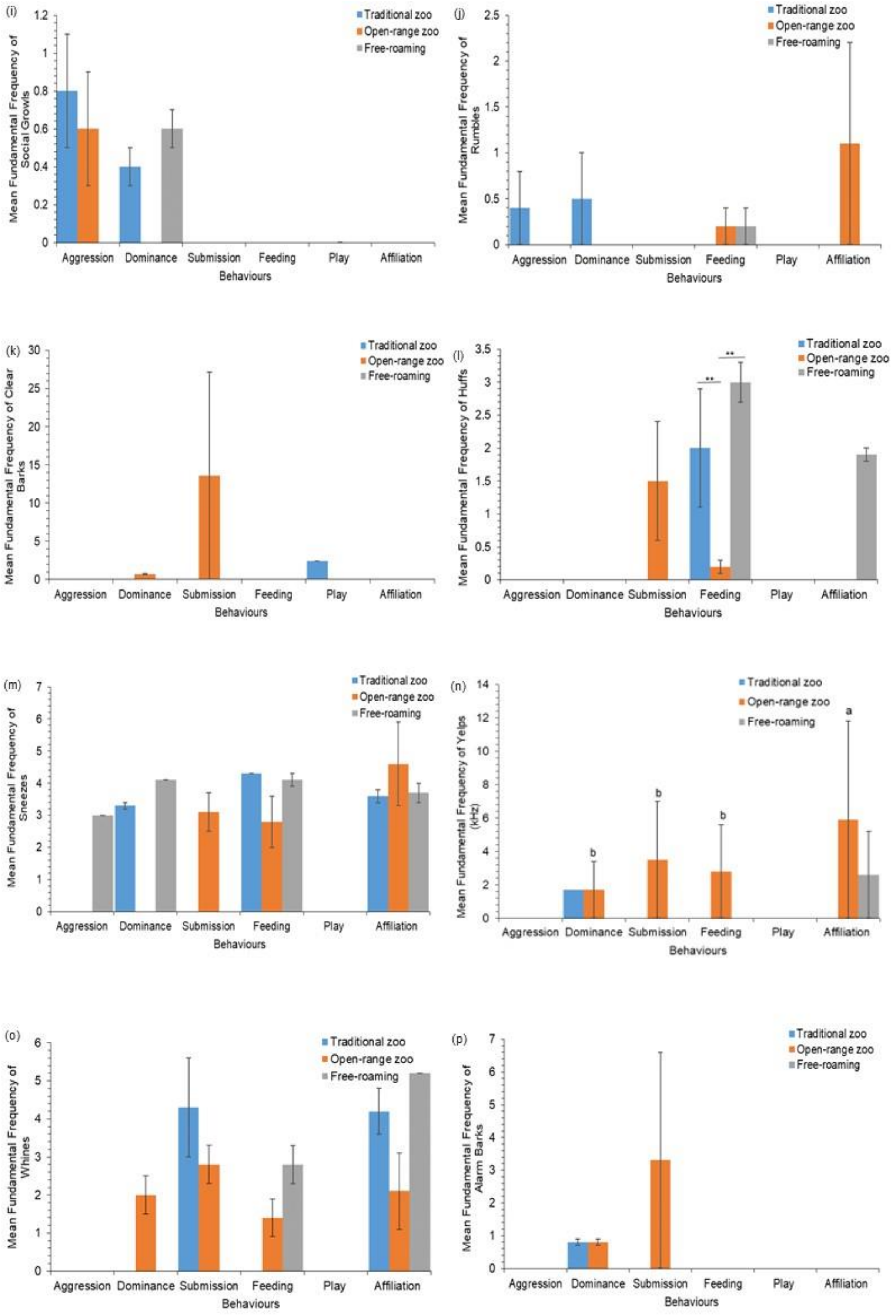


Figure 4.6. Bar graphs of the mean syllable length of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*) of different captive status in traditional zoo, open-range zoo, and free-roaming packs. ^{a,b,c} denotes a significant difference between behaviour categories within an enclosure type for a particular vocalisation type. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ denote significance between behaviour contexts.



(a)
Mean Fundamental Frequency of Social Twitters
Traditional zoo
Open-range zoo
Free-roaming
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
(b)
Mean Fundamental Frequency of Spar Twitters
(c)
Mean Fundamental Frequency of Mob Twitters
(d)
Mean Fundamental Frequency of Full Moans
(e)
Mean Fundamental Frequency of Whistle Squeals
(f)
Mean Fundamental Frequency of Begging Squeals
(g)
Mean Fundamental Frequency of Whimpers
(h)
Mean Fundamental Frequency of Begging Cries


(i)
Mean Fundamental Frequency of Social Growls
1.2
1
0.8
0.6
0.4
0.2
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(j)
Mean Fundamental Frequency of Rumbles
2.5
2
1.5
1
0.5
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(k)
Mean Fundamental Frequency of Clear Barks
30
25
20
15
10
5
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(l)
Mean Fundamental Frequency of Huffs
3.5
3
2.5
2
1.5
1
0.5
0
**
**
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(m)
Mean Fundamental Frequency of Sneezes
7
6
5
4
3
2
1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(n)
Mean Fundamental Frequency of Yelps (kHz)
14
12
10
8
6
4
2
0
a
b
b
b
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(o)
Mean Fundamental Frequency of Whines
6
5
4
3
2
1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming
(p)
Mean Fundamental Frequency of Alarm Barks
7
6
5
4
3
2
1
0
Aggression
Dominance
Submission
Feeding
Play
Affiliation
Behaviours
Traditional zoo
Open-range zoo
Free-roaming

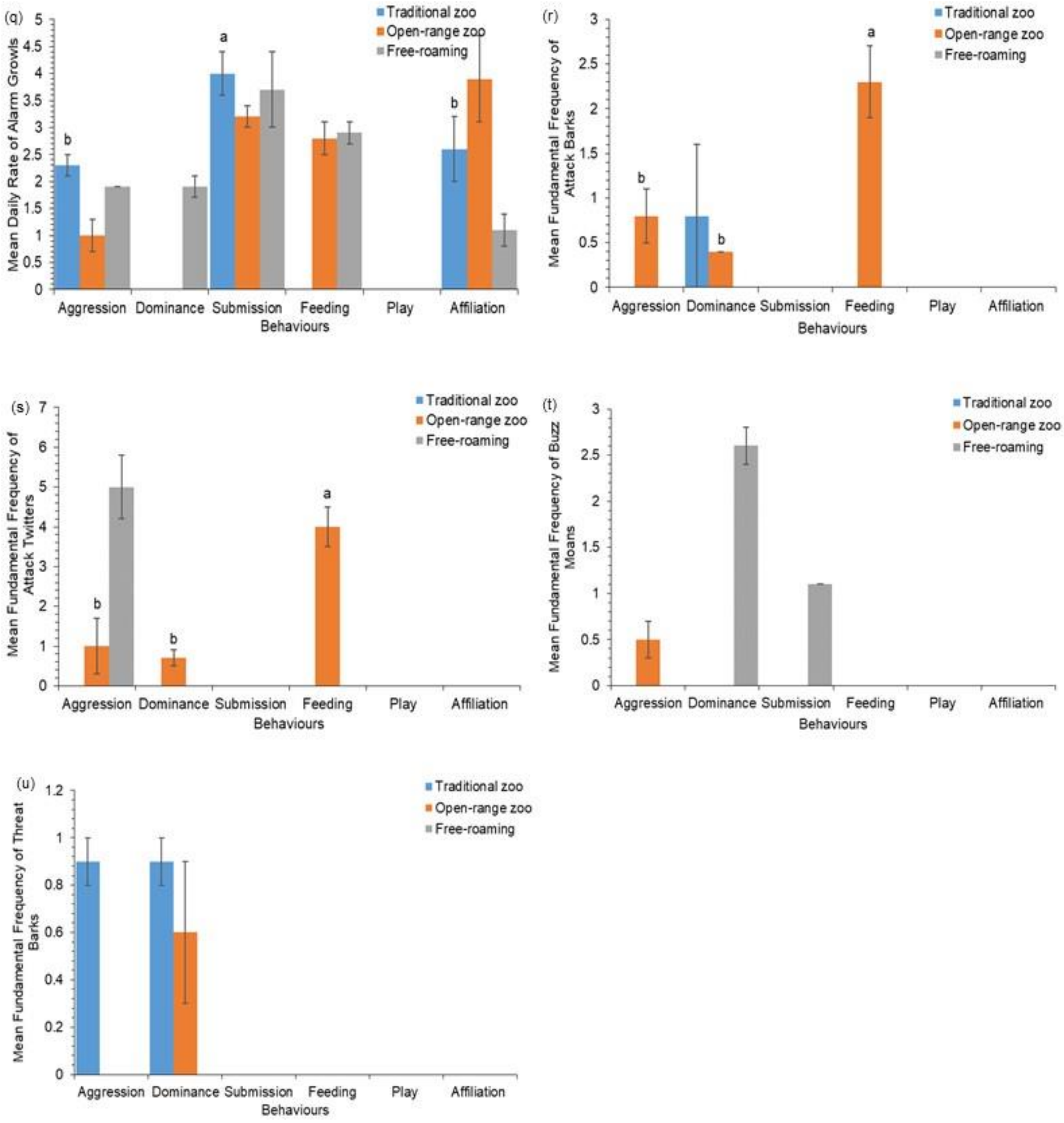


Figure 4.7. Bar graphs of the mean fundamental frequency of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*) of different captive status in traditional zoo, open-range zoo, and free-roaming packs. ^{a,b,c} denotes a significant difference between behaviour categories within an enclosure type for a particular vocalisation type. * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ denote significance between behaviour contexts.

4. Discussion

Our results showed that vocalisation range, rates and acoustic parameters are altered with different behaviours, contexts and housing, making them a potentially sensitive tool with which to measure social cohesion, and warrants further investigation. Comparison of vocalisation classes identified that most African wild dog vocalisations are used during a range of pro- and anti-social behaviours. Moreover, anti-social dominant behaviours appear to be associated with an increase in the range of vocalisations, lengthening of syllables and lower fundamental frequencies during these behaviours, while the opposite was seen with pro-social behaviours. Specifically alarm barks, threat barks, attack barks, full moans, social growls, alarm growls, buzz moans and attack twitters appeared to predominantly occur during anti-social behaviours and may provide good indicators of negative emotional states, such as experiencing chronic stress leading to anti-social aggression. The contexts of animal and human threats elicited mostly anti-social behaviours and were associated with an increased range and rate of vocalisations, longer syllables and lower fundamental frequencies than during social interactions and feeding, indicating a more negative emotional state during these contexts. When compared between housing types, free-roaming packs used less vocalisations which had shorter syllables and higher fundamental frequencies than traditional zoo and open-range zoo packs, suggesting that zoo-based packs may experience increased negative emotional states, possibly due to increased stress and a reduced ability to display natural behaviours such as dispersal.

Interestingly, during social interactions, attack twitters, alarm growls, attack barks, threat barks and alarm barks were absent but were all present during animal and human threats. Additionally, during feeding only buzz moans, alarm growls and threat barks were absent further supporting the hypothesis that these vocalisations are indicative of negative emotional states and could be good indicators for changes in social cohesion.

African wild dog vocalisations are largely multifunctional (Robbins 2000; Robbins & McCreery, 2003) and this was largely supported by our results. However, some vocalisations have been found to be more singularly functional (Robbins 2000; Marneweck et al., 2019b). For example, decreasing rates of hoo-calls has been associated with increased social cohesion during the context of artificial pack formation (Marneweck et al., 2019b). In free-roaming packs hoo-calls are typically used to locate separated pack members and in dispersal groups to locate opposite sex dispersers (Robbins, 2000). Similarly, wolves use howls to communicate with separated pack members, and it has been shown previously that howling after separation of pack members is positively associated with increased cortisol indicating heightened stress in this species (Mazzini et al., 2013). Given, the similar use of hoo-calls and howls, hoo-calls may be an indicator of heightened stress in African wild dogs. As such the measurement of rates of hoo-calls, cortisol and negative social behaviours during stressful interventions such as separation or artificial pack formation could be used to elucidate stress.

In the current study, most vocalisations were associated with a range of behaviour categories, but alarm barks, threat barks, attack barks, full moans, social growls, alarm growls, buzz moans and attack twitters occurred with either only or predominantly anti-social behaviours. Similarly, Robbins (2000) noted all these vocalisations, except for buzz moans, were associated with aggressive or frustration behaviours and all occurred within negative contexts such as intra- and interspecies aggression, frustration or high arousal greeting and begging displays. Similarly, all these vocalisations occurred with either human or animal threats or with both and feeding or social interaction contexts within our own study. Stress can be transmitted between conspecifics, whereby exposure to a stressed individual can lead to a stress response in other pack members (Brandl et al., 2022). In African wild dogs, stress is directly related to an increase in anti-social behaviours such as dominance and submission (Van den Berghe et al., 2019) that could result in reduced social bonds. In turn, such maladaptive social interactions and coordination could reduce social cohesion at great cost to the pack (Brandl et al., 2022). For example, this could result in reduced hunting ability or successful raising of young in free-roaming packs and increased aggression and fighting in captive packs. As such, the rate of alarm barks, threat barks, attack barks, full moans, social growls, alarm growls, buzz moans and attack twitters may serve as indicators of increased anti-social behaviours and as such, declining social cohesion. Furthermore, alarm growls seemed to be specifically used to indicate threats from human encroachment, or from other African wild dogs during feeding. While threat barks seem to only be used to indicate external human or animal threats. Interestingly, the most noticeable vocal changes within the anti-social behaviour grouping appear to be associated with dominant behaviours, which are predominantly used to reinforce hierarchy.

The African wild dog's large vocal repertoire presents some challenges to studies of this kind, due to the rarity of some vocalisations, such as hoo-calls and high representation of others such as spar and social twitters which appear to be used in a range of different behaviours. As was expected, our results show that most African wild dog vocalisations are used during both pro- and anti-social behaviours. Similarly, Robbins (2000) and Robbins and McCreery (2003) suggested that many African wild dog vocalisations are used with a range of behaviours and in a wide range of contexts, but changes in each vocalisation-types acoustic parameters in different behaviours and/or contexts may be indicative of changes in emotional states.

Morton's motivational-structural (MS) model suggests that the frequency of sounds, and as such the pitch perceived by receiver, may change depending on the emotional state of the animal transmitting it (Marx et al., 2021). In the MS model, vocalisations associated with affiliative behaviours should have a higher frequency, while those associated with aggression should be lower. Other canids modulate the acoustic properties of their vocalisations during negative emotional states by lowering their fundamental frequency and lengthening their syllables (Farago et al., 2010; Szenczi et al., 2023). Similarly, we found that multiple vocalisation types emitted during aggressive behaviours were more likely to be longer in duration and lower in fundamental frequency, while the same vocalisation types emitted during affiliative behaviours were typically shorter with a higher fundamental frequency, and thus, predominantly fit the MS model. Social twitters, mob twitters, begging squeals, begging cries, huffs, sneezes, and buzz moans all had longer syllables during anti-social compared to pro-social behaviours. However, some of these vocalisation's syllables were also longer during pro-social behaviours. This may be a result of large amounts of variation in pack vocalisation use, contexts and hours of recording and syllable length differences may be more prominent with increased data for each of these vocalisations. Additionally, a lower F_0 was found in social twitters, rumbles, clear barks, huffs, yelps, whines, alarm barks and alarm growls during anti-social compared to pro-social behaviours. Additionally, vocalisations that differed in syllable length between contexts were longer during animal threats and fundamental frequency lower in both human and animal threat contexts. As such, these results suggest that acoustic modulation may also indicate changes in emotional state in African wild dogs. Marx et al., (2021) have suggested that increases in the level of spectral noise of dog vocalisations leading to acoustic irregularities is also indicative of negative arousal state. This was not tested within this study but should be explored as an additional change in acoustic parameters that could indicate increased negative emotional state in African wild dogs. Overall, differences in acoustic parameters, such as syllable length and fundamental frequency, may be more sensitive tools than vocalisation rate to detect changes in emotional state of among ambivalent vocalisations of African wild dogs. Additionally, studies in which emotional states are manipulated, such as a separation and reintroduction event, could offer further insight into how vocalisations are precisely modulated in this species.

Previously, it has been shown that African wild dogs housed in enclosures have higher baseline faecal glucocorticoid metabolites than free-roaming counterparts (Crossey et al., 2020), and thus may be more susceptible to heightened negative emotional states. It has been shown that signs of increased physiological stress and aggression (glucocorticoids and androgens) are associated with increased contact dominance in African wild dogs during reintroductions (Van den Berghe et al., 2019b; Van der Weyde et al., 2015). In the current study, open-range zoo and traditional zoo packs increased their range of vocalisations associated with dominance, higher rates of mob twitters, whimpers and begging cries during anti-social and feeding behaviours than free-roaming packs. Additionally, alarm barks, threat barks, attack barks, full moans, social growls, alarm growls, buzz moans and attack twitters were recorded in open- range zoos, all except attack twitters and buzz moans in traditional zoos, but only full moans, social growls, alarm growls and buzz moans were present in free-roaming packs. Which may suggest a higher state of negative emotion in open-range zoo packs. Additionally, vocalisation parameters showed similar trends of longer and lower fundamental frequency with pro-social behaviours compared to free-roaming packs, suggesting that zoo-based packs experience increase negative emotional states and thus reduced social cohesion. Given the open-range pack consisted of adult offspring housed with their natal pack, this may reflect an inability for this pack to perform their full range of natural behaviours, including dispersal of young adults from the natal pack. Young adults typically disperse from the natal pack around two years of age (McNutt, 1996). Our detection of more anti-social vocalisations of longer syllables and lower frequency, reflect a more negative emotion state within the pack, which suggests that it may be necessary for the zoo to consider rehoming the young adult cohort of the pack to mimic natural dispersal. By so doing, social cohesion within this large packs may be improved. However, only one open-range zoo was able to be sourced for this study and thus future studies should investigate this further.

In conclusion, our results show that increased rates of alarm barks, threat barks, attack barks, full moans, social growls, alarm growls, buzz moans and attack twitters, and lengthening of syllables and lower fundamental frequency of these vocalisations may be good indicators of increased anti-social behaviours and thus reduced social cohesion in African wild dog packs. Additionally, an increase in the rate, longer syllables and lower frequency of multifunctional vocalisations, such as spar twitters, social twitters and whimpers may be indicators of increased anti-social behaviours and negative emotional states in this species. Furthermore, context and housing may impact vocalisation use, with threat contexts and zoo-based housing (regardless of enclosure size) associated with lengthening syllables and lower fundamental frequency. Changes in vocalisations could now be used as a useful tool by African wild dog managers to monitor social cohesion in existing packs and during stressful conservation interventions and their mitigation using appeasing pheromones. This could be achieved by measuring changes in the rate of calls alongside behavioural changes, paying particular attention to increased rates of barks, growls, buzz moans and attack twitters, which may indicate increased negative emotional states or stress.

Chapter 5: The effect of season, stress and dog appeasing pheromones on African wild dog vocal communication

Riddell P, Paris MCJ, Joone C, Van den Berghe F, Ganswindt A & Paris DBBP. Dog appeasing pheromones increase pro-social and reduce the rate, raise the pitch and lengthen syllables of anti-social vocalisations during the breeding season and stressful interventions of captive African wild dogs (*Lycaon pictus*). *Animal Behaviour* (In prep).

Abstract

Seasonal and stress-related changes in social unity among group-living canids are often monitored using changes in physical behaviours, but this approach overlooks a vital part of social communication: vocalisations. African wild dogs are a vocally complex species that experience heightened stress during the breeding season and reintroduction events, which can lead to higher rates of contact dominance and aggression. Dog appeasing pheromones (DAP) have been shown to reduce the hormones and behaviours associated with aggression in this species and thereby improve social cohesion during times of stress. This blinded placebo-controlled study aimed to characterise how the vocalisations of captive African wild dogs change in response to season, stress of reintroduction, and during appeasing pheromone (DAP) treatment to mitigate stress. Vocalisation data was extracted from video recordings of 5 packs obtained 3 days before, during, and 3 days after a stressful separation, immobilisation and reintroduction event in both the pre-breeding (n = 12) and breeding (n = 11) seasons. During each intervention, all males within a pack were treated with either 10 mL of DAP (n = 11) or placebo (n = 12) solution. The rate, mean pitch and syllable length of each type of vocalisation were compared before, during and after reintroduction. During the breeding season there was an increase in antisocial vocalisations, a lowering of mean pitch and lengthening of syllables observed. Stressful interventions also elicited an increase in anti-social vocalisations that were accompanied by a lower mean pitch and longer syllables. Appeasing pheromone treatment caused increased pro-social and a decreased rate, higher pitch and longer syllables among anti-social vocalisations. Collectively, these results suggest that during periods of stress (including the breeding season and reintroductions) when contact dominance, aggression and androgens are high, anti-social vocalisations more clearly enunciate highly negative states of arousal in African wild dogs. Furthermore, appeasing pheromone treatment during reintroduction appears

to alleviate these effects through improved pro-social communication, which may reduce the incidence of physical conflict.

1. Introduction

Social cohesion is an important contributor to the harmony of group-living animals. The endangered African wild dog (*Lycaon pictus*) is a socially complex canid that relies heavily on cooperation by members to ensure survival of all individuals in the pack(Creel & Creel, 2002; Van den Berghe et al., 2012; Woodroffe & Sillero-Zubiri, 2020; Paris et al., 2024;). Once the separate male and female hierarchies are established, packs tend to remain stable over time, but stress is known to disrupt this stability (Creel and Creel, 2002; Van den Berghe et al., 2012; Van den Berghe et al., 2019b). Furthermore, stress is associated with increased antisocial behaviours, such as dominance and aggression, and increased circulating glucocorticoids and androgens (Van der Weyde, 2013; Van den Berghe et al., 2019b; Crossey et al., 2020).

Behavioural changes, such as increased intra-pack aggression, are indicative of the breeding season that occurs during the peak summer months in African wild dogs (McNutt, 1996; Buettner et al., 2006; Boutelle & Bertschinger 2010; Van den Berghe 2012), but is also evident during both natural and artificial pack (re)introductions (Marneweck et al., 2019a; Van den Berghe et al., 2019b). Aggressive/dominant behaviours during the breeding season occur when dominant pairs suppress subdominant pack members from reproducing (Van Heerden and Kuhn, 1985). In contrast, stress and aggression during (re)introductions are thought to occur as a result of hierarchy instability (Van den Berghe et al., 2019b). Such aggression has been known to cause major morbidity and occasional deaths in managed populations (Van Heerden 1986, 1996; Potgieter et al., 2015).

In South Africa, *in situ* management of African wild dogs requires ongoing human-assisted translocation and artificial bonding of packs to ensure species survival, but captivity and artificial bonding are known stressors (Marneweck et al., 2019a; Marneweck et al., 2019b; Crossey et al., 2020). Such stress and aggression may be exacerbated in managed settings where there is limited space to escape from conflict with unfamiliar conspecifics (Van den Berghe et al., 2012). Changes in social behaviours have been shown to be useful indicators of pack cohesion in social canids. Success of artificial pack formation in African wild dogs and grey wolves can be measured through an increase in socially cohesive behaviours (such as close resting proximity and play), while social disunity is characterised by high rates of contact dominance, aggression and submission (McCreery, 2000; Stahler et al., 2002; Marneweck et al., 2019a; Marneweck et al., 2019b; Van den Berghe et al., 2019b).

Social canids also rely heavily on vocal communication to work cooperatively to hunt, guard territory and raise young (Robbins, 2000; Schnieder and Anderson 2011; Jordan et al., 2023). Both long-range and short-range vocalisations appear to be important in the social communication of canids. Long range vocalisations may help reunite separated pack members (Robbins, 2000; Deaux and Clarke 2013), locate dispersing animals for new pack formations (Marneweck et al., 2019b) and signal individual identity (Palacious et al., 2015), while high- and low-pitched frequencies of short-range vocalisations may be associated with pro- and anti-social vocalisations, respectively (Marx et al., 2021). These types of vocalisations may represent a sensitive tool to measure social cohesion/disunity. The African wild dog vocal repertoire consists of 11 vocalisation classes and 18 subclasses, making them one of the most vocally complex canids (Marten, 1981; Robbins, 2000). Seven of these classes are unique to this species: twitters, begging cries, hoo-calls, gurgles, buzz-moans, howl-barks and rumbles (Robbins, 2000). Long-distance hoo-calls are used to locate separated pack members (Robbins, 2000). Distinct sets of pro-social and anti-social vocalisations can trigger a cascade of effects in the pack and alter physical behaviours, thus playing an important role in social cohesion (Robbins, 2000; Potgieter et al., 2015; Table 5.2). Conversely, some vocalisations are context-specific and can indicate either pro- or anti-social emotional states such as spar twitters, begging cries and barks (Robbins, 2000; Table 5.2). Despite evidence that vocal communication is important to African wild dog interactions, there is a lack of research into how this species uses and responds to vocalisations within packs, and how vocalisations change according to season, context or emotional state.

The structure of howls show clear differences between and within canid species and variations may reflect referential or context-specific information to conspecifics (Kershenbaum et al., 2016). Different vocalisation types (howls, growls, barks, etc.) are associated with different behavioural contexts (Cohen and Fox, 1976; Tembrock, 1976, Robbins, 2000). More recent studies have suggested that particular features of howls could represent differences in arousal states or environmental contexts (Schneider and Anderson, 2011; Deux and Clarke 2013; Frargo et al., 2014). The breeding season is marked by an increase in howls in wolf and jackal species. In grey wolves (*Canis lupus*), Mexican wolves (*Canis lupus baileyi*) and golden jackals (*Canis aureus*), increased howling is associated with increased anti-social and sexual behaviours—indicating social disunity at that time (Harrington and Mech, 1979; Jaeger et al., 1996; Servín, 2000). In contrast, Mexican wolf howl rate was associated with increased pro-social behaviours (Servin, 2000). Thus vocalisations may serve to reduce anti-social behaviours and the chance of conflict between conspecifics during the breeding season (Harrington and Mech, 1979; Jaeger et al., 1996; Stahler et al., 2002). To date, no studies have examined whether vocalisations change during the breeding season in African wild dogs.

Similarly, periods of stress can also induce vocal changes in canids. Separation-induced stress is known to increase cortisol and rates of howling in both grey wolves and domestic dogs (Mazzini et al., 2013; Pongrácz et al., 2017). Studies in domestic dogs suggest that vocalisations may be good indicators of distress and social bonding. Howls, barks and whines of dogs increase during periods of separation anxiety, with a two-fold increase in whines when the owner leaves compared to dogs without separation anxiety (Pongrácz et al., 2017). Furthermore, exposure to whines of unfamiliar dogs elicits increased alert and stress-related behaviours but exposure to whines of familiar dogs reduces comfort-offering behaviours to familiar conspecifics suggesting an effect of familiarity on response to vocal communication (Quervel-Chaumette et al., 2016). Moreover, specific vocalisation parameters may also change in response to stress, with domestic dogs showing separation-related anxiety exhibiting vocalisations containing increased levels of spectral noise and vocal irregularities (Marx et al., 2021). These results would suggest that, when stressed, domestic dogs use vocalisations to not only communicate their emotional state, but also to reinforce social cohesion. As such, it would appear that vocalisations as well as behaviours could be important communication tools used to reinforce social bonds in wild canids. In African wild dogs, a decrease in long-range vocalisations can be a useful tool to indicate social cohesion during artificial pack formation, with a decline in hoo-calls being correlated with a decline in the spatial resting distance between unfamiliar animals (Potgieter et al., 2015; Marneweck et al., 2019a). Collectively, these results suggest that vocalisations might be a sensitive indicator of social cohesion in wild canids, capable of detecting the impact of season, stress and efforts to ameliorate stress on pack dynamics.

In this regard, the use of pheromones has been proposed as a tool that could be used to improve stress and aggression-related behaviour and social cohesion during conservation interventions of wild canids, including African wild dogs (Vlamings, 2011; Van den Berghe et al., 2019b; Riddell et al., 2021). Dog appeasing pheromone (DAP) is a synthetic derivative of a naturally occurring chemical message that can moderate the behaviour and physiology of conspecifics and has been reported to reduce stress and aggression-related behaviours in both puppies and adult domestic dogs and adult African wild dogs (Pageat & Gaultier, 2003; Riddell et al., 2021; Paris et al., 2024). In addition, DAP appears to modulate the effects of stress on vocalisation parameters by reducing mean barking intensity by 6.5dB in domestic dogs (Hermiston et al., 2018). Previous studies in established captive packs of African wild dogs showed that DAP was able to mitigate a surge in faecal androgen metabolite concentrations and significantly decreased rates of contact dominance and active submission behaviours after a veterinary separation immobilisation and reintroduction (SIR) event (Van den Berghe et al., 2019b). However, the beneficial effect of DAP on vocalisations during periods of stress has not yet been investigated in this species.

In summary, despite research suggesting that vocalisations are an important communication tool used during canid social interactions, there is a lack of research into whether African wild dog vocalisations change according to season or emotional state. Moreover, vocalisations could be a sensitive tool to monitor social cohesion and welfare in African wild dogs during management interventions known to induce stress and aggression, and during attempts to mitigate these effects. Thus, the aims of the current study were to (i) determine whether the rate and acoustic properties of different vocalisations change between pre-breeding and breeding seasons; (ii) determine whether vocalisation parameters change before and immediately after a veterinary separation, immobilisation and reintroduction (SIR) event; and (iii) determine whether DAP improves social cohesion after reintroduction by improving communication between pack members.

2. Materials and Methods

2.1 Animals

This retrospective study was conducted using audio extracted from pre-existing behavioural video collected during the 2014 Northern Hemisphere pre-breeding (May to early July) and breeding (August to September) seasons from five zoo-based African wild dog packs in the United States of America (BRK, Brookfield Zoo, Chicago, Illinois; ALB, Albuquerque Biopark, Albuquerque, New Mexico; BIN, Binder Park Zoo, Battle Creek, Michigan; TOP, Topeka Zoo and Conservation Centre, Topeka, Kansas; OKL, Oklahoma City Zoo, Oklahoma

City, Oklahoma) before and after a stress inducing intervention consisting of a separation, immobilisation and reintroduction (SIR) event for routine health checks (Van den Berghe et al., 2019b). The study used a placebo-controlled research design, including a cross over study for three (ALB, BRK and BIN) of the five packs (Table 5.1). The researcher who performed audio data analysis was blinded to treatment until analysis was completed. The size and composition of each pack is described briefly in Table 1; details of animal management and husbandry have been described previously (Van den Berghe et al., 2019b).

Immobilisation occurred over one or two consecutive days as described in Van den Berghe et al., (2019b). During immobilisation, application of 10 ml of either DAP or placebo spot-on solution (Research Institute for Semiochemistry and Applied Ethology, Apt, France) to the pelt at the withers (5 ml) and tail set (5 ml) of each animal. The same solution was applied to sedated pack mates (Table 5.1). The placebo and DAP solutions were of the same composition (transcutol gel) but in the placebo gel the pheromone component was absent.

Animals were held in individual holding areas or crates with visual and olfactory contact with conspecifics during recovered and until reintroduction. Immobilisation of the entire pack occurred over one to two days and reintroduction occurred the next morning, by releasing animals one at a time from each holding area into the main enclosure. For the OKL pack, all males were released and allowed to settle before the female was released. In the BRK pack, reintroduction during the pre-breeding season occurred in the communal holding area, but in the breeding season the alpha male was not immobilised and was kept in the display enclosure during SIR. In this season the three sedated males were reintroduced within the communal holding area overnight and released into the main enclosure the next day. Sedation of pack members in the ABQ and BIN packs occurred over two days during the breeding season (August – September). Reintroduction of ABQ pack members was subsequently staggered over two days, with two animals reintroduced the day after sedation and the third animal reintroduced one day later. In the BIN pack, all animals were isolated in their holding area over the two-day period before reintroduction on the evening of the second day (Van den Berghe et al., 2019b). Where possible the procedure was performed as uniformly as possible between packs, but there were some clear differences in husbandry, enclosure design, and pack composition that we tried to control for using the cross-over design described in Van den Berghe et al., (2019b).

Table 5.1. African wild dog (*Lycaon pictus*) pack composition, pheromone treatment by season and duration of observations from which vocalisations were extracted and analysed.

		Pre-breeding season*[†] *(May – July)		***Breeding season*[†] *(August – September)***	
Pack (abbreviation)	***Composition***	***Treatment***	***Mean (min-max) daily observation time (h:m)***	***Treatment***	***Mean (min-max) daily observation time (h:m)***
Albuquerque Biopark (ALB)	3 ♂	Placebo (n = 3♂)	4:23 (1:25 - 6:00)	DAP (n = 3♂)	2:03 (1:26 - 3:06)
Brookfield Zoo (BRK)	3♂; 1♀	Placebo (n = 3♂)	4:00 (2:14 - 5:11)	DAP (n = 2♂)	4:13 (3:20- 5:28)
Binder Park Zoo (BIN)	3♂	DAP (n = 3♂)	2:31 (1:15 - 4:30)	Placebo (n = 3♂)	4:00 (2:35 - 5:00)
Topeka Zoo (TOP)	3♂	DAP (n = 3♂)	4:36 (3:17 - 5:25)	-	-
Oklahoma City Zoo (OKL)	3♂; 1♀	-	-	Placebo (n = 3♂)	2:16 (1:25 - 2:33)
Mean (min-max) observation time (h:m)		***Pre-breeding:*** 3.53 ± 0:25 (1.09 - 6.00)		***Breeding:*** 2.49 ± 0:20 (1.15 - 5.49)	
Mean (min-max) observation time (h:m)		***DAP:*** 4:01 ± 0:24 (1:25 - 6:01)		***Placebo:*** 3:26 ± 0:24 (1:15 - 5:28)	

h:m = hours:minutes; no. = number; [†]*Northern Hemisphere*

2.2. Experimental design

A Sony FDR-AX100E 4K digital video camera was used to record video and audio of African wild dog behaviour and vocalisations from both public and zookeeper viewing areas. Recordings were performed from the first three days prior to sedation (pre-SIR), at reintroduction (SIR) and for three days after (post-SIR). The time window and duration of video recordings used in the current study were as described in Table 1 and Van den Berghe et al., (2019b). For the current study, all video data were provided to the researcher, but the researcher was blinded to the treatments of each group until the vocalisation analyses had been completed.

Data obtained from the pre-SIR observation period were used to analyse the effect of season on vocalisation rate, mean frequency and syllable length. Data from the placebo group obtained during the pre-SIR, SIR and post-SIR periods were used to determine the effect of a stressful intervention on vocalisation rate, mean frequency and syllable length. Lastly, data from placebo and DAP treatment groups were compared during the pre-SIR, SIR and post-SIR periods to determine whether DAP mitigates the effects of stress on vocalisation rate, mean frequency and syllable length.

2.3. Audio processing and vocal classification

Audio was extracted from the original video footage using Adobe Audition (v 22.0, Adobe Inc., California, USA) and converted to MP4 format using Audacity software (v 3.1.2, The Audacity Team, open-source development, Muse Group, Limassol, Cyprus). Twelve different vocalisations were identified (Table 5.2; Robbins, 2000). The accurate classification of each specific vocalisation was made through spectral analysis using Kaleidoscope Pro Software (v 5.4.6, Wildlife Acoustics Inc., Massachusetts, USA). If a vocalisation was ambiguous or was overlapped by noise from humans or other animals, it was cross referenced back to video footage to confirm it was emitted from an African wild dog and not background noise and not background noise from nearby enclosures or humans. Vocalisations were not assigned to individual animals due to difficulties in performing this with the pre-existing video footage. The observed rate for each type of vocalisation was tallied for each pack. Acoustic parameters for each vocalisation were measured from spectral images and consisted of mean frequency range and syllable length.

Table 5.2. African wild dog vocal ethogram and context as defined by Robbins (2000).

Vocalisation	Context for use
Social twitters	Greeting ceremonies, reinforcement of social bonds, adults approached by pack mates during rest
Whimpers	Greeting ceremonies
Hoo calls	Long distance contact call
Begging cries	Greeting ceremonies, distress, food solicitation, submission
Spar twitters	Vigorous play, social investigation, submission displays
Barks	Running side by side during intra-pack encounters, antagonistic interactions, threats/warnings, muted aggression, alarm
Mob twitters	Mobbing pack mates, pursuit of prey, chasing away hyena, adults warning approaching puppies
Yelps/squeals	Submission, anticipation of pain, frustration
Whine	Submission & frustration
Rumbles	High arousal during greeting ceremonies
Growls	Antagonistic interactions & threat warning
Moans	Frustration

2.4. Statistical analysis

Data were analysed using R Studio (v 3.6.2) for Windows. Mean ± SEM values were calculated for each pack from vocal data obtained from the three days spanning the pre-SIR period to compare seasonal differences in normal vocalisation rates. Tests for normality were performed for each type of vocalisation using histograms and a Shapiro-Wilk test. A generalised linear mixed model (GLMM) was performed to assess whether the rate, mean frequency and syllable length of each type of vocalisation from the pre-SIR period differed between the pre-breeding and breeding seasons. This model included fixed effects of pack composition, hours of analysis, and the random effect of pack. GLMMs were fitted by maximum likelihood with Laplace approximation. A two-step procedure was utilised to create parsimonious models, firstly, the Akaike's information criterion was used to determine mean random-effects structure based on the full model containing all candidate fixed effects (pack, observation day, observation time, pack size, context, housing, behaviour categories, sex ratio), and then removed statistically insignificant fixed-effect terms (Zuur et al., 2009). Secondly, variables were pruned using a backward stepwise selection based on likelihood ratio tests (Zuur et al., 2009).

Data from placebo-treated packs were also used in this study to determine whether the stress of SIR affected the bioacoustics of African wild dog vocalisations. Data were grouped as pre-SIR (three days prior to SIR), SIR (reintroduction) and post-SIR (three days post-SIR). A generalised linear mixed model was used to measure how stress influenced the rate, mean frequency and syllable length of each type of vocalisation before, during and after SIR. This model included fixed effects of intervention days, pack composition, season, hours of analysis and the random effect of pack.

Finally, data from placebo and DAP treated packs were used in this study to determine whether DAP could improve social cohesion through pro-social vocalisations during and after SIR. A linear mixed model was used to determine whether DAP influenced the rate, mean frequency and syllable length of each type of vocalisation before, during and after SIR. This model included fixed effects of intervention days, pack composition, season and hours of analysis and the random effect of pack. All results were considered significant when $P \leq 0.05$.

3. Results

In total, 230 h of audio was extracted from video recordings. Mean observation times across all observation days from which audio was extracted for vocalisation analysis within each pack are summarized in Table 5.1.

3.1. Effect of season on vocalisations

Eleven vocalisations changed in response to season. The rate of whimpers decreased significantly, while the frequency was higher and syllable length was longer in the breeding compared to pre-breeding season (Fig. 5.1a - c and Appendix 4). By contrast, the rate of social twitters significantly increased and its syllable length shortened over the same period (Fig. 5.1a & c and Appendix 4). The rate and mean frequency of begging cries decreased significantly in the breeding compared to pre-breeding season (Fig. 5.1a & b and Appendix 4). By contrast, the mean frequency and syllable length of barks significantly increased, while the syllable length of spar twitters was shorter over the same period. The rate of moans, growls, rumbles and mob twitters increased significantly, with a lower mean frequency for moans and shorter syllable length for rumbles and mob twitters in the breeding compared to pre-breeding season (Fig. 5.1a - c and Appendix 4). By contrast, the rate and mean frequency of whines and yelps/squeals decreased significantly, and syllables became longer for both over the same period.

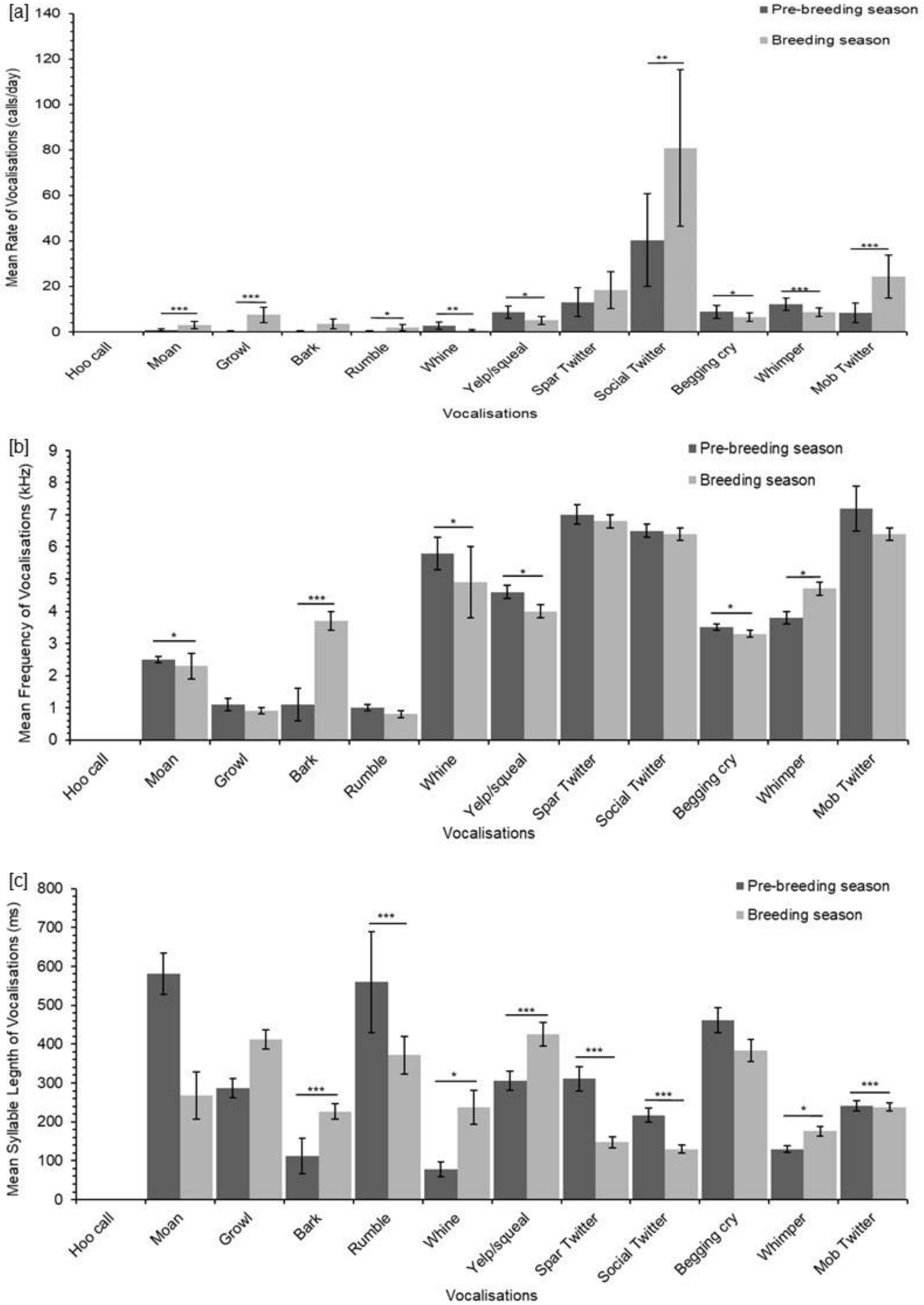
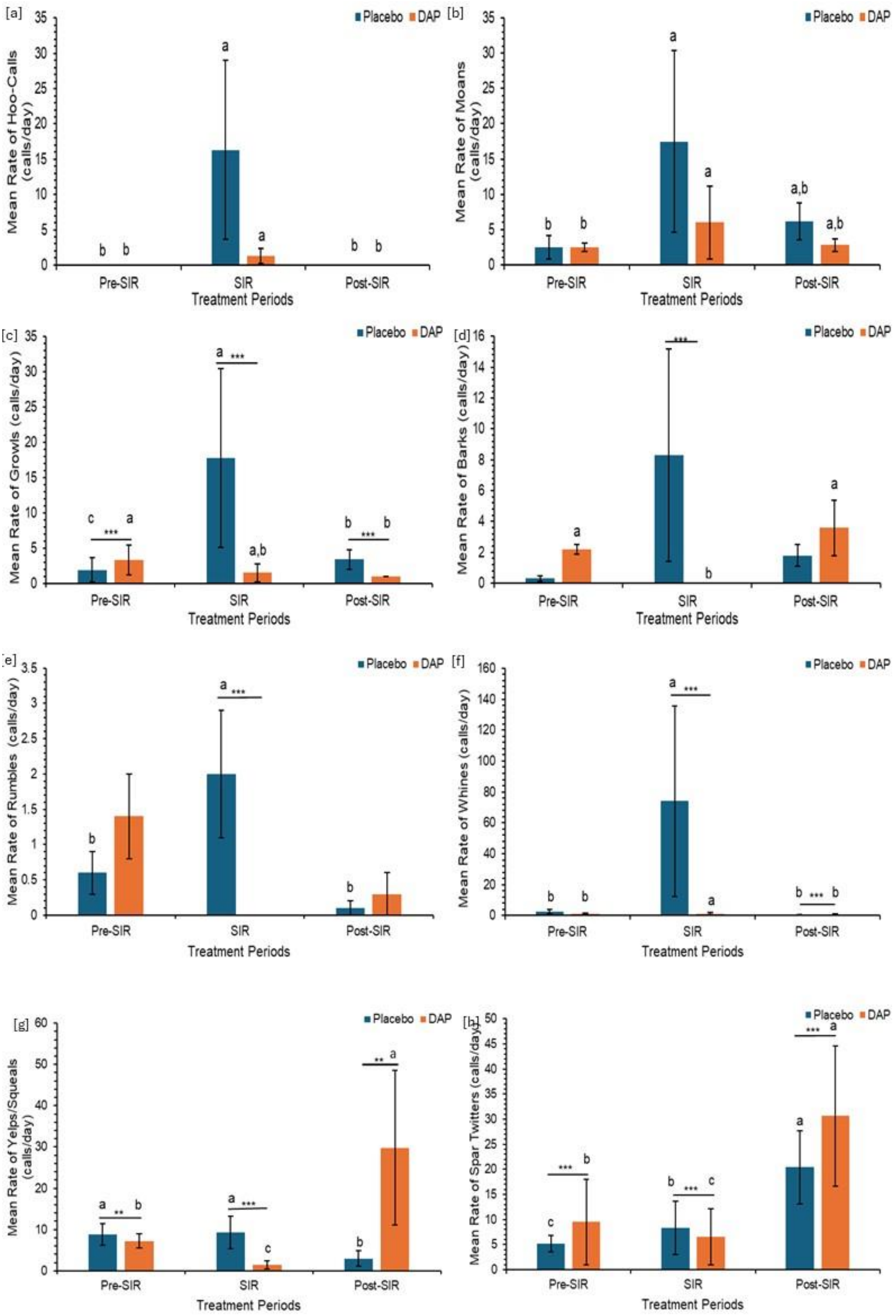


Figure 5.1. Bar graphs of mean a) rate, b) frequency and c) syllable length of pro-social and anti-social vocalisations (first three days prior to intervention) during the pre-breeding (n = 13) *vs.* breeding (n = 12) seasons in captive African wild dogs (*Lycaon pictus*). * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

3.2. Effect of stressful intervention on vocalisations

Baseline changes in the rate, mean frequency and syllable length of vocalisations in response to a stressful intervention are illustrated by placebo-treated packs in Figure 5.2a, c, e and Appendix 5. The rate of social twitters, whimpers and hoo call vocalisations increased significantly during reintroduction from pre-SIR and post-SIR. The mean frequency and syllable length of whimpers was significantly higher and longer during SIR reintroduction that persisted for syllable length post-SIR (Fig.5.2a, c, e and Appendix 5). By contrast, the syllable length of social twitters was significantly shorter during SIR and after post-SIR. The rate of spar twitters and begging cries significantly increased at SIR and post-SIR, with a significantly lower mean frequency among begging cries during SIR and post-SIR (Fig. 5.2a, c, e and Appendix 5). By contrast, barks had a significantly higher mean frequency post-SIR and syllables that were significantly longer during SIR and post-SIR. The rate of moans, growls, rumbles and whines significantly increased SIR in placebo-treated packs (Fig. 5.2a and Appendix 5). Additionally, moans were significantly increased, and yelps/squeals decreased at post-SIR. The mean frequency of rumbles, whines and yelps/squeals was significantly lower, while for growls it was significantly higher SIR and post-SIR (Fig. 5.2c and Appendix 5). Moreover, the syllable length of whines and yelps/squeals was significantly longer during SIR and after post-SIR reintroduction (Fig. 5.2e and Supplementary Table 5), while the length of growls was significantly shorter at SIR and longer at post-SIR (Fig. 5.2e and Appendix 5).

[a]
Mean Rate of Hoo-Calls (calls/day)
0
5
10
15
20
25
30
35
Placebo
DAP
b
b
a
a
b
b
Pre-SIR
SIR
Post-SIR
Treatment Periods
[b]
Mean Rate of Moans (calls/day)
0
5
10
15
20
25
30
35
Placebo
DAP
b
b
a
a
a,b
a,b
Pre-SIR
SIR
Post-SIR
Treatment Periods
[c]
Mean Rate of Growls (calls/day)
0
5
10
15
20
25
30
35
Placebo
DAP
c
***
a
a
***
a,b
b
***
b
Pre-SIR
SIR
Post-SIR
Treatment Periods
[d]
Mean Rate of Barks (calls/day)
0
2
4
6
8
10
12
14
16
Placebo
DAP
a
***
b
a
Pre-SIR
SIR
Post-SIR
Treatment Periods
[e]
Mean Rate of Rumbles (calls/day)
0
0.5
1
1.5
2
2.5
3
3.5
Placebo
DAP
b
a
***
b
Pre-SIR
SIR
Post-SIR
Treatment Periods
[f]
Mean Rate of Whines (calls/day)
0
20
40
60
80
100
120
140
160
Placebo
DAP
b
b
a
***
a
b
***
b
Pre-SIR
SIR
Post-SIR
Treatment Periods
[g]
Mean Rate of Yelps/Squeals (calls/day)
0
10
20
30
40
50
60
Placebo
DAP
a
**
b
a
***
c
b
**
a
Pre-SIR
SIR
Post-SIR
Treatment Periods
[h]
Mean Rate of Spar Twitters (calls/day)
0
5
10
15
20
25
30
35
40
45
50
Placebo
DAP
c
***
b
b
***
c
a
***
a
Pre-SIR
SIR
Post-SIR
Treatment Periods

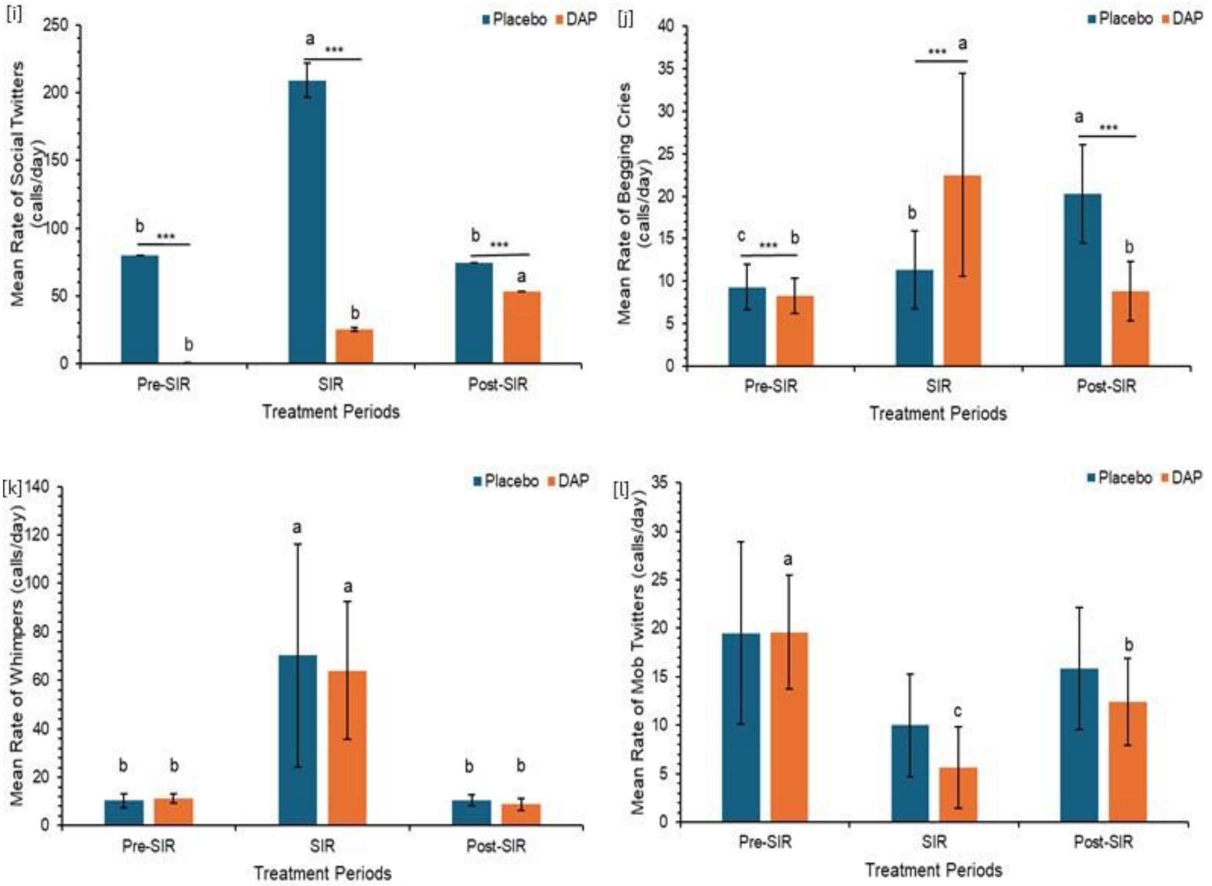
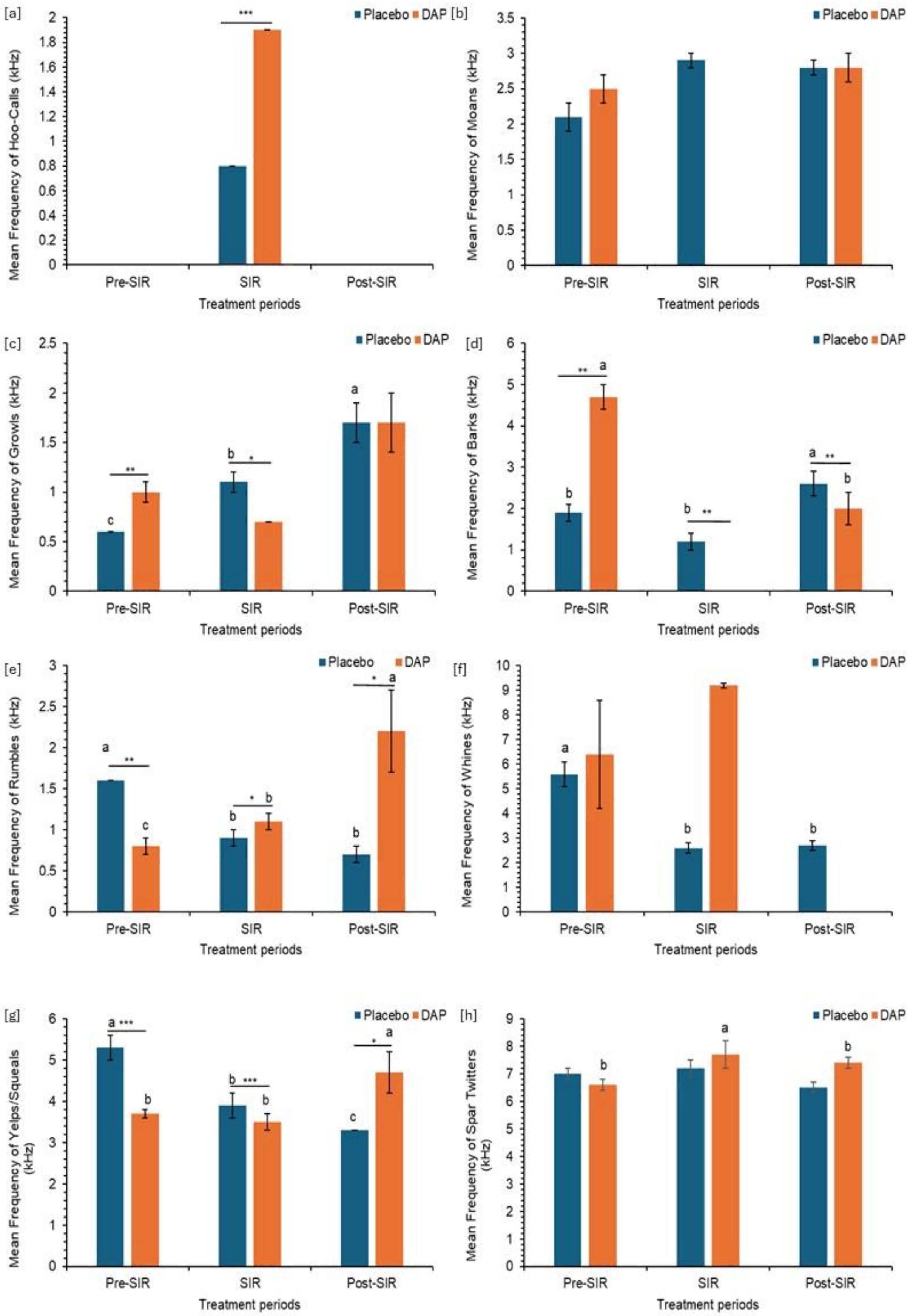


Figure 5.2. Bar graphs of the mean rate of vocalisations before, during and after a stressful intervention in appeasing pheromone- (DAP; n = 11) *vs.* placebo-treated (n = 12) captive African wild dogs (*Lycaon pictus*). SIR, separation, immobilisation and reintroduction. ^{a,b,c} denote a significant difference between observation periods within a treatment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ denotes significant differences between treatments during the same observation period.


[a]
Mean Frequency of Hoo-Calls (kHz)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
1.8
2
***
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[b]
Mean Frequency of Moans (kHz)
0
0.5
1
1.5
2
2.5
3
3.5
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[c]
Mean Frequency of Growls (kHz)
0
0.5
1
1.5
2
2.5
c
**
b
*
a
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[d]
Mean Frequency of Barks (kHz)
0
1
2
3
4
5
6
**
a
b
b
**
a
**
b
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[e]
Mean Frequency of Rumbles (kHz)
0
0.5
1
1.5
2
2.5
3
a
**
c
b
*
b
b
*
a
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[f]
Mean Frequency of Whines (kHz)
0
1
2
3
4
5
6
7
8
9
10
a
b
b
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[g]
Mean Frequency of Yelps/Squeals (kHz)
0
1
2
3
4
5
6
a ***
b
b ***
b
c
*
a
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods
[h]
Mean Frequency of Spar Twitters (kHz)
0
1
2
3
4
5
6
7
8
9
b
a
b
Placebo
DAP
Pre-SIR
SIR
Post-SIR
Treatment periods

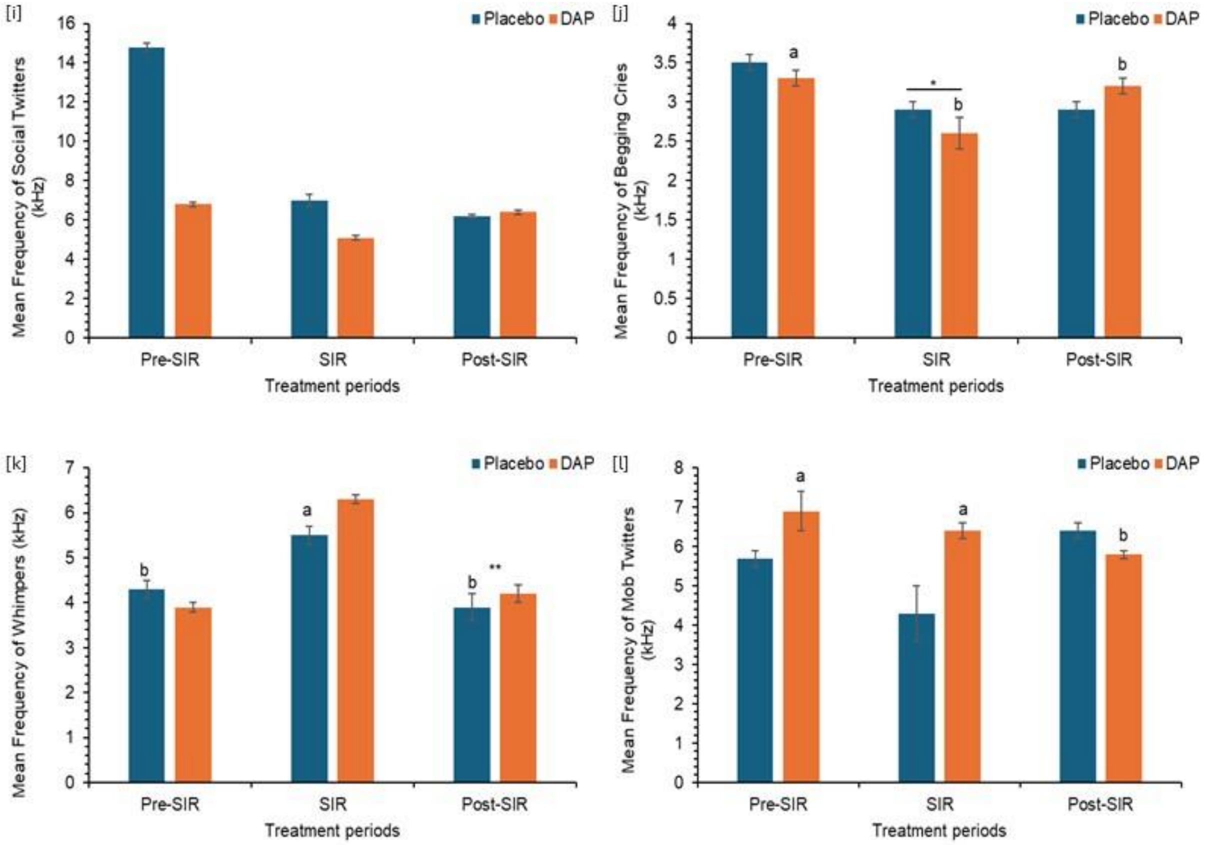
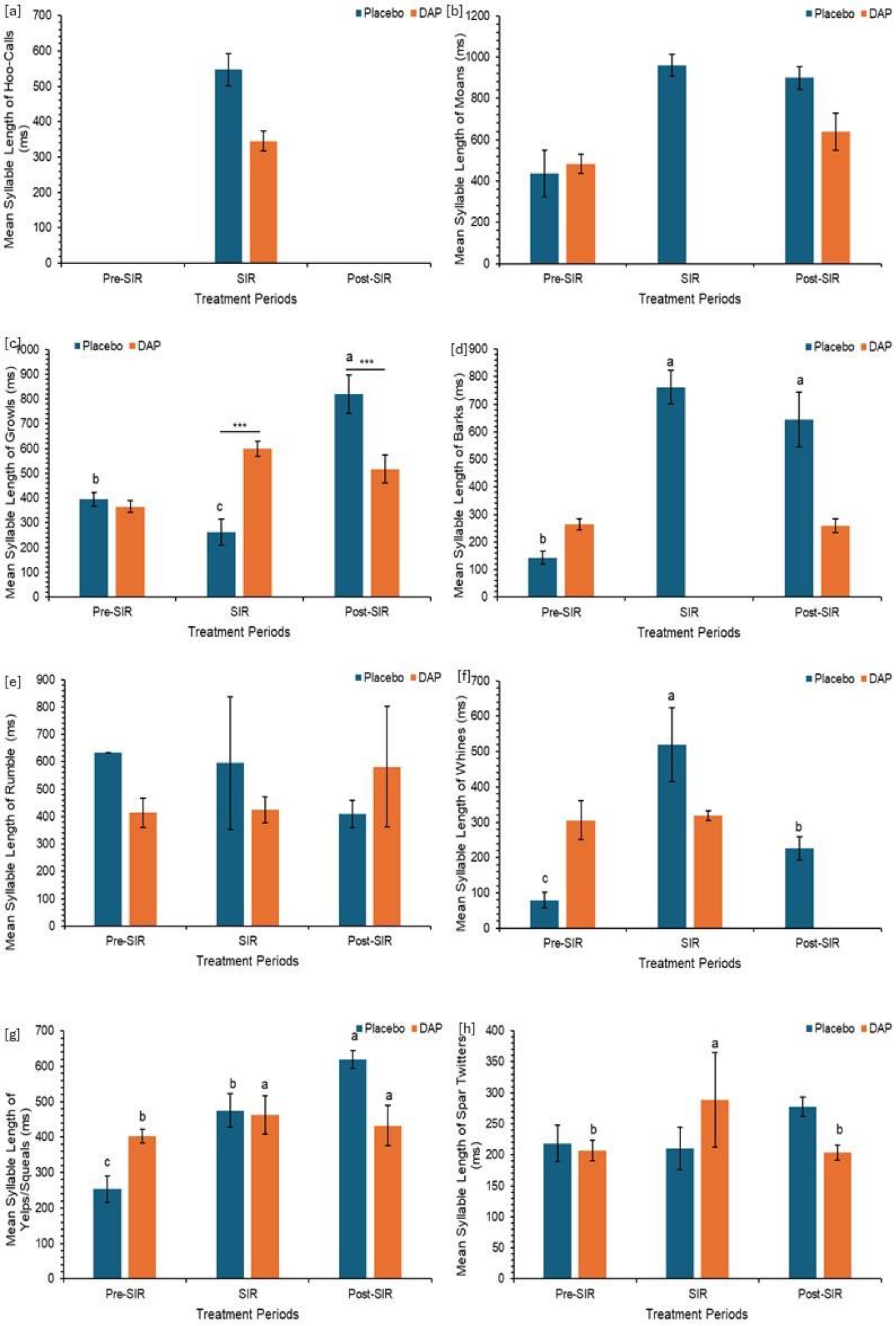


Figure 5.3. Bar graphs of the mean frequency of vocalisations before, during and after a stressful intervention in appeasing pheromone- (DAP; n = 11) *vs.* placebo-treated (n = 12) captive African wild dogs (*Lycaon pictus*). SIR, separation, immobilisation and reintroduction. ^{a,b,c} denote a significant difference between observation periods within a treatment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ denotes significant differences between treatments during the same observation period.


[a]
Placebo
DAP
Mean Syllable Length of Hoo-Calls (ms)
0
100
200
300
400
500
600
700
Pre-SIR
SIR
Post-SIR
Treatment Periods
[b]
Placebo
DAP
Mean Syllable Length of Moans (ms)
0
200
400
600
800
1000
1200
Pre-SIR
SIR
Post-SIR
Treatment Periods
[c]
Placebo
DAP
Mean Syllable Length of Growls (ms)
0
100
200
300
400
500
600
700
800
900
1000
b
c
***
a
***
Pre-SIR
SIR
Post-SIR
Treatment Periods
[d]
Placebo
DAP
Mean Syllable Length of Barks (ms)
0
100
200
300
400
500
600
700
800
900
b
a
a
Pre-SIR
SIR
Post-SIR
Treatment Periods
[e]
Placebo
DAP
Mean Syllable Length of Rumble (ms)
0
100
200
300
400
500
600
700
800
900
Pre-SIR
SIR
Post-SIR
Treatment Periods
[f]
Placebo
DAP
Mean Syllable Length of Whines (ms)
0
100
200
300
400
500
600
700
c
a
b
Pre-SIR
SIR
Post-SIR
Treatment Periods
[g]
Placebo
DAP
Mean Syllable Length of Yelps/Squeals (ms)
0
100
200
300
400
500
600
700
c
b
b
a
a
a
Pre-SIR
SIR
Post-SIR
Treatment Periods
[h]
Placebo
DAP
Mean Syllable Length of Spar Twitters (ms)
0
50
100
150
200
250
300
350
400
b
a
b
Pre-SIR
SIR
Post-SIR
Treatment Periods

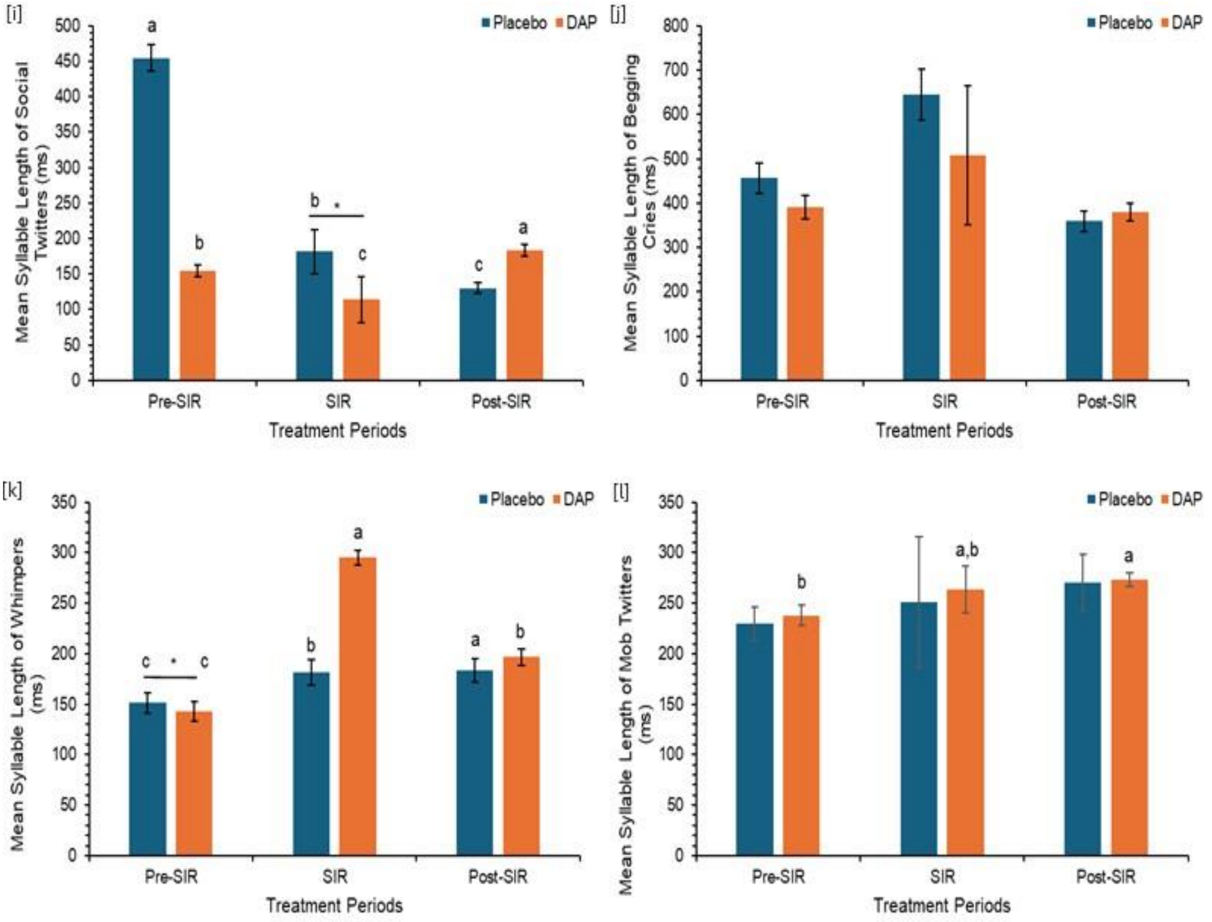


Figure 5.4. Bar graphs of the mean syllable length of vocalisations before, during and after a stressful intervention in appeasing pheromone- (DAP; n = 11) *vs.* placebo-treated (n = 12) captive African wild dogs (*Lycaon pictus*). SIR, separation, immobilisation and reintroduction. ^{a,b,c} denote a significant difference between observation periods within a treatment. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$ denotes significant differences between treatments during the same observation period.

3.3. Effect of appeasing pheromones on vocalisations

Changes over time in the rate, mean frequency and syllable length of vocalisations among DAP-treated packs in response to a stressful intervention are illustrated in Figure 5.2b, d, f and Appendix 5. The rate of social twitters significantly increased SIR and post-SIR, with a transitory but significant shortening of syllable length SIR. By contrast, the rate of whimpers increased at SIR compared to pre- and post-SIR, which was accompanied by significantly longer syllables SIR and post-SIR. When vocalisations were compared between treatment groups, the rate of social twitters was significantly lower in DAP-treated compared to placebo-treated packs during pre-SIR and at SIR but higher at post-SIR (Fig. 5.2a-b and Appendix 5). Additionally, syllable length of social twitters was significantly lower in DAP-treated packs at

SIR (Fig. 5.2e-f and Appendix 5). Finally, the mean frequency of whimpers was significantly higher in the DAP-treated packs at post-SIR (Fig. 5.2c-d and Appendix 5) and syllable lengths significantly lower pre-SIR (Fig. 5.2e-f and Appendix 5).

The rate of begging cries, barks and spar twitters significantly increased during SIR (begging cries) or post-SIR (begging cries, barks and spar twitters). The rate of spar twitters and barks decreased significantly at SIR (Fig. 5.2a-b and Appendix 5). The mean frequency of barks and spar twitters was significantly lower and higher respectively SIR and post-SIR. Spar twitters had a transitory but significant lengthening of syllables SIR. When these vocalisations were compared between treatment groups, the rate, mean frequency and syllable length of spar twitters increased significantly in DAP-treated compared to placebo-treated packs at SIR (Fig. 5.2a-f and Appendix 5). The rate of begging cries also significantly increased but rate of barks decreased in DAP-treated compared to placebo-treated packs at this time. The rate of barks and spar twitters significantly decreased in the DAP-treated compared to placebo-treated packs during SIR. Additionally, barks, begging cries and spar twitters significantly decreased in the DAP-treated compared to placebo-treated packs at SIR; this persisted to post-SIR for barks (Fig. 5.2c-d and Appendix 5).

The rate of rumbles, yelps/squeals, mob twitters, whines and moans significantly increased either at pre-SIR (rumbles, yelps/squeals, mob twitters) or SIR (growls, whines and moans) reintroduction among DAP-treated packs (Fig. 5.2b and Appendix 5). The mean frequency of rumbles was significantly higher at SIR that persisted to post-SIR (Fig. 5.2d and Appendix 5). By contrast, mean frequency was significantly higher for mob twitters and yelps/squeals respectively post-SIR. Moreover, syllable length was significantly longer for both yelps/squeals and mob twitters at SIR and post-SIR (Fig. 5.2f and Appendix 5). When vocalisations were compared between treatment groups the rate of growls, rumbles, whines and yelps/squeals significantly decreased in DAP-treated compared to placebo-treated packs

SIR, in growls these remained decreased and increased for whines and yelps/squeals at post-SIR (Fig. 5.2a, b and Appendix 5). The mean frequency of rumbles was significantly higher in DAP-treated compared to placebo-treated packs at SIR and post-SIR, while lower in growls and yelps/squeals at SIR. The syllables of growls were significantly longer in DAP-treated compared to placebo-treated packs at this time (Fig. 5.2c-f and Appendix 5).

4. Discussion

Our study has demonstrated that multiple vocalisations increase in rate during the breeding season, and these were typically accompanied by a lower mean frequency and either lengthening or shortening of syllable length for several of these vocalisations. Similarly, we showed that the stress of reintroductions increased the rate of most vocalisations, but mean frequency and syllable length changes appear to be specific to each vocalisation, with all vocalisations that changed other than growls showing decreased mean frequency and lengthening syllables. Growls had increased mean frequency and decreased syllable lengths. Finally, DAP treatment was associated with an increase in the mean frequency of several vocalisations over time. Moreover, DAP-treated packs had an increased rate, lower mean frequency and shorter syllable length of some vocalisations and a reduced rate, lower mean frequency and shorter syllable length of some anti-social vocalisations compared to those in placebo-treated packs. These results and those of the previous study by Van den Berghe et al., (2019b), provide evidence that both behaviours and vocalisations can be influenced by season, intervention and pheromones, and play an important role in African wild dog social communication and cohesion.

Previously, the field of canid vocalisation has focused heavily on the use of long-distance communication such as howls and hoo-calls to monitor animal territory and movements or indicate distress and seasonality. For example, Mexican wolves increase rates of howling in chorus and the syllable length of howls during the breeding season (Servín, 2000). By comparison, pack and individual howls in grey wolves increase in the pre-breeding and remain elevated in the breeding season; reflecting higher rates of territorial and mating behaviours (McIntyre et al., 2017). The African wild dog breeding season shows marked changes in social behaviours, including increased dominance and fighting during mate guarding months and stress likely increases during this time (Van den Berghe et al., 2012). It is thought that the stress of captivity may impact reproduction success, with high rates of infanticide in captive packs (Yordy & Mossotti, 2016). Thus, the breeding season highlights the social complexities and the importance of social unity to the continuation of important genetic lines (Paris et al., 2024). During the breeding season, alpha animals use dominant behaviours to reproductively suppress subordinate animals (Van den Berghe et al., 2019a). The results of our present study suggest that concurrent with an increase in intra-pack dominance, there is an increase in the rate, lowering of mean frequency and lengthening of syllables of vocalisations during the breeding season. Together these results suggest that the breeding season is a time of relative pack instability, in which dominant animals must expend more energy to maintain social structures. This study was limited by the fact that data was only collected during the pre-breeding and breeding seasons from stable packs consisting of familiar individuals. Further insight into how African wild dogs use vocal communication could be gained by collecting data via an annual study in both captive and free-roaming populations within a natural setting, as well as during the formation of new packs when hierarchies are initially established among unfamiliar individuals.

Long-distance contact calls are a common feature of canid packs experiencing separation and thought to indicate distress (Robbins, 2000, Déaux and Clarke, 2013). For example, the stress of separation in grey wolves results in increased howling (Mazzini et al., 2013). In African wild dogs, a decrease in long distance calls has been associated with social unity, with a decline in hoo-calls as social cohesion and acceptance increases during artificial bonding (Marneweck et al., 2019a). In our study hoo-calls occurred more often in the placebo-treated compared to DAP-treated packs (though this was not significant) and were only significantly increased at reintroduction in the placebo treated packs providing further support that hoo-calls are indicative of social cohesion, with increased rates of hoo-calls being indicative of reduced social unity with unfamiliar individuals (Marneweck et al., 2019a). Interestingly, the frequency of hoo-calls was higher in the DAP-treated compared to placebo-treated packs at reintroduction.

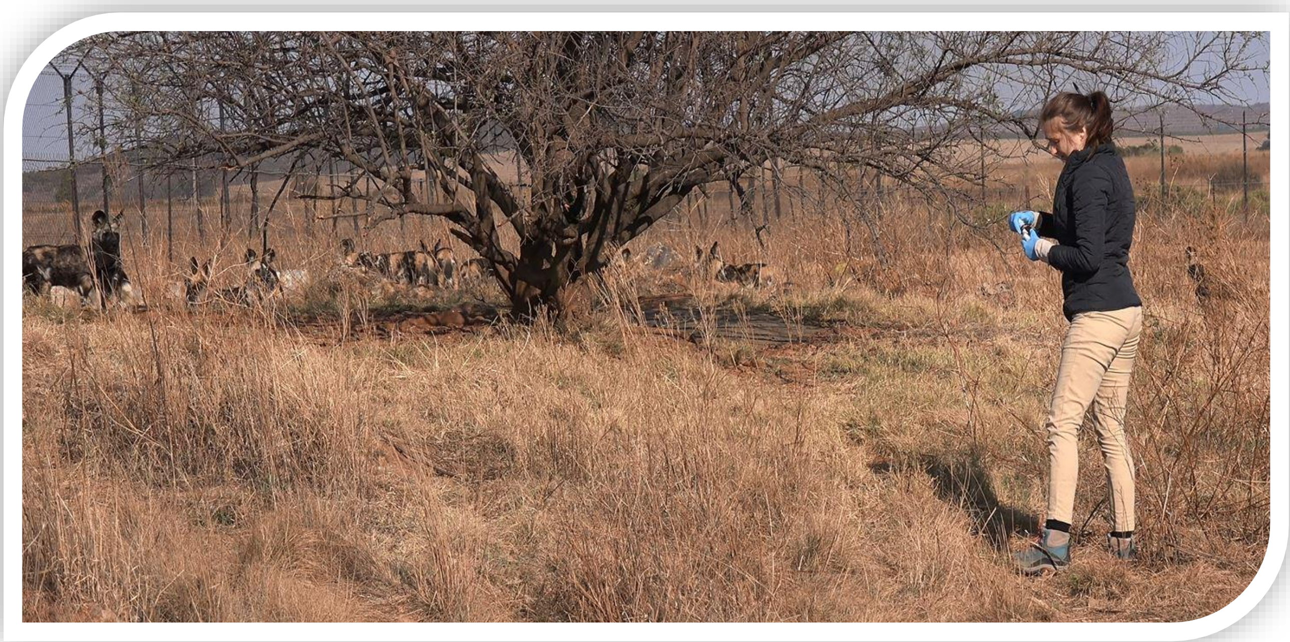
Stress causes both behavioural and physiological adaptations in individuals. In African wild dogs, signs of stress include an increase in both circulating glucocorticoids and androgens and a concurrent increase in rates of dominance and agonistic behaviours (Creel et al., 1997a; de Villiers et al., 1997; Van der Weyde, 2013; Van den Berghe et al., 2019b; Crossey et al., 2020). The outcome of stress could impact pack functionality and performance, leading to reduced cooperation in hunting and reproduction (Barndl et al., 2022). For example, high cortisol and testosterone in humans reduces performance in group tasks (Akinola et al., 2016). As such, it is likely that increased stress and aggression hormones in African wild dogs experiencing stress could result in behaviours which reduce pack coordination and as such reduce pack cohesion. Additionally, Long-term chronic stress in this species could compromise the health and welfare of animals, with increased aggressive conflicts within packs increasing the chance of injury and mortality (Riddell et al., 2021). In the current study, we found that the rate, frequency and syllable length of many vocalisations were affected by stress. Interestingly, when stressed at the time of reintroduction, the rate increases (moans, growls, rumbles, whines), mean frequency lowers (rumbles, whines, yelps/squeals, begging cries), and syllables lengthen (growls, whines, yelps/squeals, barks) for many vocalisations in placebo-treated packs. This corresponds to an increase in contact dominance behaviours found in the same placebo-treated packs after reintroduction (Van den Berghe et al., 2019b).

Morton's motivational-structural (MS) model postulates that high frequency, harmonic sounds are most likely to be positively correlated with affiliative behaviours and low frequency, noisy sounds are more likely to be correlated with aggression (Marx et al., 2021). The lowering of frequency described for many anti-social vocalisations during stressful reintroduction demonstrated in our study appear to fit the MS model. However, previous work suggests that African wild dog vocalisations do not always fit the MS model well, with some noisy and high-frequency vocalisations being emitted during aggressive interactions (Robbins, 2000). Oddly, our seasonal findings appear to both fit and contradict the MS model. Low-frequency bark vocalisations became higher frequency during the breeding season, while low-frequency moans and begging cries became lower frequency in the same season. Similarly, high-frequency whimper vocalisations became higher frequency in the breeding season, while high frequency whines and yelps/squeals became lower frequency in the same season. Compared to the MS model, the source-filter framework (Fant, 1971, Titze and Martin, 1998) proposes that the tension states of organs necessary for sound production are affected by arousal states and the valence (positive or negative emotional loads) of individuals causing specific vocal inflections that advertise the emotional state of the caller (Marx et al., 2021). This phenomenon is observed in both domestic dogs and Australian dingoes in which the pitch of barks and alert calls modulate the response of receivers, such that higher pitches elicit a larger response (Déaux et al., 2016; Root-Gutteridge et al., 2021). As such, the lower frequency observed in some vocalisations of African wild dog during periods of stress may be caused by physiological changes (increased androgens or cortisol) or behavioural changes (increased aggression or stress), during the breeding season or other stressful events (Creel et al., 1997a; Newell-Fugate et al., 2012; Van den Berghe et al., 2019a), and help amplify communication of their emotional state. Coupled with increased dominance behaviours during periods of stress this may more strongly enunciate to conspecifics that a caller is currently in a state of high arousal and negative valence.

Dog appeasing pheromones are a potential management tool that may reduce the harmful effects of stress and aggression on the health and social cohesion of African wild dogs (Van den Berghe et al., 2019b; Riddell et al., 2021). Additionally, these pheromones appear to modulate stress and aggression-related behaviours in domestic dogs (Mills and Hargrave, 2004; Gandia Estellés and Mills, 2006; Landsberg et al., 2015; Puglisi et al., 2022), with initial evidence suggesting that dog appeasing pheromones may reduce mean barking intensity 6.5dB compared to placebo-treated dogs (Hermiston et al., 2018). Similarly, our study in African wild dogs predominantly showed an increased rate of several pro-social vocalisations and decreased rate of several anti-social vocalisations in DAP-treated relative to placebo-treated packs. The same DAP-treated packs also displayed no surge in androgen and reduced contact dominance behaviours during reintroduction (Van den Berghe et al., 2019b). Furthermore, despite evidence of frequency and syllable length modulation on the day of reintroduction in DAP treated packs there was no conclusive pattern of increased/decreased frequency or lengthening/shortening of vocalisation. Thus, we postulate that DAP-treated packs use increased vocal communication instead of resorting to dominant behaviours to regulate and mitigate social disruption among pack members. Collectively, these results suggest that DAP is a useful tool to improve the welfare of African wild dogs by social communication having a reducing effect on contact dominance behaviours, reducing rates of vocalisations and frequency modulation which act to improve social cohesion during stressful interventions.

In conclusion, African wild dog vocalisations appear to be sensitive indicators of seasonality, stress and social cohesion. Our study showed that both the breeding season and stressful events are marked by an increase in the rate, lowering of frequency and lengthening of syllables of predominantly anti-social vocalisations. As such, for keepers of African wild dogs it would be advantageous to have a baseline measure of vocalisation rate, syllable length and frequency with which to measure changes which might indicate increased stress. This could then be used to monitor packs at regular intervals or during Similarly, this could be used during artificial pack formation of free-ranging individuals during meta-population management, by monitoring each single-sex group in adjacent pens for anti-social vocalisations with higher frequency, shorter syllables and reduced rates prior to introduction. Encouragingly, domestic dog appeasing pheromones appear to improve social communication during stressful reintroductions by increasing some multifunctional vocalisations and reducing anti-social behaviours and vocalisations as a result of improved social communication and thus unity. Given the ongoing need for human intervention to conserve and manage this species, our results demonstrate that vocal analysis and appeasing pheromones are valuable tools to monitor and improve social cohesion during stressful reintroductions and thereby improve animal welfare.

Chapter 6: Comparison of traditional *vs.* pharmacophore-derived dog appeasing pheromone perception for the management of stress and aggression in African wild dogs (*Lycaon pictus*)



Riddell P, Paris MCJ, Joone CJ, Ganswindt A, Pageat P & Paris DBBP. Pheromone perception behaviours increase but some pro-social behaviours decline when African wild dog (*Lycaon pictus*) enclosures are treated with different appeasing pheromone analogues. *Applied Animal Behaviour Science* (In prep).

Abstract

Translocation and artificial pack formation are current practice in South Africa to facilitate dispersal of African wild dogs. However, such strategies are known to induce stress in this species, which can lead to increased aggression, conflict and pack instability. Dog appeasing pheromone (DAP) has shown promise in reducing stress and aggression in African wild dogs in captivity. A novel enhanced dog appeasing pheromone (phDAP) has recently been formulated by our team which was reverse engineered to more closely match the olfactory receptor sequences of domestic dogs and hence elicit a more robust behavioural response in the domestic dog, but its effectiveness for other canids is currently unknown. Preliminary investigation of African wild dog secretions containing natural appeasing pheromones determined that chemical peaks were potentially similar to those in phDAP. As such, this study aimed to compare whether phDAP causes a stronger behavioural response and elicits increased pro-social behaviour in African wild dogs compared to the original domestic DAP formulation. Baseline rates of behaviour were determined by daily 1-hour observations of seven captive packs in South Africa over 3 consecutive days. Using a double-blinded, baseline-controlled competitive design, packs were then exposed to a 1 m^2 area of their enclosure treated with either (i) original DAP (Day 4 and 23) or (ii) phDAP (Day 8 and 27), and behaviour observed for 1 hour immediately post-treatment. After a three-day rest period, blinded treatments were reversed and applied to a different location within each enclosure. The pheromone exposure protocol above was then replicated 15 days later. Investigative behaviours (classified as general investigation which did not occur immediately after interacting with pheromones) and pheromone response behaviours (classified as immediate investigation of treatment areas) increased significantly or showed increased trends respectively in DAP-treated and to a lesser extent phDAP-treated sites compared to baseline. Unexpectedly, some pro-social behaviours (play and affiliation) declined significantly or showed a declining trend respectively in animals

exposed to phDAP treated sites. The preliminary results suggest that the original DAP analogue elicits a more favourably response by African wild dogs than the novel phDAP when sprayed within enclosures in unstressed animals. However, it remains to be determined whether longer-latency pro-social effects may be better detected through the prolonged topical application of these pheromones during stressful interventions.

1. Introduction

Increased habitat loss and fragmentation, hunting, snaring, poisoning and infectious diseases are leading causes for the continued decline of the African wild dog (*Lycaon pictus;* Woodroffe et al., 2007; Woodroffe and Sillero-Zubiri, 2020). Currently this enigmatic social canid is listed as endangered by the IUCN, and only an estimated 6,600 individuals remain in the wild consisting of only 1500 adult animals (Woodroffe and Sillero-Zubiri, 2020). Packs inhabit extensive territories and disperse great distances to find unrelated individuals with which to form new packs, often passing through human-affected areas (Pomilia et al. 2015; Cozzi et a., 2020). The fencing of nature reserves in order to protect wild animals ironically further limits natural dispersal and the survival of wild dogs (McCreery and Robbins, 2001). In South Africa, a meta-population management approach has been adopted to overcome these issues, which includes translocation and artificial pack formation of dogs between reserves (Davies-Mostert et al., 2009).

Meta-population management methods have relatively high levels of success (86%) within fenced reserves and have enabled population growth but a relatively slow rate of gene dispersal (Gusset et al., 2008; Marneweck et al., 2019a; Tensen et al., 2024). These initiatives involve the human-assisted movement of wild individuals between habitats, translocation (Armstrong and Seddon, 2008), and the artificial joining of unrelated individuals/groups to form new packs (Gusset et al., 2008). The success of translocation and artificial pack formation can be improved by holding animals in temporary captivity to facilitate social integration and acclimatisation to the reintroduction site (Miller et al., 1999; Gusset et al., 2006; Briers-Louw et al., 2019). The length of time required for pack formation can vary, with increased time in temporary holding enclosures, occurrence of breeding behaviours, smaller group sizes and a female-biased sex-ratio of the new group all known to play a positive role in improving pack formation success and decreasing the length of time for social integration (Gusset et al., 2008;

O'Riain et al., 2015; Marneweck et al., 2019a). One inadvertent cost associated with the well-intentioned management of African wild dogs is stress, triggered by disruption of social bonds when animals are separated, immobilised and (re)introduced (Dickens et al., 2010). Forced cohabitation of unfamiliar African wild dogs in confined areas can increase stress and aggression between individuals and delay hierarchy establishment (Potgieter et al., 2015; Marneweck et al., 2019a). Furthermore, the experience of captivity itself is a known stressor (Van den Berghe et al., 2019b; Crossey et al., 2020).

Short-term, acute stress lasting only minutes to hours, may be beneficial to animals in certain situations, such as flight from a predator (Dhabhar, 2000). Acute stress results in a release of glucocorticoids, which in the short-term increase immunity at the level of the skin, prevent immune overreaction, supress aggression and divert energy towards immediate survival (Dhabhar, 2000). However, long-term chronic stress, lasting days or months, can be detrimental to animal health (Dhabhar, 2000). Prolonged captivity, over days, months or even years, can induce a state of chronic stress in African wild dogs above that of free-living individuals (Crossey et al., 2020). Such chronic stress causes hypothalamic-pituitary-adrenal (H-P-A) axis dysfunction and increased circulating glucocorticoids which can increase the chance of anti-social aggressive interactions or suppress important physiological pathways, such as the immune, reproductive and digestive systems (Dickens et al., 2010; Sheriff et al., 2011; Van den Berghe et al., 2019b).

Pheromones are groups of naturally occurring volatile semiochemicals (often contained in complex blends secreted from glands as sweat, sebum, tears, urine or saliva) that send signals to the brain causing physiological and/or behavioural changes, which are becoming popular tools for behaviour modification in animals (Liberles, 2014; Wyatt, 2017; Riddell et al., 2021). Pheromone perception relies on the highly specific nature of chemoreceptors found within the olfactory organs of mammals, whose activation can modulate endocrine responses, signal individual identity and evoke a range of complex behaviours (Wyatt, 2017; Riddell et al., 2021).

Appeasing pheromones are currently used as a popular therapeutic intervention for the reduction of stress-related behaviours in domestic dogs, showing positive behavioural modification in a range of both acute and chronic stressful situations including transport, veterinary intervention or in dog rescue shelters (Mills and Hargrave, 2004; Gaultier et al., 2005; Gandia Estellés and Mills, 2006; Taylor and Mills, 2007; Siracusa et al., 2010; Landsberg et al., 2015; Hermiston et al., 2018). Given the negative effects of translocation, (re)introduction and captivity on stress in African wild dogs, it has been suggested that appeasing pheromones could be used to improve animal welfare (Riddell et al., 2021). Preliminary research has shown that a synthetic analogue of the domestic dog appeasing pheromone (DAP) can be perceived by African wild dogs and shows a trend of increased investigative behaviours when sprayed in their enclosure (Vlamings, 2011). Vlamings (2011) exposed four packs of African wild dogs to a 1 m^2 area of ground treated with DAP or placebo solution and their behavioural responses directed towards each site were analysed within the first hour of exposure. Vlamings (2011) reported a trend towards increased resting, sniffing, urinating, licking mandibular and panting behaviours, but significance could not be demonstrated due to the limited sample size. Additionally, this study showed a trend of an 18-33% reduction in faecal glucocorticoid metabolites in animals treated with dog appeasing pheromone collars, suggesting a putative suppressive effect on the H-P-A axis (Vlamings, 2011). In a follow-up double-blinded placebo-controlled study, application of DAP during stressful veterinary intervention reduced aggression hormones (faecal androgen metabolites) and antagonistic behaviours (contact dominance, active submission), and increased non-contact dominance behaviours, suggesting a putative suppressive effect on the hypothalamic-pituitary-gonadal axis (Van den Berghe et al., 2019b). Interestingly, DAP did not decrease the hormonal stress response at reintroduction in this study, suggesting further research is required to understand the precise mechanism of action of appeasing pheromones on the H-P-A axis. Collectively however, these results suggest that DAP may reduce hormones and behaviours associated with aggression during pack re-establishment in captivity. As such, DAP represents a promising tool to facilitate the establishment of the hierarchy and social cohesion during translocation and artificial pack formation of African wild dogs.

The appeasing pheromone message has also been identified in cerumen secretions from the ears of African wild dogs and chemical peaks in these secretions closely resemble those contained in a recently formulated novel pharmacophore dog appeasing pheromone (phDAP; Patrick Pageat, personal communication), suggesting that the African wild dog appeasing pheromone may be similar to that of the domestic dog. Emerging research in domestic dogs demonstrates that phDAP, incorporated into long-acting gels, positively modifies behaviour in animals experiencing stress (Puglisi et al 2022; Nicolas et al., 2022). Given the highly specific nature of pheromone transduction (Riddell et al., 2021) we hypothesised that the novel phDAP would be more readily perceived by African wild dogs, and elicit stronger investigative responses and increased pro-social and affiliative behaviours than DAP, due to its potential to elicit a greater behavioural modification response in domestic dogs and the similarities in its chemical structure and the chemical peaks identified on initial analysis of African wild dog secretions. Thus, the aim of this study was to compare the perception of original DAP *vs.* enhanced phDAP and any behaviours elicited in captive packs of African wild dogs by measuring changes in the rate of behaviours among pack members upon exposure to each compound.

2. Materials and methods

2.1. Animals

Seven packs of n = 46 healthy African wild dogs (n = 33 male; n = 13 female) were used in this study, located across four captive facilities in South Africa (Bothongo Rhino & Lion Nature Reserve, BOT; The Ann Van Dyke Cheetah Centre, AVDCC; Hoedspruit Endangered Species Centre, HESC; SANBI National Zoological Gardens, Pretoria, PRET; Table 6.1). The age of animals ranged from one to eleven years, and they were housed in either single-sex packs (PRET, AVDCC1 and AVDCC3) or mixed-sex packs (AVDCC2, BOT, HESC and AVDCC4) in permanent outdoor enclosures. Animals were fed daily, or every second to third day, on either beef, venison, chicken or equid (depending on institution and meat available) and had access to fresh water *ad libitum*. To avoid confounding effects on behaviour, no observations were performed during or just prior to feeding times. This study was approved by the James Cook University Animal Ethics Committee (A2710) and by the local in-country Ethics Committee (PRET; P2022-09) or Research Management Team (BOT, AVDCC and HESC) of each participating institution.

Table 6.1. Demographics, public access and observation times of captive African wild dog (*Lycaon pictus*) packs used in this study.

Zoo/Pack ID	*Pack composition*	*Age (y)*	*Public Access*	*Mean (min-max) daily observation time (min)*	*Observation month(s)*
AVDCC1	2F	8	PVL	49 (45-56)	July-August
AVDCC2	2F,2M	3	PVL	48 (40-56)	July-August
AVDCC3	5M	3	PVL	46 (45-48)	July-August
AVDCC4	2F,7M	1-11	PVL	53 (44-57)	July-August
BOT	5F,10M	2-10	SD	52 (50-62)	August-September
PRET	3M	5	PVL	59 (45-80)	September-October
HESC	2F,6M	5	GD	55 (50-65)	September-October

F, female; M, male; PVL, public viewing lookout; SD, self-drive vehicle; GD, game drive vehicle; AVDCC1, Ann Van Dyke Cheetah Centre Pack 1; AVDCC2, Ann Van Dyke Cheetah Centre Pack 2; AVDCC3, Ann Van Dyke Cheetah Centre Pack 3; AVDCC4, Ann Van Dyke Cheetah Centre Pack 4; BOT, Bothongo Rhino & Lion Nature Reserve; PRET, SANBI National Zoological Gardens Pretoria; HESC, Hoedspruit Endangered Species Centre.

2.2. Treatments

Using a double-blinded placebo-controlled design, packs were exposed to two dog appeasing pheromone analogues, DAP, phDAP and a placebo which contained the same base solution but omitted the pheromone component (all IRSEA, Apt, France). All spray solutions were provided to the primary investigator as blinded treatments in identical bottles labelled A, B, F and H (Placebo), C and G (DAP) and E and D (phDAP), along with a blinded application protocol to ensure that either DAP or phDAP was correctly administered to each treatment site when required (Fig. 6.1). The composition of each labelled bottle was only revealed to the primary investigator after data collection and behavioural analyses were complete. African wild dog packs were exposed to pheromone analogues on different days with at least 3 rest days between to ensure changes in behaviours were specific to each treatment.

Each of two 1 m^2 areas within a pack's enclosure (separated by at least 3 m) was treated on days 4 and 23 (DAP *vs.* placebo) or days 8 and 27 (phDAP *vs.* placebo) of the observation period with 30 ml of either appeasing pheromone or a placebo treatment by spraying the treatment on the ground within a pre-selected area based on enclosure use so as to avoid common resting areas (Fig. 6.1). Treatments on days 23 and 27 were the same as on days 4 and 8 respectively except that the site of the specific pheromone/placebo combination were swapped to control for location bias.

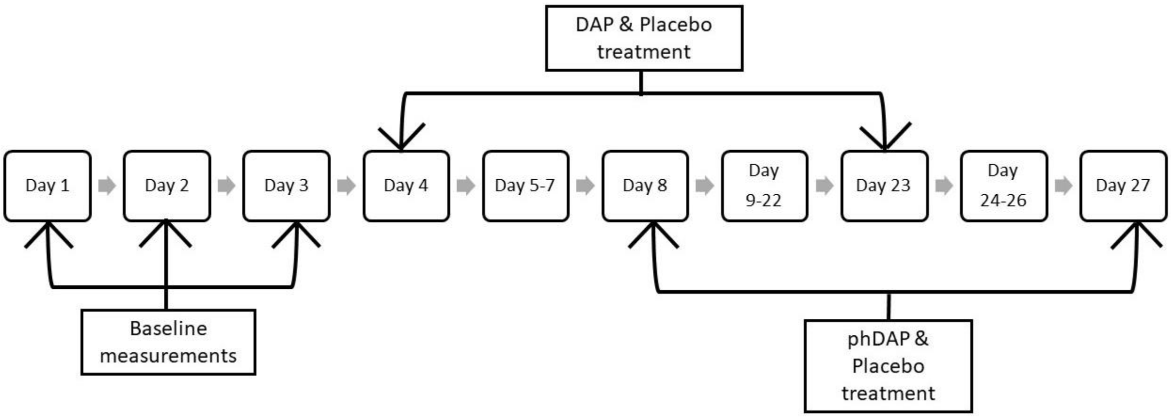


Figure 6.1. Behavioural observations and treatment schedule for each pack of captive African wild dogs (*Lycaon pictus*).

2.3. Behavioural observations

Study animals were video-recorded within their enclosures using a Sony FDR-AX100E 4K digital video camera from either a public viewing area or vehicle. Baseline rates of normal behaviours were observed for the initial first three days before treatments (Fig. 6.1). Thereafter, subsequent observations were performed on treatment Day 4, 8, 23 and 27 (Fig. 6.1), either immediately after pheromones were sprayed (all packs except PRET) or within 5 minutes of sprays being applied to the ground (PRET). Recordings were captured over a continuous one-hour period each day between 7:30 am and 3:00 pm depending on when animals were most active as determined by animal managers. Thereafter, both time and duration of recording remained consistent for each individual pack across all observation days in line with keeper routines.

The total rate of specific behaviours directed at or within one adult dog length of each treatment site were tallied during the one-hour observation period according to a modified ethogram derived from Vlamings (2011) and Van den Berghe *et al.,* (2019b; Table 6.2). The daily rate of vocalisations were then calculated as calls per minute for each day of recording.

Table 6.2. African wild dog (*Lycaon pictus*) ethogram showing social and investigative behaviours evaluated in our study. Modified from Vlamings (2011) and Van den Berghe et al., (2019b).

Behaviour	**Description**
Affiliation	
Greet conspecific	The actor stands or walks in parallel with the recipient, tries to contact their muzzle, and performs a complex set of behaviours, including food-solicit or inspection behaviour.
Groom conspecific	The actor licks and cleans the ears, face and/or body of the recipient.
Rub-on conspecific	The actor establishes intensive latero-lateral contact with the recipient. This may occur while both animals are in motion.
Dominance	
Dominance	Due to the low rate of dominant interactions during the observation period all dominant behaviours were categorised under one heading. These included actor engaging in: scruff orientation, stalk approach, food approach, intervention by approach, stand or threat, fixating, freezing, inguino-genital inspection, point, mount, stand-over position, approaches in high posture, and high posture snout or high posture face lick as defined by by Van den Berghe et al., (2019b) and summarised in Chapter 4, Table 4.2.
Area defence	The actor physically defends a treatment site using aggressive behaviours such as nipping and biting and/or growls to move recipient away from the site to maintain individual access.
Submission	
Submission	Due to low rate of submissive interactions during the observation period all submissive behaviours were categorised under one heading. These included: active or passive submission, approaching in low posture, low posture snout contact and present body as defined by Van den Berghe et al (2019b) and summarised in Chapter 4, Table 4.2.
Grin	This is the only clear facial expression shown by African wild dogs. The mouth corners remain retracted and the mouth may be held slightly open so that teeth become visible.
Play	Due to low rate of these interactions all play behaviours were categorised under one heading. These included: play solicitation, fur bite, play fighting, play chase, play wrestle, play sniff and social play as defined by Van den Berghe et al., (2019b).
Investigation	

Sniffing	Lowering of the head to an object and drawing up air audibly through the nose to detect an odour.
Digging	Breaking up or moving earth by scratching with paws and/or snout
Licking ground	Licks at the ground with the tongue, usually performed to increase detection of pheromones or particular odours.
Pheromone response	
Licking mandibular	Repetitive tongue and jaw movement, whereby the tongue is rapidly thrust out of the mouth and licks around the lips and lower jaw of the individual performing the action.
Yawns	Spontaneous action involving deep inspiration of air, opening the jaw wide and the rapid exhalation of the inspired air with jaw closing action.
Panting	Moderate to rapid open-mouthed respiration. Involves the animal opening jaw and the tongue hanging slightly forward out of the mouth with obvious respiration action in the chest.
Urine Marking	Purposeful urination (often containing pheromone components to signal an individual’s sex and hierarchy) on an object and/or the rubbing of the side of the face on an object (also with the purpose of excreting pheromone secretions onto an object).
Flehmen Response	Curling of the upper lip and chattering of teeth with the specific purpose of inspirating chemical odours through the vomeronasal organ and main olfactory epithelium for pheromone perception.
Rolling	Animal rolls onto its back with its legs in the air and rubs its body on the ground.
Defaecating	Squatting position and discharge of faeces from the body.
Rest	Individual lays alone or with pack members on the ground with head resting on the ground or on a pack mate without moving for longer than five seconds.

2.4. Statistical analysis

Due to the low rate of individual behaviours scored during the 1h observation period, behaviours were grouped and analysed within distinct categories (affiliative, dominant, submissive, play, investigation, pheromone response, and resting behaviours) as defined in Table 6.2. Moreover, due to concerns that behaviours, caused by inhalation of pheromones at the pheromone-treated site, could be delayed and exhibited at the placebo-treated site, the behaviours at both sites were pooled in daily counts. Comparison between pre-treatment (Days 1-3), DAP treatment (Days 4 and 23) and phDAP treatment (Days 8 and 27) was performed with a generalized linear mixed model for repeated measures (GLMM). In the GLMM model each behaviour category was compared between treatments (pre-treatment, DAP and phDAP). Random effects of pack and observation day were included in the model and pack size and observation time were included as offset terms. All GLMM's were run in Poisson distribution and dispersion was evaluated using the Pearson/x^2 criterion. If the Pearson/x^2 criterion was less than 2 the Poisson model was suitable for the data (submissive behaviours), if greater than 2 over dispersion had occurred and the negative binomial distribution was used (affiliative, dominant, play, investigation, pheromone investigation and resting behaviours). GLMMs were fitted by maximum likelihood with Laplace approximation. A two-step procedure was utilised to create parsimonious models, firstly, the Akaike's information criterion (AIC) was used to determine mean random-effects structure based on the full model containing all candidate fixed effects (pack, observation day, observation time, pack size, context, housing, behaviour categories, sex ratio), and then removed statistically insignificant fixed-effect terms (Zuur et al., 2009). Secondly, variables were pruned using a backward stepwise selection based on likelihood ratio tests (Zuur et al., 2009). All statistics were performed in RStudio (version 3.6.2) for Windows. Significance was set at $P \leq 0.05$ for all analyses.

3. Results

Rates of investigation were higher at both DAP and phDAP treatment sites than were observed pre-treatment (X^2=32.18, df=2, $P \leq 0.0001$), with DAP inducing significantly higher rates of investigation than phDAP (Fig. 6.2a). Similarly, rates of pheromone investigation tended to be higher at DAP treatment sites than were observed at both the phDAP site and during pre-treatment (approaching significance at X^2=5.74, df=2, P = 0.056; Fig. 6.2b). Unexpectedly, rates of play behaviour were significantly lower at phDAP treatment sites than were observed at both DAP sites and during pre-treatment (X^2=6.97, df=2, P = 0.031; Fig. 6.2e). Additionally, rates of affiliative behaviour tended to decline at DAP (intermediate) and phDAP (lowest) treatment sites than were observed pre-treatment (approaching significance at X^2=4.71, df=2, P = 0.098; Fig. 6.2f). No significant differences were observed between any treatment for rates of dominant (X^2=1.24, df=2, P = 0.538) or submissive behaviours (X^2=0.37, df=2, P = 0.832; Fig. 6.2c & d). Similarly, while rates of resting behaviour appeared to be reduced at DAP treatment sites, this was not significant (X^2=2.92, df=2, P = 0.233; Fig. 6.2g).

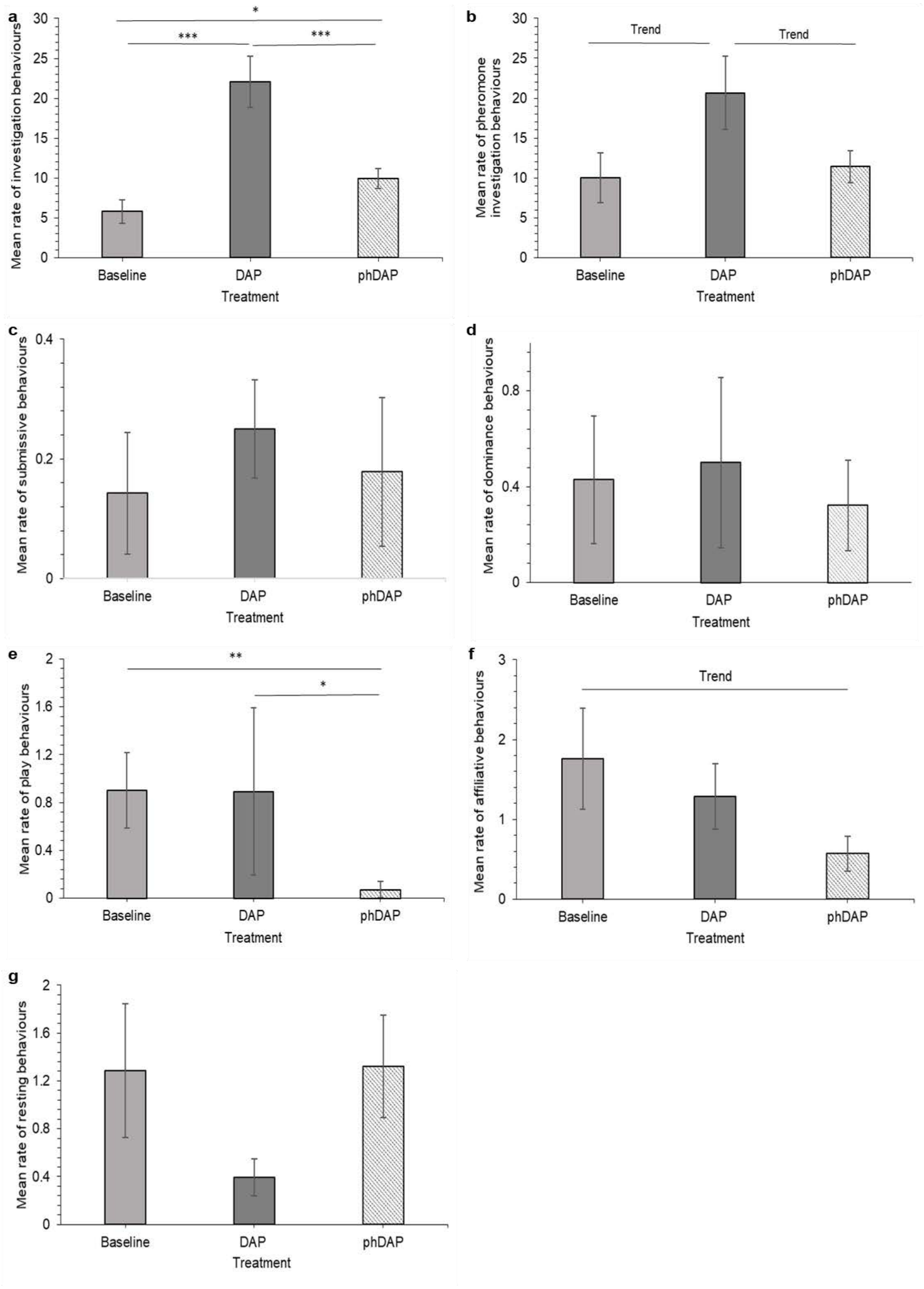


Figure 6.2. Mean ± SEM rate of a) investigative, b) pheromone investigation, c) submissive, d) dominant, e) play, f) affiliative, and g) resting behaviours within one adult dog length of treatment sites among seven captive packs of African wild dogs (n = 46) in South Africa. DAP, dog appeasing pheromone; phDAP, pharmacophore dog appeasing pheromone. Significance: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.0001$ between treatments.

4. Discussion

Appeasing pheromones have been proposed as a useful tool for the management of antisocial behaviours in African wild dogs during stressful interventions. Moreover, novel pheromone formulations that more closely mimic the composition of appeasing pheromones from the target species, are hypothesized to induce a more robust prosocial response. Our results in captive African wild dog packs suggest that exposure to dog appeasing pheromones in the environment can impact both investigative and some social behaviours. Significantly and near significantly higher rates of investigation and pheromone investigation respectively toward both pheromone treatments demonstrates that DAP and to a lesser extent phDAP can be readily perceived by African wild dogs. Unexpectedly however, this translated into a significant reduction in play and a trend of declining affiliative behaviours, particularly toward the phDAP treatment. Further research is needed to determine whether the mode of application (ground *vs.* skin), duration of exposure/observation (short *vs.* long), differences in stability (DAP *vs.* phDAP) or level of stress (high *vs.* low) might explain the more limited and counter-effective response to phDAP.

Investigation behaviours, which were most pronounced in the DAP treatment group, suggested that the DAP analogue spray was more easily detected in the environment compared to the phDAP analogue spray. The DAP pheromone composition was the same as a commercial product (Adaptil®, Ceva Sante Animale, Libourne, France) which has been shown to induce positive behavioural changes in domestic dogs using sprays, spot-on solutions and imbibed collars. The only difference was that the DAP formulation used here was supplied in a new enhanced carrier solution that improves longevity as a spot-on solution. The new phDAP has been formulated as a solution to be applied to the skin and fur and as such both pheromones may disperse more quickly in the environment than when supplied in previous carriers (Patrick Pageat, personal communication).

Naturally occurring appeasing pheromones contain a core chemical signature of a 1:1:1 fatty acid ratio of oleic, palmitic and linoleic acid across species in which this pheromone has been identified. Additional to this core appeasing message are other species-specific chemical components which differ between even closely-related species (Pageat and Gaultier, 2003). Chemoreceptors that are excited by pheromone perception must be activated in precise combinations to cause specific behavioural responses in animals (Wyatt, 2017). The complex chemical composition of pheromones can make determining exact active compounds important for recognition in different species difficult. Body secretions can contain multiple pheromones and elucidating which semiochemicals within these secretions are active components of the African wild dog appeasing pheromone is a complex task (Riddell et al., 2021). The appeasing pheromones tested in this experiment were synthesised based on the known structure of the domestic dog pheromone, with phDAP appearing to be an even more dog-specific analogue than DAP. This enhanced species-specificity of phDAP may explain why African wild dogs responded less favourably to this analogue.

More recently, semiochemical discovery has moved towards using approaches in which the binding ability of olfaction-related proteins are used to determine their potential to effect behaviours; this process is referred to as pharmacophore modelling (Liu et al., 2016). The phDAP analogue used in this experiment was developed using pharmacophore modelling to determine the precise chemoreceptor ability to dock with specific chemicals to create an optimised composition of the dog appeasing pheromone (Patrick Pageat, personal communication). A recent study provides evidence that neuronal clusters on the VNO epithelium within the vomeronasal system of the Iberian wolf are different to those of the domestic dog and are more highly differentiated within the accessory olfactory bulb (Ortiz-Leal et al., 2024). This suggests that domestic dogs may have different neuronal configurations to wild canids and thus may produce different species-specific behaviours in the presence of the same manufactured pheromones. As such, the pharmacophore developed phDAP may have produced a highly specific and specialised DAP analogue for the domestic dog, which may elicit a poorer response in African wild dogs due to activation of even fewer African wild dog olfactory receptors than the original DAP formulation. Development of a more species-specific appeasing pheromone would require further investigation including further exploration of the semiochemical components of the natural/pure African wild dog appeasing pheromone and further investigation into the singularities of the olfactory system of African wild dogs.

Given the potential for substantial physiological differences in the olfactory systems of African wild dogs and domestic dogs, unexpected decreases in play and affiliative behaviours observed in the current study may be explained. Our results showed that the phDAP caused a decrease in play behaviours compared to either DAP or pre-treatment rates. While there was a small reduction in these behaviours when exposed to the DAP treatment, this was not significantly different to pre-treatment rates of play. Additionally, in both pheromone treatments there was a non-significant decreasing trend in affiliative behaviours compared to pre-treatment rates. However, this appears more pronounced in relation to the phDAP treatment and increased sample size may see a significant decrease in affiliative behaviours when exposed to this treatment compared to pre-treatment rates. Our results suggest that when no stressor is present, dog appeasing pheromones (particularly phDAP) sprayed within the environment may have a small negative impact on social behaviours. Thus, there is a need to optimise artificial pheromone analogues for this species or to use analogues which are less species-specific.

Pheromones are classified as one of two classes: releasers or primers. Releaser pheromones are responsible for short-latency behavioural responses, whereas primer pheromones produce delayed neuroendocrine responses linked to hormonal, physiological and delayed behavioural changes (Tirindelli et al., 2009). The appeasing pheromone appears to act as both a releaser and primer pheromone, producing measurable changes in short-term behaviours such as investigation related behaviours and long-term stress and aggression mitigation related behaviours, which may occur in response to upregulation of prolactin and downregulation of androgen hormones after stressful interventions (Pageat and Gaultier, 2003; Siracusa et al., 2010; Van den Berghe et al., 2019b). As such, low pheromone dose, short length of pheromone exposure and relatively short observation periods may have limited the observed outcomes of this study.

The method of application of DAP to modify stress related behaviours may influence efficacy. Appeasing pheromones are naturally produced from glands on the body of animals and no evidence has shown they are expressed in markings left in the environment (Pageat and Gaultier, 2003). As such, social behavioural responses may be more pronounced when pheromones are applied on the body of individuals. Pheromone infused collars appear to produce a more marked response in behaviour changes than diffusers in studies performed over longer periods of time (Mills and Hargrave, 2004; Mills et al., 2006). Additionally, short-term exposure to DAP has been highlighted as a possible reason for a lack of significant results in a study involving a new gel formulation of dog appeasing pheromone applied during veterinary visits just prior to examination (Hermiston et al., 2018; Puglisi et al., 2022). In light of this, the method of pheromone deployment and the length of observations could all have been variables that impacted the strength of results in our current study. Going forward it may be more advantageous to measure both behavioural and physiological responses with an increased exposure time, controlled pheromone doses and undertake observations over longer periods of time to better determine appeasing pheromone actions.

On a positive note, despite reductions in play and affiliative behaviours neither dominance nor submission behaviours were negatively altered beyond levels normally exhibited, suggesting that phDAP does not disrupt behaviours important for pack stability. In a similar study, where African wild dogs were exposed to DAP and placebo treatments sprayed in captive enclosures, none of the measured behaviours were significantly different between treatments either within 5 minutes of exposure or at 1 hour post exposure (Vlamings, 2011). The lack of results here could be due to small sample sizes compared to our own study (*n*=10 *vs* *n*=46 respectively). Additionally, in the Vlamings (2011) study affiliative, dominant or submissive behaviours were not measured. By contrast, when the same treatments were applied to the skin and fur of pack members experiencing a stressful reintroduction event, DAP-treated animals did not show a rise in contact dominance above baseline compared to placebo-treated animals, suggesting that DAP modulates a rise in dominance behaviours during periods of acute stress in this species reducing the chance of increased aggression and increasing pack stability. Comparatively, DAP-treated animals appeared to shift their behaviour to a trend for higher non-contact dominance, while placebo-treated animals tended to have higher rates of contact dominance and active submission, though these results were not significant within or between treatments but were different to baseline (Van den Berghe et al., 2019b). Given the high levels of variation in the behavioural responses of animals upon exposure to appeasing pheromone treatments in our own and other studies, it is possible that the beneficial effects of these pheromones are only detectable statistically when animals are challenged with a significant stressor. Given that the action of appeasing pheromones is to reduce stress and aggression in dogs and other species, it might be difficult to determine its effects when stress is not present or is low. As such, we believe that ongoing optimisation and investigation into appeasing pheromones for the modification of stress and aggression in this species is warranted.

In conclusion, the DAP analogue thus far, elicits strong investigation related behaviours when no stressors are present, as well as reduction in behaviours and hormones associated with aggression during stressful interventions making it thus far the most promising appeasing pheromone for use in African wild dog management. From our results we cannot at this stage recommend the use of phDAP in African wild dog management due to potential concerns about decreases in play and affiliative behaviours. However, length of pheromone exposure, method of application and duration of observation and their effects on outcomes should be further investigated. Additional examination of the African wild dog olfactory system would provide great insight into the precise chemosensory receptors activated in this species and allow for further appeasing pheromone optimisation.

Chapter 7: General Discussion

Increasingly around the globe, populations of wildlife are becoming threatened, endangered or are on the brink of extinction, necessitating hands on management (Moehrenschlager & Somers, 2004). Development of species management methods, such as translocation, reintroduction and artificial pack formation, are of great additive importance (Moehrenschlager & Somers, 2004; Macdonald et al., 2015; Buk et al., 2018; Marneweck, et al., 2019a). These management approaches are highly relevant for species that face increased habitat fragmentation, human persecution, and limited natural dispersal opportunities, such as canids, that causes inbreeding and genetic decline, leading to reduced species fitness (Marneweck, et al., 2019a; Tensen et al., 2024). However, these management procedures come with inherent risks to animal welfare that include significant stress and aggression and its associated consequences on social cohesion and immune function (Van der Weyde, 2013; Van den Berghe et al., 2019b; Crossey et al., 2020).

Metapopulation management of the African wild dog in South Africa requires intensive translocation, artificial pack formation and reintroduction (Marneweck, et al., 2019a; Van den Berghe et al., 2019b; Riddell et al., 2021). Recent genetic modelling suggests that continued metapopulation management will require the translocation of at least two new animals every two years into each fragmented population to effectively maintain this population above 95% heterozygosity for the next 150 years (Tensen et al., 2024; Paris et al., 2024). However, animals experience significant stress and related social disunity during these management procedures (Riddell et al., 2021). As such, it is important to ensure animal health and welfare is monitored effectively throughout any interventions, which can be achieved through the measurement of changes in behaviour and physiology. However, non-invasive sample collection is often needed to avoid sampling stress associated with more traditional blood-based monitoring techniques (Van der Weyde et al., 2015; Crossey et al., 2018; Riddell et al., 2021; Paris et al., 2024). Development of non-invasive methods to measure the effects of stress and aggression on physiology and social cohesion are important to provide researchers and wildlife managers with tools to monitor animal health and welfare during management procedures. Additionally, it is important to develop strategies to reduce the experience of stress and aggression in this species. Despite evidence that the current commercial DAP analogue could be a useful tool to mitigate reduced social cohesion during management interventions, a new pharmacophore DAP (phDAP) has been developed that shows greater potential to manage stress and aggression behaviours in domestic dogs (Patrick Pageat, 2022, personal communication). As such, phDAP was thought to have the potential to be an even more potent tool for African wild dogs. Thus, this thesis aimed to develop tools to measure and improve immunity and social cohesion in African wild dogs for improved welfare during stressful but necessary ongoing human management of this species. This was achieved through the following objectives: (i) Develop a non-invasive, field-applicable assay for immune function (Chapter 3); (ii) Determine how African wild dog vocalisations change with different behaviours, contexts and housing (Chapter 4); (iii) Determine how African wild dog vocalisations change with season, stress and dog appeasing pheromone mitigation (Chapter 5); (iv) Compare the perception of DAP *vs.* phDAP by African wild dogs (Chapter 6).

Disease related mortality is high in African wild dogs, with 33% of deaths in free-roaming populations attributed directly to disease outbreaks (Creel & Creel, 1998; Woodroffe et al., 2007; Marneweck et al., 2022). However, immune monitoring is difficult with sample collection often causing stress to animals, which can disrupt pack cohesion making longitudinal study's difficult (Anderson, 2018). In Chapter 3 an enzyme-linked immunosorbent assay was developed to measure immunoglobulins in the faeces of African wild dogs and other canids. This assay now effectively removes the need to collect blood or saliva samples to measure immune function in wild canids, thus removing sampling stress and making repeated long-term monitoring of immune function possible in African wild dogs for the first time. Our results indicated that baseline fIgM concentrations were similar across species, but that fIgG is significantly lower in unvaccinated African wild dogs compared to vaccinated Australian dingoes and domestic dogs. Faecal immunoglobulin ELISA's have shown that age and sex do not impact faecal immunoglobulin concentrations in adult domestic dogs (Peters et al., 2004), but may be different in juvenile and adult dogs (German et al., 1998). However, faecal immunoglobulins may be different in vaccinated and unvaccinated domestic dogs (Decaro et al., 2004). Additionally, in this chapter it was shown that faecal immunoglobulins of African wild dogs are relatively stable post-defaecation meaning that immune function can be monitored in non-fresh samples collected from tracked animals in the field where collection of fresh samples is difficult. This removes the need to dart and obtain fresh blood samples from animals in real time, meaning sample collection could be delayed if the identity of the defaecating animal is known, or if combined with genotyping to link immune function and identity in unknown samples. Identification of wild individuals for the purpose of sampling could be improved by utilising camera traps at shared marking sites and latrines, which are known to be used by African wild dog packs with relative consistency to communicate between packs (Apps et al., 2021; Claase et al. 2022; 2024). This would allow for regular fresh sample collection from known individuals for immune monitoring in free-roaming packs.

Safe vaccinations have been developed to pathogens of concern; however these often appear to confer limited long-term protection in African wild dogs, with some translocated packs succumbing to pathogens soon after release despite vaccination (Burrows et al., 1994; Burroughs et al., 2002; Connolly et al., 2013, 2015; Anderson & Smith, 2019; Woodroffe, 2001, 2021; Mulreany et al., 2021). The seemingly limited long-term protection conferred by vaccination of African wild dogs, may be caused by animal stress during management, which may impact immune function and thus reduce vaccine efficacy and long-term immunity (Anderson & Smith, 2019; Riddell et al., 2021; Paris et al., 2024). Faecal immunoglobulin

ELISAs have shown that canine parvovirus and coronavirus infections can be diagnosed in domestic dog faecal IgM and IgG (Teramoto et al., 1984; Decaro et al., 2004). Furthermore, these assays have shown that vaccination related immunoglobulin changes can be measured in domestic dogs (Decaro et al., 2004). In Chapter 3 we also showed that the immune response to vaccinations can be measured in the faeces of canids, with a clear response to booster vaccinations able to be detected within 8 weeks of vaccination in domestic dogs and Australian dingoes. A similar response was observed for both canids in fIgM and fIgG, but an IgG rise occurred more rapidly after vaccination in dingoes. This may reflect a more robust and responsive immune system in captive dingoes compared to domestic dogs as a result of more semi-natural housing and greater potential exposure to environmental pathogens, but this would require further testing. These faecal immune assays can now be used to measure the effect of stress during management interventions on immune function and vaccination response in African wild dogs. Faecal immune assays coupled with faecal hormone and behaviour monitoring could be used to determine the association between elevated cortisol, stress behaviour and suppressed immune function in managed African wild dogs. These assays could be also used to determine if appeasing pheromones could improve immune function by reducing immune suppression through the alleviation of chronic stress and aggression. Additionally, these ELISAs could be used for longitudinal non-invasive monitoring of African wild dogs to determine the duration of immunity to CDV, rabies and CPV post-vaccination for the first time; giving vets insight into appropriate vaccination intervals for the species (Anderson & Smith, 2019).

Reduced social cohesion in captive African wild dogs' results in increased contact dominance and aggressive behaviours (Van den Berghe et al., 2019a; Van den Berghe et al., 2019b; Cock et al., 2023;), with 23% of mortalities and 62% of morbidities occurring as a result of aggression within packs (Cock et al., 2023). Despite evidence in other canids that changes in the rate and acoustic parameters of vocalisations can indicate changes in emotional states (Faragó et al., 2010; Déaux & Clarke, 2013; Déaux et al., 2016; Quervel-Chaumette et al., 2016; Pongrácz et al., 2017; Marx et al., 2021), little is known about how African wild dogs use vocalisations to communicate.

In Chapter 4, we investigated how African wild dogs use vocalisations with pro- and anti-social behaviours, in different contexts, and between traditional *vs.* open-range zoos *vs.* free-ranging packs. We found that much of the African wild dog vocal repertoire is used during both pro- and anti-social behaviours. However, anti-social behaviours are often associated with vocalisation that have an increased rate, longer syllables and lower frequencies than vocalisations emitted during pro-social behaviours. Furthermore, during anti-social contexts, such as human and animal threats that were often tightly linked with anti-social behaviours, higher rates of vocalisations occurred which had longer syllables and lower frequency. Finally, captive enclosures in which natural behaviours were restricted, such as the inability of young adult offspring to disperse, caused an increased in the range and rate of anti-social vocalisations, which had longer syllables and lower frequencies.

These finding are in keeping with the motivational-structural model which postulates that emotional states influence vocalisation production, with negative emotional states inducing low frequency vocalisations and positive emotional states inducing high frequency vocalisations (Marx et al., 2021), as observed in domestic dogs (Faragó et al., 2010). African wild dogs held in long-term zoo-based or temporary captivity during metapopulation management, have increased rates of faecal glucocorticoids compared to free-ranging packs (Crossey et al., 2020). As such, it is unsurprising that the vocalisations of captive packs appear to indicate a more negative emotional state. Additionally, threat barks, attack barks, full moans, alarm growls, buzz moans, attack twitters, social growls and alarm barks were only associated with anti-social behaviours and may be good indicators of negative emotional state. Our results coupled with those of previous studies suggest that rates of specific vocalisations, such as barks, growls, moans and hoo-calls may provide a sensitive tool for monitoring the effect of stress on social cohesion within African wild dogs (Robbins, 2000; Robbins & McCreery, 2003; Marneweck et al., 2019b), and our results further highlight that changes in syllable length and frequency of vocalisations appear to be good indicators of changes in emotional state, thus could be a good indicator of social cohesion during periods of increased stress.

Stressful interventions and the breeding season cause an increase in contact dominant, submissive and aggressive behaviours, which occur in parallel with rises in stress, aggression and reproductive hormone (Van den Berghe et al., 2019a; Van den Berghe et al., 2019b; Van den Berghe et al., 2023). In Chapter 5, we determined whether vocalisations change in response to stress, season and during the application of DAP to reduce stress and aggression during a stressful intervention. During the breeding season, anti-social vocalisations increased, had longer syllables and lower frequency, indicating an increased negative emotional state and potentially reduced social cohesion. This was in keeping with the results of Chapter 4 and observations in domestic dogs (Faragó et al., 2013). Similarly, during the stressful intervention event, placebo-treated animals experienced an increase in the rate of vocalisations, length of syllables and lowering of frequency. These observations further support the hypothesis that negative emotional states cause longer syllables and lower frequency in vocalisations (Faragó et al., 2013; Marx et al., 2021).

Unfortunately, the manual recording, extraction and acoustic analysis of African wild dog vocalisations is a timely and labour-intensive exercise—limiting its current potential for real-time diagnosis of social cohesion and welfare. Future efforts should aim to automate the process via artificial intelligence to detect, classify and analyse changes in vocalisation range, rate and acoustic parameters (Stowell, 2022). This would speed up the real-time monitoring of changes in social cohesion and animal welfare thereby enabling managers to respond proactively rather than reactively prior to significant social deterioration in packs (Clark & Dunn, 2022; Stowell, 2022). This tool could also be used to monitor and improve our knowledge about pack social dynamics during stressful translocations and artificial pack formations to improve welfare practices during management of this socially complex species (Jones et al., 2021; Clark & Dunn, 2022). Moreover, vocalisation monitoring could be used to detect subtle changes in pack dynamics in the lead-up to the breeding season or may even indicate other reproductive behaviours such as the onset of oestrous or parturition in this species (Jones et al., 2021; Clark & Dunn, 2022).

Appeasing pheromones have shown initial promise as an aggression management tool in African wild dogs, with synthetic DAP reducing aggression hormones and contact dominance during stressful interventions in captive packs (Van den Berghe et al., 2019b). In Chapter 5, it was shown that African wild dog packs treated with a DAP spot-on solution had reduced vocalisation rates in all vocalisations except social twitters, begging cries, mob twitters and moans, shortening of syllables in growls and social twitters and lower frequencies in spar twitters, begging cries, barks and growls compared to placebo-treated packs when experiencing stress. In a previous study, this same DAP-treatment also mitigated the rise in androgens associated with reintroduction of individuals to their pack (Van den Berghe et al., 2019b). In domestic dogs, reduced stress and aggression behaviours after DAP treatment is well documented (Gaultier et al., 2005; Mills et al., 2006; Landsberg et al 2015). Similarly, a reduction in the mean frequency of barks has been reported in shelter dogs treated with DAP (Hermiston et al., 2018). Interestingly, there is limited evidence that DAP effects cortisol in dogs during stressful interventions but does reduce prolactin concentrations (Siricusa et al., 2010; Riddell et al., 2021). However, when taken together, these positive changes in vocalisations, behaviours and hormone levels suggest that DAP is an effective tool to improve social cohesion during stressful management interventions in African wild dogs. Future studies should be performed to determine the effectiveness of DAP in reducing vocalisation rates and reduce the effect of stress on acoustic modulation, reduce anti-social behaviours and stress/aggression-related hormones in free-roaming packs during stressful translocation and artificial pack formation procedures associated with metapopulation management.

Lastly, the development of a new pharmacophore DAP analogue for domestic dogs raised the potential for us in Chapter 6 to test whether this new analogue would be positively perceived and induce an enhanced response in African wild dogs. During this blinded cross-over designed perception/response study, phDAP elicited less investigative and play behaviours than DAP when applied in the enclosures of African wild dogs. Play behaviour in response to phDAP-treatment was significantly lower than baseline, and a trend of decreased affiliative behaviours below baseline was also observed. As such, it would appear that phDAP elicits a less favourable appeasing pheromone response compared to DAP in African wild dogs. Furthermore, the reduction in play and affiliative behaviours induced by this pheromone was concerning and in contrast to our predictions. Pharmacophore developed pheromones are highly specific, with pheromone compositions determined according to how well volatile olfactory proteins bind to olfactory receptors (Liu et al., 2016). Evidence of differences in the neuronal clustering on the sensory epithelium of the VNO and differentiation of the accessory olfactory bulb between the Iberian wolf and domestic dog (Ortiz-Leal et al., 2024), could be a possible explanation as to why phDAP is not as well perceived by African wild dogs. African wild dogs genetically diverged from wolves around 1- 2 million years ago into their own genus and from domestic dogs 3 million years ago and as such are likely to have even less genetic similarities to the domestic dog (Girman et al., 1993). Thus, even though the core appeasing message is identical across multiple species (Table 2.2., Chapter 2; Riddell et al., 2021; Pageat et al., 2002), tweaks to the volatile chemicals in the core message coupled with changes to volatile species-specific components may have greatly diminished phDAP's ability to excite the olfactory receptors and by so doing, transcend into other species, even within the Canidae. It has been shown that the original, and some now might say, more generic domestic dog DAP is an effective behaviour modification tool which also reduces androgens during stress across species in African wild dogs (Vlamings, 2011; Van den Berghe et al., 2019b). Interestingly, other original appeasing pheromone formulations appears to have beneficial effects across several species. Rabbit appeasing pheromone collars applied to anxious domestic dog's modulated heart rate and behaviours as effectively as DAP (Thompson, 2013). Furthermore, application of domestic cat appeasing pheromones in the enclosures of tigers (*Panthera tigris*) and lions appeared to reduce head rubbing in both species and also spray marking behaviours in tigers (Dehnhard, 2011).

The method of application of appeasing pheromones may impact the measurable effect they have on animals. It would appear that appeasing pheromones applied to the skin and fur of animals elicit a stronger effect than their application to enclosures or in aerosol diffusers, especially during stress (reviewed in Riddell et al., 2021). Exposure of dogs to DAP through diffusers appear to have limited effect on reducing stress and aggression behaviours (Mills et al., 2006), but DAP collars do show a modulating effect on these behaviours (Mills & Hargave, 2004). One limitation to our study was that pheromones were only applied to the ground of African wild dog enclosures in the absence of stress. As such, future research should investigate whether phDAP would be more effective at mitigating stress and aggression in African wild dogs during a stressful intervention when applied directly to the animals itself. Additionally, using the recently published African wild dog genome sequence, it would be useful to compare the receptor sequences and neuronal clusters associated with the vomeronasal organ sensory epithelium and accessory olfactory bulb between domestic dogs and African wild dogs to explain the lack of efficacy of phDAP. Such sequence differences could help progress the development of an African wild dog-specific appeasing pheromone, which we believe could be more potent at alleviating stress and aggression in this species.

In conclusion, in this thesis several non-invasive species-specific tools have been developed and tested to monitor and improve the health and welfare of African wild dogs. The development of a faecal immunoglobulin assay allows for future studies to measure the impact of stress and aggression on immune function/suppression as well as the efficacy of vaccination in this species. Additionally, characterisation of African wild dog vocalisation changes across multiple contexts have shown that this is a sensitive parameter to detect changes in social unity. In the future, this could be coupled with automated artificial intelligence tools to monitor changes in African wild dog social cohesion in real-time, allowing for early detection of pack disunity or annulment by managers. Finally, the original DAP formulation was able to significantly improve vocal communication of African wild dogs during stressful interventions for the first time. By contrast, phDAP appears to be much more specific to domestic dogs, potentially precluding its use in distantly related canids like the African wild dog. Future research should test whether this analogue may still be as effective as the original DAP analogue under stressful interventions. However, it may prove more feasible to use the same bioinformatic pharmacophore approach to engineer an African wild dog-specific phAWDAP based on the recently available genome sequence for this species.

Appendices

Appendix 1: Supplementary Table 4.1: Mean ± SEM baseline daily rate, syllable length and fundamental frequency of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*).

Total daily rate of vocalisations

	Aggression	*Dominance*	*Submission*	*Feeding*	*Play*	*Affiliation*	*P value*
Social twitter	10.7 ± 3.8	11.7 ± 2.5	19.3 ± 2.3	32.3 ± 4.3	4.3 ± 1.0	19.7 ± 4.5	0.581
Spar twitter	7.0 ± 1.0	2.7 ± 0.7	7.3 ± 1.0	18.7 ± 3.6	1.7 ± 0.5	2.7 ± 0.6	0.330
Mob twitter	5.3 ± 1.7	9.0 ± 1.3	11.0 ± 2.9	24.7 ± 6.1	16.3 ± 3.7	3.0 ± 0.6	0.691
Full moan	6.3 ± 1.6	0.7 ± 0.1	1.0 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.7 ± 0.1	0.438
Begging squeal	2.3 ± 0.4	0.3 ± 0.1	11.3 ± 2.0	5.0 ± 1.1	0.7 ± 0.2	0.7 ± 0.1	0.174
Whistle squeal	0.7 ± 0	1.0 ± 0.2	6.7 ± 1.1	3.3 ± 0.5	4.0 ± 0.7	0.3 ± 0.1	0.656
Whimper	0.7 ± 0.7	11.7 ± 1.0	23.0 ± 3.1	30.0 ± 2.8	18.0 ± 4.1	5.7 ± 1.1	0.241
Begging cry	4.7 ± 1.1	0.7 ± 0.1	22.3 ± 5.3	11.3 ± 1.6	-	4.0 ± 0.5	0.088
Social growl	0.7 ± 0.2^{b}	8.7 ± 0.2^{a}	-	-	0.3 ± 0.1^{b}	-	**0.012**
Rumble	0.7 ± 0.2	2.0 ± 1.1	-	0.7 ± 0.1	-	0.3 ± 0.1	0.682
Clear bark	-	0.7 ± 0.2	0.3 ± 0.1	-	0.3 ± 0.1	-	0.435
Huff	-	-	0.7 ± 0.2^{b}	4.3 ± 0.7^{a}	-	0.7 ± 0.2^{b}	**0.021**
Sneeze	-	1.0 ± 1.0^{c}	2.3 ± 0.4^{c}	6.3 ± 1.0^{b}	-	7.0 ± 1.0^{a}	**0.015**
Yelp	-	0.7 ± 0.1	4.3 ± 1.2	4.0 ± 1.0	-	0.7 ± 0.1	0.604
Whine	-	1.0 ± 0.3	4.7 ± 1.1	5.3 ± 1.2	-	3.0 ± 0.4	0.416
Alarm bark	-	4.7 ± 0.8^{a}	0.3 ± 0.1^{b}	-	-	-	**0.041**
Alarm growl	-	2.3 ± 0.4^{a}	1.0 ± 0.2^{b}	-	-	-	**0.040**
Attack bark	1.7 ±0.5	78.0 ± 24.4	-	1.0 ± 0.3	-	-	0.459
Attack twitter	2.7 ± 0.7	5.3 ± 1.7	-	3.7 ± 1.2	-	-	0.497
Buzz moan	1.3 ± 0.4	2.7 ± 0.8	0.3 ± 0.1	-	-	-	0.605
Threat bark	3.0 ± 0.9	3.7 ± 0.6	-	-	-	-	0.419

Syllable length (s) of vocalisations

	Aggression	*Dominance*	*Submission*	*Feeding*	*Play*	*Affiliation*	*P value*
social twitter	0.5 ± 0.0^{a}	0.1 ± 0.0^{d}	0.2 ± 0.0^{c}	0.2 ± 0.0^{c}	0.3 ± 0.0^{b}	0.2 ± 0.0^{c}	**0.000**
spar twitter	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.389
mob twitter	0.5 ± 0.0^{a}	0.3 ± 0.0^{bc}	0.1 ± 0.0^{cd}	0.1 ± 0.0^{cd}	0.5 ± 0.0^{a}	0.2 ± 0.0^{c}	**0.000**
full moan	0.7 ± 0.1	1.0 ± 0.0	0.3 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.5 ± 0.2	0.561
begging squeal	0.7 ± 0.1^{a}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	0.7 ± 0.1^{a}	0.4 ± 0.1^{b}	**0.000**
whistle squeal	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.838
whimper	0.1 ± 0.0^{bc}	0.1 ± 0.0^{bc}	0.1 ± 0.0^{bc}	0.1 ± 0.0^{bc}	0.3 ± 0.0^{ab}	0.2 ± 0.1^{abc}	**0.000**
begging cry	0.7 ± 0.1^{a}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	-	0.5 ± 0.1^{ab}	**0.000**
social growl	0.5 ± 0.1	0.5 ± 0.1	-	-	0.8 ± 0.0	-	0.558
rumble	0.3 ± 0.0^{b}	0.3 ± 0.0^{b}	-	0.2 ± 0.0^{c}	-	0.9 ± 0.0^{a}	**0.000**
clear bark	-	0.1 ± 0.0	0.2 ± 0.0	-	0.6 ± 0.0	-	0.058
huff	-	-	0.3 ± 0.0^{a}	0.2 ± 0.0^{b}	-	0.1 ± 0.0^{b}	**0.011**
sneeze	-	0.2 ± 0.0^{b}	0.5 ± 0.2^{a}	0.2 ± 0.0^{b}	-	0.2 ± 0.0^{b}	**0.006**
yelp	-	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	-	0.1 ± 0.0	0.132
whine	-	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.2 ± 0.0^{ab}	-	0.3 ± 0.0^{a}	**0.021**
alarm bark	-	0.3 ± 0.1	0.1 ± 0	-	-	-	0.407
alarm growl	-	0.5 ± 0.2	0.4 ± 0.1	-	-	-	0.907

attack bark	0.1 ± 0.0	0.1 ± 0.0	-	0.1 ± 0.0	-	-	0.800
attack twitter	0.3 ± 0.1	0.1 ± 0.0	-	0.1 ± 0.0	-	-	0.107
buzz moan	0.7 ± 0.0 ^{a}	0.3 ± 0.0^{c}	0.5 ± 0.0 ^{b}	-	-	-	**0.000**
threat bark	02 ± 0.0	0.3 ± 0.1	-	-	-	-	0.153

Fundamental Frequency (kHz) of vocalisations

	Aggression	*Dominance*	*Submission*	*Feeding*	*Play*	*Affiliation*	*P value*
social twitter	2.9 ± 0.1^{c}	6.0 ± 0.7^{a}	5.0 ± 0.3^{ab}	4.7 ± 0.3^{ab}	4.3 ± 0.4^{abc}	3.9 ± 0.2^{bc}	**0.000**
spar twitter	4.8 ± 0.5	3.6 ± 0.3	3.9 ± 0.5	4.4 ± 0.2	5.2 ± 0.5	4.3 ± 0.8	0.400
mob twitter	3.1 ± 0.2	3.1 ± 0.2	3.9 ± 0.3	4.2 ± 0.2	4.3 ± 0.3	4.8 ± 0.8	0.143
full moan	2.0 ± 0.1	1.4 ± 0.6	1.8 ± 0.2	2.0 ± 1.6	1.6 ± 0.4	1.9 ± 0.5	0.858
begging squeal	2.2 ± 0.1	4.6 ± 0.0	3.8 ± 0.3	3.6 ± 0.6	1.8 ± 0.1	4.7 ± 1.2	0.132
whistle squeal	2.7 ± 1.5	4.8 ± 1.2	3.1 ± 0.3	4.2 ± 0.5	3.5 ± 0.5	5.4 ± 0.0	0.359
whimper	1.9 ± 0.3	4.0 ± 0.4	3.8 ± 0.3	5.8 ± 0.3	4.9 ± 0.2	6.8 ± 0.4	0.714
begging cry	2.1 ± 0.2	1.9 ± 0.2	3.3 ± 0.2	2.8 ± 0.2	-	3.0 ± 0.4	0.881
social growl	0.8 ± 0.3^{a}	0.4 ± 0.1^{a}	-	-	0.0 ± 0.0^{b}	-	**0.000**
rumble	0.4 ± 0.1^{b}	0.5 ± 0.0^{b}	-	0.6 ± 0.3^{ab}	-	0.6 ± 0.0^{a}	**0.000**
clear bark	-	0.7 ± 0.1^{c}	13.7 ± 0.0^{a}	-	2.4 ± 0.0^{b}	-	**0.000**
huff	-	-	1.5 ± 0.9^{b}	2.3 ± 0.4^{a}	-	1.9 ± 0.1^{ab}	**0.022**
sneeze	-	3.6 ± 0.2	3.2 ± 0.7	3.6 ± 0.4	-	3.8 ± 0.3	0.769
yelp	-	1.7 ± 0.0^{b}	3.2 ± 0.3^{a}	2.8 ± 0.3^{a}	-	4.3 ± 1.2^{a}	**0.018**
whine	-	2.0 ± 0.5^{b}	3.1 ± 0.6^{a}	2.6 ± 0.5^{ab}	-	3.6 ± 0.7^{a}	**0.000**
alarm bark	-	6.0 ± 0.1^{a}	3.3 ± 0.1^{b}	-	-	-	**0.000**
alarm growl	-	0.9 ± 0.2^{a}	0.3 ± 0.0^{b}	-	-	-	**0.014**
attack bark	0.8 ± 0.3	0.4 ± 0.0	-	2.3 ± 0.4	-	-	0.918
attack twitter	4.5 ± 0.8	0.6 ± 0.2	-	4.0 ± 0.5	-	-	0.467
buzz moan	0.5 ± 0.2	2.6 ± 0.2	1.1 ± 0.0	-	-	-	0.437
threat bark	0.9 ± 0.1	0.8 ± 0.1	-	-	-	-	0.500

$^{a,\ b,\ c,}$ values not sharing the same letter differ significantly between behaviour categories within a specific vocalisation type.

Appendix 2: Supplementary Table 4.2: Mean ± SEM daily rate, syllable length and fundamental frequency of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*) within the context of feeding, social interaction, animal threat and human threat.

Behaviour category	***Daily rate***							***Syllable length (s)***							***Fundamental frequency (kHz)***						
	Agg	*Dom*	*Sub*	*Feed*	*Play*	*Aff*	***P***	*Agg*	*Dom*	*Sub*	*Feed*	*Play*	*Aff*	***P***	*Agg*	*Dom*	*Sub*	*Feed*	*Play*	*Aff*	***P***
Social twitter																					
Feeding	-	8.0 ± 1.1^{1}	17.6 ± 1.1^{1}	32.3 ± 2.4	-	12.0 ± 1.5^{1}	0.052	-	0.1 ± 0.0	0.2 ± 0.0^{2}	0.2 ± 0.0	-	0.2 ± 0.0	0.113	-	7.9 ± 0.8^{a1}	4.8 ± 0.3^{b2}	4.7 ± 0.3^{b}	-	4.0 ± 0.3^{c1}	**0.000**
Social interaction	-	3.0 ± 0.5^{2}	1.3 ± 0.2^{2}	-	1.3 ± 0.3	6.0 ± 0.9^{2}	0.626	-	0.1 ± 0.0^{b}	0.4 ± 0.1^{a1}	-	0.3 ± 0.0^{a}	0.1 ± 0.0^{b}	**0.000**	-	4.2 ± 0.4^{b1}	6.9 ± 1.2^{a1}	-	4.3 ± 0.4^{b}	4.7 ± 0.4^{b1}	**0.042**
Animal threat	10.7 ± 1.7	-	-	-	-	-	-	0.5 ± 0.0	-	-	-	-	-	-	2.9 ± 0.1	-	-	-	-	-	-
Human threat	-	3.7 ± 0.6^{2}	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	0.1 ± 0.0	0.495	-	1.9 ± 0.4^{a2}	-	-	-	0.5 ± 0.2^{b2}	**0.037**
P value	-	***0.000***	***0.000***	-	-	***0.000***		-	*0.199*	***0.000***	-	-	*0.292*		-	***0.000***	***0.045***	-	-	***0.000***	
Spar twitter																					
Feeding	4.3 ± 0.7^{1}	2.7 ± 0.4	4.3 ± 0.4^{1}	15.3 ±1.5	-	1.3 ± 0.1	0.203	0.1 ± 0.0^{2}	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	-	0.2 ± 0.0	0.052	6.1 ± 0.6^{a1}	3.6 ± 0.3^{c}	4.4 ± 0.5^{b1}	4.4 ± 0.2^{b}	-	3.6 ± 0.4^{c}	**0.019**
Social interaction	-	-	1.7 ± 0.2^{2}	-	1.7 ± 0.3	1.3 ± 0.2	0.615	-	-	0.2 ± 0.0	-	0.3 ± 0.0	0.2 ± 0.0	0.152	-	-	2.0 ± 0.4^{2}	-	5.2 ± 0.5	5.0 ± 1.5	0.064
Animal threat	2.7 ± 0.4^{2}	-	-	-	-	-	-	0.4 ± 0.0^{1}	-	-	-	-	-	-	2.7 ± 0.1^{2}	-	-	-	-	-	-
Human threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	***0.000***	-	***0.000***	-	-	*0.443*		***0.000***	-	*0.931*	-	-	*0.703*		***0.000***	-	***0.034***	-	-	*0.476*	
Mob twitter																					
Feeding	-	0.7 ± 0.1^{3}	9.3 ± 1.5^{1}	24.7 ± 3.2	-	1.0 ± 0.2^{2}	0.312	-	0.2 ± 0.0^{2}	0.1 ± 0.0	0.1 ± 0.0	-	0.2 ± 0.0^{1}	0.066	-	2.8 ± 0.3	4.2 ± 0.3^{1}	4.2 ± 0.2	-	1.8 ± 0.1^{2}	0.126
Social interaction	-	3.3 ± 0.5^{2}	1.7 ± 0.2^{2}	-	16.3 ± 2.0	2.0 ± 0.3^{1}	0.218	-	0.4 ± 0.0^{b1}	0.1 ± 0.0^{c}	-	0.5 ± 0.0^{a}	0.1 ± 0.0^{c2}	**0.000**	-	3.8 ± 0.3^{bc}	2.0 ± 0.8^{c2}	-	4.3 ± 0.3^{b}	6.3 ± 0.3^{a1}	**0.002**

Animal threat	5.3 ± 0.9	-	-	-	-	-	-	0.5 ± 0.0	-	-	-	-	-	-	3.1 ± 0.2	-	-	-	-	-	-
Human threat	-	5.0 ± 0.6^{1}	-	-	-	-	-	-	0.2 ± 0.0^{2}	-	-	-	-	-	-	2.8 ± 0.3	-	-	-	-	-
P value	-	***0.000***	***0.000***	-	-	***0.000***		-	***0.000***	*0.773*	-	-	***0.028***		-	*0.057*	***0.017***	-	-	***0.005***	
Full moan																					
Feeding	0.3 ± 0.1^{2}	0.3 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	-	0.3 ± 0.1	0.652	0.2 ± 0.0^{b}	1.0 ± 0.0^{a}	0.3 ± 0.1^{b}	0.6 ± 0.2^{b}	-	0.2 ± 0.0^{b}	**0.000**	1.3 ± 0.0	2.2 ± 0.0	1.8 ± 0.2	2.0 ± 0.2	-	1.1 ± 0.0	0.078
Social interaction	-	-	-	-	0.7 ± 0.1	0.3 ± 0.1	-	-	-	-	-	0.5 ± 0.1	0.8 ± 0.0	-	-	-	-	-	1.6 ± 0.4	2.7 ± 0.0	-
Animal threat	6.0 ± 0.9^{1}	-	-	-	-	-	-	0.7 ± 0.1	-	-	-	-	-	-	2.0 ± 0.1	-	-	-	-	-	-
Human threat	-	0.3 ± 0.1	-	-	-	-	-	-	0.9 ± 0.0	-	-	-	-	-	-	0.6 ± 0.0	-	-	-	-	-
P value	***0.050***	-	-	-	-	-		*0.466*	-	-	-	-			*0.093*	-	-	-	-		
Begging squeal																					
Feeding	-	-	8.7 ± 1.0^{1}	5.0 ± 0.6	-	-	0.213	-	-	0.3 ± 0.0	0.2 ± 0.0	-	-	0.337	-	-	3.8 ± 0.3	3.6 ± 0.6	-	-	0.745
Social interaction	-	0.3 ± 0.0	2.7 ± 0.4^{2}	-	0.7 ± 0.0	-	0.326	-	0.2 ± 0.0^{b}	0.2 ± 0.0^{b}	-	0.7 ± 0.0^{a}	-	**0.003**	-	4.6 ± 0.0	3.8 ± 0.5	-	1.8 ± 0.0	-	0.114
Animal threat	2.0 ± 0.2	-	-	-	-	-	-	0.7 ± 0.1	-	-	-	-	-	-	2.2 ± 0.1	-	-	-	-	-	-
Human threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	-	***0.000***	-	-	-		-	-	*0.744*	-	-	-		-	-	*0.986*	-	-	-	
Whistle squeal																					
Feeding	0.7 ± 0.1	0.3 ± 0.1^{2}	4.7 ± 0.5^{1}	3.3 ± 0.3	-	-	0.164	0.1 ± 0.0	0.3 ± 0.3	0.3 ± 0.0	0.2 ± 0.0	-	-	0.363	0.7 ± 1.5	0.3 ± 2.0	4.7 ± 0.4	3.3 ± 0.5	-	-	0.199
Social interaction	-	1.0 ± 0.1^{1}	2.0 ± 0.2^{2}	-	3.7 ± 0.4	0.3 ± 0.1	0.163	-	0.3 ± 0.1	0.2 ± 0.1	-	0.2 ± 0.0	0.1 ± 0.1	0.433	-	0.7 ± 0.3	3.7 ± 0.5	-	3.5 ± 0.5	5.4 ± 5.4	0.106
Animal threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

P value	**-**	***0.002***	***0.001***	-	-	-		-	*0.927*	*0.181*	-	-	-		-	*0.098*	*0.070*	-	-	-	
Whimper																					
Feeding	-	3.3 ± 0.3^{c2}	11.3 ± 1.4^{b1}	30.0 ± 1.6^{a}	-	2.0 ± 0.2^{c2}	**0.039**	-	0.1 ± 0.0^{b2}	0.1 ± 0.0^{b3}	0.1 ± 0.0^{b}	-	0.2 ± 0.0^{a}	**0.012**	-	4.0 ± 1.0^{b1}	4.0 ± 0.4^{b}	5.8 ± 0.3^{ab}	-	6.4 ± 1.0^{a}	**0.001**
Social interaction	-	0.7 ± 0.1^{3}	6.0 ± 0.4^{2}	-	15.7 ± 2.2	3.3 ± 0.4^{1}	0.349	-	0.2 ± 0.1^{1}	0.2 ± 0.2^{2}	-	0.3 ± 0.0	0.3 ± 0.1	0.133	-	9.7 ± 9.7^{ab123}	3.0 ± 0.7^{b}	-	4.9 ± 0.2^{b}	7.1 ± 0.3^{a}	**0.000**
Animal threat	0.7 ± 0.1	4.3 ± 0.5^{1}	4.0 ± 0.6^{3}	-	-	-	0.355	0.1 ± 0.0^{c}	0.2 ± 0.0^{b1}	0.3 ± 0.0^{a1}	-	-	-	**0.000**	1.9 ± 0.3	4.7 ± 0.3^{2}	4.2 ± 1.0	-	-	-	0.078
Human threat	-	3.7 ± 0.3^{12}	-	-	-	-	-	-	0.1 ± 0.0^{2}	-	-	-	-	-	-	2.5 ± 0.4^{3}	-	-	-	-	-
P value	-	***0.000***	***0.000***	-	-	***0.000***		-	***0.007***	***0.000***	-	-	*0.625*		-	***0.011***	*0.385*	-	-	*0.797*	
Begging cry																					
Feeding	0.3 ± 0.1^{2}	0.3 ± 0.1	19.0 ± 2.7^{1}	11.3 ± 0.9	-	0.3 ± 0.1^{2}	0.299	0.3 ± 0.0	0.4 ± 0.0	0.3 ± 0.0^{1}	0.3 ± 0.0	-	0.2 ± 0.0	0.818	1.9 ± 0.0	1.6 ± 0.0	3.3 ± 0.2	2.8 ± 0.2	-	2.7 ± 0.0	0.083
Social interaction	-	0.3 ± 0.1	2.7 ± 0.3^{2}	-	-	2.3 ± 0.2^{1}	0.059	-	0.3 ± 0.0^{b}	0.1 ± 0.0^{c2}	-	-	0.5 ± 0.1^{a}	**0.012**	-	2.2 ± 0.0	2.1 ± 0.3	-	-	3.1 ± 0.5	0.268
Animal threat	4.3 ± 0.6^{1}	-	-	-	-	-	-	0.7 ± 0.1	-	-	-	-	-	-	2.1 ± 0.2	-	-	-	-	-	-
Human threat	-	-	-	-	-	0.3 ± 0.1^{2}	-	-	-	-	-	-	0.5 ± 0.0	-	-	-	-	-	-	2.5 ± 0.0	
P value	***0.000***	-	***0.000***	-	-	***0.000***		*0.441*	-	***0.030***	-	-	*0.718*		*0.863*	-	*0.098*	-	-	*0.936*	
Social growl																					
Feeding	-	-	-	-	0.3 ± 0.1	-	-	-	-	-	-	0.8 ± 0.0	-	-	-	-	-	-	0.0 ± 0.0	-	-
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	-	0.3 ± 0.1^{12}	-	-	-	-	-	-	0.4 ± 0.1	-	-	-	-	-	-	0.6 ± 0.4	-	-	-	-	-
Human threat	0.7 ± 0.1	7.0 ± 0.6^{1}	-	-	-	-	0.053	0.5 ± 0.1	0.6 ± 0.1	-	-	-	-	0.693	0.8 ± 0.3	0.4 ± 0.1	-	-	-	-	0.139
P value	-	***0.002***	-	-	-	-		-	*0.310*	-	-	-	-		-	*0.380*	-	-	-	-	
Rumble																					

Feeding	0.3 ± 0.1	-	-	0.7 ± 0.1	-	-	-	0.3 ± 0.0	-	-	0.2 ± 0.0	-	-	-	0.5 ± 0.0	-	-	0.3 ± 0.3	-	-	-
Social interaction	-	-	-	-	-	0.3 ± 0.1	-	-	-	-	-	-	0.9 ± 0.0	-	-	-	-	-	-	0.6 ± 0.0	-
Animal threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human threat	0.3 ± 0.1	2.0 ± 0.3	-	-	-	-	-	0.3 ± 0.0	0.3 ± 0.0	-	-	-	-		0.3 ± 0.0	0.5 ± 0.0	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-		
Clear bark																					
Feeding	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Social interaction	-	-	-	-	0.3 ± 0.1	-	-	-	-	-	-	0.6 ± 0.0	-	-	-	-	-	-	2.4 ± 0.0	-	-
Animal threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human threat	-	0.7 ± 0.1	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	0.7 ± 0.1	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Huff																					
Feeding	-	-	0.7 ± 0.1^{b}	5.3 ± 0.3^{a}	-	0.7 ± 0.1^{b}	**0.000**	-	-	0.3 ± 0.0^{a}	0.2 ± 0.0^{a}	-	0.1 ± 0.0^{b}	**0.011**	-	-	1.5 ± 0.9	2.3 ± 0.4	-	1.9 ± 0.1	0.769
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Sneeze																					
Feeding	0.3 ± 0.1	-	1.3 ± 0.2^{1}	5.0 ± 0.5	-	0.7 ± 0.1^{2}	0.107	0.0 ± 0.0	-	0.4 ± 0.0	0.2 ± 0.0	-	0.3 ± 0.0	0.176	3.0 ± 3.0	-	2.6 ± 0.5	3.7 ± 0.4	-	4.0 ± 0.6	0.533
Social interaction	-	-	0.3 ± 0.1^{2}	-	-	3.0 ± 0.3^{1}	-	-	-	0.8 ± 0.0	-	-	0.2 ± 0.0	-	-	-	5.6 ± 0.0	-	-	4.0 ± 0.6	-

Animal threat	-	0.3 ± 0.1	-	-	-	-	-	-	0.2 ± 0.0	-	-	-	-	-	-	3.2 ± 0.0	-	-	-	-	-
Human threat	-	0.3 ± 0.1	-	-	-	4.7 ± 0.6^{1}	-	-	0.2 ± 0.0	-	-	-	0.3 ± 0.0	0.497	-	3.8 ± 0.3	-	-	-	3.6 ± 0.3	0.832
P value	-	-	***0.000***	-	-	***0.007***		-	-	*0.382*	-	-	*0.568*		-	-	*0.103*	-	-	*0.824*	
Yelp																					
Feeding	-	-	3.7 ± 0.5^{1}	4.0 ± 0.5	-	0.3 ± 0.1	0.426	-	-	0.1 ± 0.0	0.1 ± 0.0	-	0.1 ± 0.0	0.085	-	-	3.5 ± 0.3	2.8 ± 0.3	-	5.9 ± 0.0	0.126
Social interaction	-	-	0.7 ± 0.1^{2}	-	-	0.3 ± 0.1	-	-	-	0.1 ± 0.0	-	-	0.1 ± 0.0	-	-	-	1.9 ± 0.5	-	-	0.6 ± 0.0	-
Animal threat	-	0.3 ± 0.1	-	-	-	-	-	-	0.2 ± 0.0	-	-	-	-	-	-	1.7 ± 0.0	-	-	-	-	-
Human threat	-	0.3 ± 0.1	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	1.7 ± 0.0	-	-	-	-	-
P value	-	-	***0.001***	-	-	-		-	-	*0.836*	-	-	-		-	-	*0.065*	-	-	-	
Whine																					
Feeding	-	-	6.7 ± 0.6^{a1}	5.3 ± 0.5^{b}	-	0.3 ± 0.1^{2}	**0.000**	-	-	0.1 ± 0.0	0.2 ± 0.0	-	0.1 ± 0.1	0.119	-	-	2.9 ± 0.5	2.6 ± 0.5	-	5.7 ± 5.7	0.663
Social interaction	-	-	1.7 ± 0.2^{2}	-	-	2.0 ± 0.2^{1}	0.208	-	-	0.1 ± 0.0	-	-	0.3 ± 0.1	0.099	-	-	3.5 ± 1.1	-	-	3.5 ± 0.8	0.967
Animal threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human threat	-	1.0 ± 0.2	-	-	-	0.3 ± 0.1^{2}	-	-	0.1 ± 0.0	-	-	-	0.2 ± 0.2	-	-	2.0 ± 0.5	-	-	-	2.7 ± 2.7	-
P value	-	-	***0.017***	-	-	***0.001***		-	-	*0.332*	-	-	*0.640*		-	-	*0.642*	-	-	*0.602*	
Alarm bark																					
Feeding	-	-	0.3 ± 0.1	-	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	3.3 ± 0.0	-	-	-
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	-	1.7 ± 0.3	-	-	-	-	-	-	0.9 ± 0.0^{1}	-	-	-	-	-	-	0.3 ± 0.0^{2}	-	-	-	-	-

Human threat	-	4.3 ± 0.3	-	-	-	-	-	-	0.3 ± 0.1^{2}	-	-	-	-	-	-	0.5 ± 0.1^{1}	-	-	-	-	-
P value	-	*0.399*	-	-	-	-		-	***0.043***	-	-	-	-		-	***0.000***	-	-	-	-	
Alarm growl																					
Feeding	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Human threat	-	2.7 ± 0.2	1.0 ± 0.1	-	-	-	0.132	-	0.5 ± 0.2	0.4 ± 0.1	-	-	-	0.907	-	0.9 ± 0.2	0.3 ± 0.0	-	-	-	0.143
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Attack bark																					
Feeding	-	0.3 ± 0.1^{2}	-	1.0 ± 0.2	-	-	-	-	0.1 ± 0.0	-	0.1 ± 0.0	-	-	-	-	0.8 ± 0.0	-	2.3 ± 0.4	-	-	-
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	-	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	0.8 ± 0.3	-	-	-	-	-	-
Human threat	-	78 ± 12.6^{1}	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	0.4 ± 0.0	-	-	-	-	-
P value	-	***0.000***	-	-	-	-		-	*0.947*	-	-	-	-		-	*0.409*	-	-	-	-	
Attack twitter																					
Feeding	-	-	-	3.7 ± 0.6	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	4.1 ± 0.5	-	-	-
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	1.0 ± 0.1	-	-	-	-	-	-	0.3 ± 0.1	-	-	-	-	-	-	4.5 ± 0.8	-	-	-	-	-	-
Human threat	-	5.3 ± 0.9	-	-	-	-	-	-	0.1 ± 0.0	-	-	-	-	-	-	0.7 ± 0.2	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Buzz moan																					
Feeding	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Social interaction	-	2.7 ± 0.4	0.3 ± 0.1	-	-	-	-	-	0.3 ± 0.0	0.5 ± 0.0	-	-	-	-	-	2.6 ± 0.2	1.1 ± 0.0	-	-	-	-
Animal threat	1.3 ± 0.2	-	-	-	-	-	-	0.7 ± 0.0	-	-	-	-	-	-	0.5 ± 0.2	-	-	-	-	-	-

Human threat	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Threat bark																					
Feeding	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Social interaction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Animal threat	-	0.3 ± 0.1^{2}	-	-	-	-	-	-	1.2 ± 0.0^{1}	-	-	-	-	-	-	0.3 ± 0.0	-	-	-	-	-
Human threat	3.0 ± 0.5	3.3 ± 0.3^{1}	-	-	-	-	0.990	0.2 ± 0.0	0.3 ± 0.0^{2}	-	-	-	-	0.090	0.9 ± 0.1	0.8 ± 0.1	-	-	-	-	0.570
P value	**-**	***0.004***	-	-	-	-		-	***0.000***	-	-	-	-		-	*0.148*	-	-	-	-	-

Agg, aggression; Dom, dominance; Sub, submission; Feed, feeding; Aff, affiliation. ^{a,b,c} denotes a significant difference between behaviour categories within a context for a particular vocalisation type. ^{1,2,3} denotes a significant difference between contexts within a behaviour category for a particular vocalisation type.

Appendix 3: Supplementary Table 4.3: Mean ± SEM daily rate, syllable length and fundamental frequency of vocalisations associated with different categories of behaviour in African wild dogs (*Lycaon pictus*) of different captive status.

Behaviour category	***Daily rate***							***Syllable length (s)***							***Fundamental frequency (kHz)***						
	Agg	Dom	Sub	*Feed*	*Play*	*Aff*	***P***	*Agg*	*Dom*	*Sub*	*Feed*	*Play*	*Aff*	***P***	*Agg*	*Dom*	*Sub*	*Feed*	*Play*	*Aff*	***P***
Social twitter																					
Traditional zoo	10.6 ± 3.4	-	-	-	4.3 ± 1.4	-	0.486	0.5 ± 0^{a}	-	-	-	0.3 ± 0^{b}	-	**0.000**	2.0 ± 0.1^{b}	-	-	-	4.3 ± 0.4^{a}	-	**0.000**
Open-range zoo	-	4.0 ± 3.3	5.3 ± 2.9^{2}	6.7 ± 4.3^{2}	-	2.0 ± 1.6^{2}	0.583	-	0.1 ± 0^{2}	0.1 ± 0^{2}	0.1 ± 0^{2}	-	0.1 ± 0	**0.000**	-	2.0 ± 0.4^{b2}	4.8 ± 0.4^{a}	4.3 ± 0.4^{a}	-	1.0 ± 0.5^{c2}	**0.000**
Free-roaming	-	7.7 ± 3.0	12.7 ± 2.2^{1}	25.7 ± 5.4^{1}	-	11.7 ± 4.0^{1}	0.117	-	0.2 ± 0^{1}	0.2 ± 0^{1}	0.2 ± 0^{1}	-	0.2 ± 0	**0.006**	-	7.8 ± 0.8^{1}	5.1 ± 0.3	4.8 ± 0.4	-	4.2 ± 0.2^{1}	**0.000**
P value	-	*0.894*	***0.000***	***0.000***	-	***0.000***		-	***0.150***	***0.017***	***0.007***	-	0.151		-	***0.000***	*0.630*	*0.551*	-	***0.000***	
Spar twitter																					
Traditional zoo	2.3 ± 0.7^{1}	0.3 ± 0.1	-	-	1.7 ± 0.5	-	0.602	0.4 ± 0.1^{1}	0.3 ± 0	-	-	0.3 ± 0	-	0.152	2.5 ± 0.2^{2}	4.3 ± 0	-	-	5.2 ± 0.5	-	**0.003**
Open-range zoo	0.3 ± 0.3^{c2}	-	3.0 ± 1.7^{b}	14.0 ± 5.7^{a}	-	-	**0.040**	0.1 ± 0^{2}	-	0.2 ± 0^{1}	0.2 ± 0	-	-	0.622	2.8 ± 0^{2}	-	3.7 ± 0.6	4.2 ± 0.2^{2}	-	-	0.447
Free-roaming	4.3 ± 2.0^{1}	2.3 ± 1.1	1.3 ± 0.5	1.3 ± 0.4	-	1.3 ± 0.4	0.819	0.1 ± 0^{b2}	0.2 ± 0^{a}	0.1 ± 0^{b2}	0.2 ± 0^{a}	-	0.2 ± 0^{a}	**0.001**	6.1 ± 0.6^{a1}	3.5 ± 0.3^{b}	4.3 ± 0.8^{b}	6.8 ± 0.2^{a1}	-	4.3 ± 0.8^{b}	**0.028**
P value	***0.017***	-	***0.000***	***0.000***	-	-		***0.000***	*0.341*	***0.014***	*0.981*	-	-		***0.000***	-	*0.544*	***0.005***	-	-	
Mob twitter																					
Traditional zoo	5.3 ± 1.7	6.0 ± 1.3	-	-	16.3 ± 3.7	-	0.184	0.5 ± 0	0.3 ± 0	-	-	0.5 ± 0	-	**0.017**	3.1 ± 0.2	3.4 ± 0.2	-	-	4.3 ± 0.3	-	**0.001**

Open-range zoo	-	2.3 ± 1.9	9.3 ± 7.6	24.0 ± 14.6	-	-	0.306	-	0.1 ± 0	0.1 ± 0	0.1 ± 0	-	-	0.144	-	2.6 ± 0.4	4.0 ± 0.3	4.2 ± 0.2	-	-	0.099
Free-roaming	-	0.7 ± 0.3	1.0 ± 0.5	0.7 ± 0.3	-	1.0 ± 0.5	0.198	-	0.2 ± 0	0.1 ± 0	0.3 ± 0	-	0.2 ± 0	0.063	-	2.8 ± 0.3	2.5 ± 1.1	5.2 ± 1.4	-	4.8 ± 0.9	0.466
P value	-	***0.000***	***0.000***	***0.000***		-		-	*0.077*	*0.291*	*0.883*	-	-		-	*0.184*	*0.196*	*0.184*	-	-	
Full moan																					
Traditional zoo	0.7 ± 0.2	-	-	-	-	-	-	0.9 ± 0.2	-	-	-	-	-	-	2.2 ± 0.3	-	-	-	-	-	-
Open-range zoo	5.3 ± 4.4	0.3 ± 0.3	0.3 ± 0.3	0.7 ± 0.5	-	0.3 ± 0.3^{1}	0.532	0.6 ± 0.1	0.9 ± 0.9	0.2 ± 0.2	0.6 ± 0.2	-	0.8 ± 0.8	0.837	1.8 ± 0.2	0.6 ± 0.6	2.1 ± 2.1	2.0 ± 0.2	-	2.7 ± 2.7	0.267
Free-roaming	0.3 ± 0.2	0.3 ± 0.2	0.7 ± 0.2	-	0.7 ± 0.2	0.3 ± 0.2^{2}	0.647	0.2 ± 0	1.0 ± 1.0	0.4 ± 0.4	-	0.5 ± 0.1	0.2 ± 0.2	0.073	1.3 ± 1.3	2.2 ± 2.2	1.6 ± 0.2	-	1.6 ± 0.4	1.1 ± 1.1	0.776
P value	***0.341***	-	-	-	-	-		*0.433*	-	-	-	-	-		*0.606*	-	-	-	-	-	
Whistle squeal																					
Traditional zoo	-	-	-	-	0.3 ± 0.1^{2}	-	-	-	-	-	-	0.7 ± 0	-	-	-	-	-	-	3.3 ± 0	-	-
Open-range zoo	-	-	4.7 ± 3.0	1.0 ± 0.8	0.7 ± 0.5^{2}	0.3 ± 0.3	0.342	-	-	0.2 ± 0	0.3 ± 0.1	0.2 ± 0	0.1 ± 0	0.712	-	-	2.2 ± 0.2^{d} ^{2}	3.0 ± 0^{b}	2.8 ± 0.2^{c}	5.4 ± 0^{a}	**0.012**
Free-roaming	0.7 ± 0.3	0.3 ± 0.2	1.3 ± 0.4	2.3 ± 0.6	3.0 ± 1.0^{1}	-	0.278	0.1 ± 0	0.3 ± 0	0.2 ± 0	0.2 ± 0	0.2 ± 0	-	0.763	2.7 ± 1.5	4.8 ± 1.2	4.7 ± 0.4^{1}	4.7 ± 0.7	3.7 ± 0.6	-	0.513
P value	**-**	-	*0.226*	*0.688*	***0.000***	-		-	-	*0.790*	*0.605*	*0.545*	-		-	-	***0.000***	*0.157*	*0.568*	-	
Begging squeal																					
Traditional zoo	1.3 ± 0.4	-	-	-	0.7 ± 0.2	-	0.546	0.8 ± 0.1^{1}	-	-	-	0.7 ± 0.1	-	0.839	2.4 ± 0.1^{1}	-	-	-	1.8 ± 0.1	-	0.072

Open-range zoo	0.7 ± 0.5	-	7.0 ± 4.9	4.3 ± 2.8	-	0.3 ± 0.3	0.370	0.5 ± 0^{2}	-	0.2 ± 0^{2}	0.2 ± 0^{2}	-	0.3 ± 0	**0.024**	1.9 ± 0.1^{2}	-	3.7 ± 0.4	2.9 ± 0.2^{2}	-	3.0 ± 0	0.218
Free-roaming	0.3 ± 0.2	0.3 ± 0.2	1.7 ± 0.5	0.7 ± 0.4	-	0.3 ± 0.2	0.195	0.2 ± 0^{3}	0.2 ± 0	0.3 ± 0^{1}	0.4 ± 0^{1}	-	0.5 ± 0	0.128	2.2 ± 0^{2}	4.6 ± 0	4.0 ± 0.4^{b}	9.0 ± 0.5^{a1}	-	6.4 ± 0	**0.003**
P value	***0.741***	-	*0.729*	*0.397*	-	*0.280*		***0.000***	-	***0.000***	***0.043***	-	-		*0.043*	-	*0.221*	*0.028*	-	-	
Whimper																					
Traditional zoo	-	4.0 ± 0.8^{1}	5.7 ± 1.4^{1}	3.6 ± 0.9^{3}	15.7 ± 4.2^{1}	0.7 ± 0.1	0.418	-	0.2 ± 0^{1}	0.3 ± 0^{1}	0.3 ± 0^{1}	0.3 ± 0	0.2 ± 0.1^{2}	**0.000**	-	5.6 ± 0.5^{1}	4.2 ± 0.3	7.7 ± 0.5^{1}	4.8 ± 0.2	6.4 ± 0.5	**0.000**
Open-range zoo	0.7 ± 0.5	1.3 ± 1.1^{2}	9.7 ± 6.7^{1}	8.3 ± 4.0^{2}	-	0.7 ± 0.5	0.410	0.1 ± 0^{b}	0.1 ± 0^{b2}	0.1 ± 0^{b2}	0.1 ± 0^{b2}	-	0.3 ± 0^{a1}	**0.002**	1.9 ± 0.3	1.7 ± 0.2^{3}	3.7 ± 0.4	3.9 ± 0.5^{2}	-	5.8 ± 0.3	0.095
Free-roaming	-	3.3 ± 0.8^{b1}	1.7 ± 0.8^{b2}	18.0 ± 3.0^{a1}	2.3 ± 1.1^{b2}	1.7 ± 0.5^{b}	**0.016**	-	0.1 ± 0^{b2}	0.1 ± 0^{b2}	0.1 ± 0^{b2}	0.3 ± 0^{a}	0.2 ± 0.1^{a2}	**0.026**	-	3.7 ± 0.7^{c2}	3.8 ± 0.7^{c}	6.2 ± 0.3^{b1}	5.6 ± 0.8^{b}	7.2 ± 0.5^{a}	**0.000**
P value	-	***0.000***	***0.000***	***0.000***	***0.000***	*0.455*		-	***0.028***	***0.000***	***0.009***	*0.978*	***0.000***		-	***0.002***	*0.810*	***0.000***	***0.871***	*0.551*	
Begging cry																					
Traditional zoo	3.7 ± 1.2^{1}	-	2.3 ± 0.5^{2}	-	-	1.3 ± 0.7	0.567	0.8 ± 0.2^{1}	-	0.3 ± 0	-	-	0.6 ± 0.1^{1}	0.144	2.3 ± 0.2^{b}	-	4.0 ± 0.4^{a}	-	-	2.6 ± 0.6^{b}	**0.037**
Open-range zoo	0.7 ± 0.5^{2}	-	17.3 ± 13.7^{1}	7.0 ± 2.9	-	0.3 ± 0.3	0.365	0.5 ± 0.1^{2}	-	0.3 ± 0	0.3 ± 0	-	0.3 ± 0^{2}	0.559	1.0 ± 0.3	-	3.2 ± 0.2	2.8 ± 0.3	-	3.9 ± 0.8	0.106
Free-roaming	0.3 ± 0.2^{2}	0.3 ± 0.2	1.7 ± 0.4^{2}	4.3 ± 1.5	-	1.0 ± 0.5	0.268	0.3 ± 0^{3}	0.3 ± 0	0.3 ± 0.1	0.3 ± 0.1	-	0.2 ± 0.1^{3}	0.941	1.9 ± 0	1.9 ± 0.2	3.7 ± 0.7	2.9 ± 0.2	-	1.1 ± 0.3	0.246
P value	***0.000***	-	***0.000***	*0.571*		*0.862*		***0.601***	-	*0.830*	*0.803*	-	***0.039***		*0.124*	-	*0.339*	*0.777*	-	*0.549*	
Social growl																					
Traditional zoo	0.7 ± 0.2^{b}	6.0 ± 1.2^{a1}	-	-	-	-	**0.013**	0.5 ± 0.1^{1}	0.7 ± 0.1^{1}	-	-	-	-	0.432	0.8 ± 0.3	0.4 ± 0.1	-	-	-	-	0.085

Open-range zoo	2.3 ± 1.0^{a}	-	-	-	0.3 ± 0.3^{b}	-	**0.039**	0.3 ± 0.1^{2}	-	-	-	0.8 ± 0	-	-	0.6 ± 0.3	-	-	-	0.0 ± 0	-	-
Free-roaming	-	0.3 ± 0.2^{2}	-	-	-	-	-	-	0.3 ± 0^{2}	-	-	-	-	-	-	0.6 ± 0.1	-	-	-	-	-
P value	0.635	***0.008***	-	-	-	-		*0.476*	***0.013***	-	-	-	-		*0.221*	*0.254*	-	-	-	-	
Rumble																					
Traditional zoo	0.7 ± 0.2	2.0 ± 0.6	-	-	-	-	0.523	0.3 ± 0	0.3 ± 0	-	-	-	-	0.436	0.4 ± 0.1	0.5 ± 0	-	-	-	-	0.213
Open-range zoo	-	-	-	0.3 ± 0.3	-	0.3 ± 0.3	-	-	-	-	0.9 ± 0	-	0.6 ± 0	-	-	-	-	0.2 ± 0	-	1.1 ± 0	-
Free-roaming	-	-	-	0.3 ± 0.2	-	-	-	-	-	-	0.2 ± 0	-	-	-	-	-	-	0.2 ± 0	-	-	-
P value	-	-	-	-	-	-	-	-	-	-	-	-	-		-	-	-	-	-	-	
Clear bark																					
Traditional zoo	-	-	-	-	0.3 ± 0.1	-	-	-	-	-	-	0.6 ± 0	-	-	-	-	-	-	2.4 ± 0	-	-
Open-range zoo	-	0.7 ± 0.5	0.3 ± 0.3	-	-	-	-	-	0.1 ± 0	0.2 ± 0.2	-	-	-	-	-	0.7 ± 0.1	13.6 ± 13.6	-	-	-	-
Free-roaming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	-
Huff																					
Traditional zoo	-	-	-	1.0 ± 0.2	-	-	-	-	-	-	0.2 ± 0	-	-	-	-	-	-	2.0 ± 0.9	-	-	-
Open-range zoo	-	-	0.7 ± 0.5	1.0 ± 0.8	-	-	0.561	-	-	0.3 ± 0	0.2 ± 0	-	-	0.068	-	-	1.5 ± 0.9	0.2 ± 0.1	-	-	0.287

Free-roaming	-	-	-	3.3 ± 0.6^{a}	-	0.7 ± 0.4^{b}	**0.025**	-	-	-	0.1 ± 0	-	0.1 ± 0	0.206	-	-	-	3.0 ± 0.3	-	1.9 ± 0.1	0.089
P value	-	-	-	*0.060*	-	-		-	-	-	*0.170*	-	-		-	-	-	***0.004***	-	-	
Sneeze																					
Traditional zoo	-	0.7 ± 0.1	-	0.3 ± 0.1	-	3.3 ± 0.9	0.354	-	0.2 ± 0	-	0.2 ± 0	-	0.3 ± 0^{1}	0.349	-	3.3 ± 0.1	-	4.3 ± 0	-	3.6 ± 0.2	0.605
Open-range zoo	-	-	2.0 ± 1.2	3.0 ± 2.4	-	1.3 ± 1.1	0.607	-	-	0.4 ± 0.2	0.2 ± 0	-	0.3 ± 0^{1}	0.195	-	-	3.1 ± 0.6	2.8 ± 0.8	-	4.6 ± 1.3	0.458
Free-roaming	0.3 ± 0.2	0.3 ± 0.2	-	1.7 ± 0.5	-	0.7 ± 0.2	0.284	0.0 ± 0	0.2 ± 0	-	0.2 ± 00	-	0.2 ± 0^{2}	0.060	3.0 ± 0	4.1 ± 0	-	4.1 ± 0.2	-	3.7 ± 0.3	0.353
Between context P value	-	-	-	*0.142*	-	*0.773*		-	-	-	*0.218*	**-**	***0.021***		-	-	-	*0.515*	-	*0.111*	
Yelp																					
Traditional zoo	-	0.3 ± 0.1	-	-	-	-	-	-	0.2 ± 0	-	-	-	-	-	-	1.7 ± 0	-	-	-	-	-
Open-range zoo	-	0.3 ± 0.3	3.7 ± 2.6	4.0 ± 2.5	-	0.3 ± 0.3	0.428	-	0.1 ± 0	0.1 ± 0	0.1 ± 0	-	0.1 ± 0	0.188	-	1.7 ± 0^{c}	3.5 ± 0.3^{b}	2.8 ± 0.3^{b}	-	5.9 ± 0^{a}	**0.021**
Free-roaming	-	-	-	-	-	0.3 ± 0.2	-	-	-	-	-	-	0.1 ± 0	-	-	-	-	-	-	2.6 ± 0	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Whine																					
Traditional zoo	-	-	1.3 ± 0.4	-	-	1.0 ± 0.2	0.427	-	-	0.1 ± 0	-	-	0.3 ± 0.1	0.219	-	-	4.3 ± 1.3	-	-	4.2 ± 0.6	0.955
Open-range zoo	-	1.0 ± 0.8	3.0 ± 2.4	1.0 ± 0.5	-	1.7 ± 1.4	0.637	-	0.1 ± 0	0.1 ± 0	0.1 ± 0	-	0.2 ± 0.1	**0.033**	-	2.0 ± 0.5	2.8 ± 0.5	1.4 ± 0.5	-	2.1 ± 1.0	0.595
Free-roaming	-	-	-	4.7 ± 1.5	-	0.3 ± 0.2	0.252	-	-	-	0.2 ± 0	-	0.1 ± 0	-	-	-	-	2.8 ± 0.5	-	5.2 ± 0	-

P value	-	-	*0.354*	***0.036***	-	*0.188*		-	-	*0.474*	*0.289*	-	*0.495*		-	-	*0.203*	*0.304*	-	*0.714*	
Alarm bark																					
Traditional zoo	-	2.7 ± 0.5	-	-	-	-	-	-	0.3 ± 0.1	-	-	-	-	-	-	0.8 ± 0.1	-	-	-	-	-
Open-range zoo	-	2.0 ± 1.2	0.3 ± 0.3	-	-	-	-	-	0.4 ± 0.1	0.1 ± 0.1	-	-	-	-	-	0.8 ± 0.1	3.3 ± 3.3	-	-	-	-
Free-roaming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	*0.142*	-	-	-	-		-	*0.378*	-	-	-	-		-	*0.077*	-	-	-	-	
Alarm growl																					
Traditional zoo	-	1.0 ± 0.2	0.3 ± 0.1	-	-	1.3 ± 0.7	0.567	0.8 ± 0.2^{1}	-	0.3 ± 0.0	-	-	0.6 ± 0.1^{1}	0.144	2.3 ± 0.2^{b}	-	4.0 ± 0.4^{a}	-	-	2.6 ± 0.6^{b}	**0.037**
Open-range zoo	-	1.3 ± 1.1	-	7.0 ± 2.9	-	0.3 ± 0.3	0.365	0.5 ± 0.1^{2}	-	0.3 ± 0.0	0.3 ± 0.0	-	0.3 ± 0.0^{2}	0.559	1.0 ± 0.3	-	3.2 ± 0.2	2.8 ± 0.3	-	3.9 ± 0.8	0.106
Free-roaming	-	-	0.7 ± 0.3	4.3 ± 1.5	-	1.0 ± 0.5	0.268	0.3 ± 0.0^{3}	0.3 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	-	0.2 ± 0.1^{3}	0.941	1.9 ± 0.0	1.9 ± 0.2	3.7 ± 0.7	2.9 ± 0.2	-	1.1 ± 0.3	0.246
P value	-	0.061	-	*0.571*		*0.862*		*0.601*	-	*0.830*	*0.803*	-	***0.039***		*0.124*	-	*0.339*	*0.777*	-	*0.549*	
Attack bark																					
Traditional zoo	-	0.3 ± 0.1	-	-	-	-	-	-	0.1 ± 0.1	-	-	-	-	-	-	0.8 ± 0.8	-	-	-	-	-
Open-range zoo	1.7 ± 1.4	76.0 ± 62.1	-	1.0 ± 0.8	-	-	0.465	0.1 ± 0	0.1 ± 0	-	0.1 ± 0	-	-	**0.009**	0.8 ± 0.3^{b}	0.4 ± 0^{b}	-	2.3 ± 0.4^{a}	-	-	**0.000**
Free-roaming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	-	-	-	-	-		-	*0.981*	-	-	-	-		-	*0.458*	-	-	-	-	
Attack twitter																					
Traditional zoo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Open-range zoo	0.3 ± 0.2	5.3 ± 4.4	-	3.7 ± 3.0	-	-	0.580	1.1 ± 0^{a}	0.1 ± 0^{b}	-	0.1 ± 0^{b}	-	-	**0.000**	1.0 ± 0.7^{b}	0.7 ± 0.2^{c}	-	4.0 ± 0.5^{a}	-	-	**0.000**
Free-roaming	2.3 ± 1.1	-	-	-	-	-	-	0.1 ± 0	-	-	-	-	-	-	5.0 ± 0.8	-	-	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Buzz moan																					
Traditional zoo	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Open-range zoo	1.3 ± 1.1	-	-	-	-	-	-	0.7 ± 0	-	-	-	-	-	-	0.5 ± 0.2	-	-	-	-	-	-
Free-roaming	-	2.7 ± 1.2	0.3 ± 0.2	-	-	-	0.484	-	0.3 ± 0	0.5 ± 0	-	-	-	-	-	2.6 ± 0.2	1.1 ± 0	-	-	-	-
P value	-	-	-	-	-	-		-	-	-	-	-	-		-	-	-	-	-	-	
Threat bark																					
Traditional zoo	3.0 ± 0.9	2.7 ± 0.6	-	-	-	-	0.426	0.2 ± 0^{a}	0.3 ± 0^{b}	-	-	-	-	**0.002**	0.9 ± 0.1	0.9 ± 0.1	-	-	-	-	0.674
Open-range zoo	-	1.0 ± 0.5	-	-	-	-	-	-	0.5 ± 0.3	-	-	-	-	-	-	0.6 ± 0.3	-	-	-	-	-
Free-roaming	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
P value	-	*0.571*	-	-	-	-		-	*0.410*	-	-	-	-		-	*0.239*	-	-	-	-	

Agg, aggression; Dom, dominance; Sub, submission; Feed, feeding; Aff, affiliation

^{a,b,c} denotes a significant difference between behaviour categories within an enclosure type for a particular vocalisation type. ^{1,2,3} denotes a significant difference between enclosure types within a behaviour category for a particular vocalisation type.

Appendix 4: Supplementary Table 5.1: Mean ± SEM baseline rate, frequency and syllable length of vocalisations (first three days prior to intervention) during the pre-breeding *vs*. breeding seasons in captive African wild dogs (*Lycaon pictus*).

Vocalisation	Pre-breeding season (n=13)	Breeding season (n=12)	*P* value
Vocalisation rate			
Hoo call	-	-	-
Moan	0.8 ± 0.6	3.0 ± 1.6	**0.000*****
Growl	0.3 ± 0.2	7.4 ± 3.4	**0.000*****
Bark	0.3 ± 0.2	3.5 ± 2.1	0.095
Rumble	0.3 ± 0.2	1.9 ± 1.4	**0.012***
Whine	2.6 ± 1.6	0.6 ± 0.4	**0.000*****
Yelp/squeal	8.6 ± 2.7	5.0 ± 1.7	**0.014***
Spar Twitter	13 ± 6.3	18.3 ± 8.0	0.944
Social Twitter	40.3 ± 20.3	80.8 ± 34.5	**0.000*****
Begging cry	8.8 ± 2.8	6.4 ± 2.0	**0.002***
Whimper	12.2 ± 2.7	8.6 ± 1.9	**0.000*****
Mob Twitter	8.3 ± 4.3	24.3 ± 9.4	**0.000*****
Fundamental frequency (kHz)			
Hoo call	-	-	-
Moan	2.5 ± 0.1	2.3 ± 0.4	**0.011***
Growl	1.1 ± 0.2	0.9 ± 0.1	0.118
Bark	1.1 ± 0.5	3.7 ± 0.3	**0.000*****
Rumble	1.0 ± 0.1	0.8 ± 0.1	0.417
Whine	5.8 ± 0.5	4.9 ± 1.1	**0.048***
Yelp/squeal	4.6 ± 0.2	4.0 ± 0.2	**0.041***
Spar Twitter	7.0 ± 0.3	6.8 ± 0.2	0.116
Social Twitter	6.5 ± 0.2	6.4 ± 0.2	0.265
Begging cry	3.5 ± 0.1	3.3 ± 0.1	**0.003***
Whimper	3.8 ± 0.2	4.7 ± 0.2	**0.030***
Mob Twitter	7.2 ± 0.7	6.4 ± 0.2	0.420
Syllable length (ms)			
Hoo call	-	-	-
Moan	580.2 ± 53.2	267.3 ± 60.3	0.131
Growl	286.3 ± 24.4	411.7 ± 24.1	0.706
Bark	112.3 ± 45.4	226.3 ± 19.9	**0.000*****
Rumble	559.3 ± 130.1	372.0 ± 48.5	**0.000*****
Whine	77.9 ± 18.7	237.2 ± 44.4	**0.008***
Yelp/squeal	304.9 ± 24.8	425.9 ± 30.6	**0.000*****
Spar Twitter	310.5 ± 31.5	147.5 ± 14.3	**0.000*****
Social Twitter	216.6 ± 18.1	129.5 ± 10.6	**0.000*****
Begging cry	461.8 ± 32.2	383.3 ± 28.5	0.333
Whimper	129.9 ± 9.1	175.7 ± 11.9	**0.035***
Mob Twitter	241.0 ± 13.2	238.2 ± 11.1	**0.000*****

* $P \leq 0.05$, ** $P \leq 0.01$, ** $P \leq 0.001$.

Appendix 5: Supplementary Table 5.2: Mean ± SEM rate, frequency and syllable length of vocalisations before, during and after stressful intervention in appeasing pheromone (DAP-; n = 11) *vs.* placebo-treated (n = 12) captive African wild dog (*Lycaon pictus*) packs.

Vocalisation	Treatment	Pre-SIR	SIR	***Post-SIR***	***p*** **value**
Vocalisation rate					
Hoo call	Placebo	0.0 ± 0.0^{b}	16.3 ± 12.7^{a}	0.0 ± 0.0^{b}	**0.000**
	DAP	0.0 ± 0.0^{b}	1.3 ± 1.1^{a}	0.0 ± 0.0^{b}	0.038^{*}
p value treatments		-	*0.998*	-	
Moan	Placebo	2.5 ± 1.7^{b}	17.5 ± 12.9^{a}	$6.2 \pm 2.6^{a,b}$	**0.026**
	DAP	2.5 ± 0.6b	6.0 ± 5.2^{a}	$2.8 \pm 0.9^{a,b}$	**0.000**
p value treatments		*0.364*	*0.211*	*0.149*	
Growl	Placebo	1.9 ± 1.7^{c}	17.8 ± 12.7^{a}	3.4 ± 1.4^{b}	**0.000**
	DAP	$3.3 \pm 2.1^{a,b}$	1.5 ± 1.3^{b}	1.0 ± 0.6^{c}	0.104
p value treatments		***0.000***	***0.000***	***0.000***	
Bark	Placebo	0.3 ± 0.2	8.3 ± 6.9	1.8 ± 0.7	0.838
	DAP	2.2 ± 0.3^{b}	0.0 ± 0.0^{b}	3.6 ± 1.8^{a}	**0.001**
p value treatments		*0.989*	***0.000***	*0.990*	
Rumble	Placebo	0.6 ± 0.3^{b}	2.0 ± 0.9^{a}	0.1 ± 0.1^{b}	**0.044**
	DAP	1.4 ± 0.6^{a}	0.0 ± 0.0^{b}	0.3 ± 0.3^{a}	**0.013**
p value treatments		*0.961*	***0.000***	*0.962*	
Whine	Placebo	2.6 ± 1.6^{b}	74.0 ± 61.5^{a}	0.3 ± 0.2^{b}	**0.000***
	DAP	1.0 ± 0.4^{b}	1.3 ± 0.8^{a}	0.7 ± 0.6^{b}	**0.000**
p value treatments		*0.695*	***0.000***	***0.000***	
Yelp/squeal	Placebo	8.8 ± 2.6^{a}	9.3 ± 4.0^{a}	3.0 ± 1.8^{b}	**0.000**
	DAP	7.2 ± 1.7^{b}	1.5 ± 1.0^{c}	29.8 ± 18.7^{a}	**0.000**
p value treatments		***0.001***	***0.000***	***0.003***	
Spar Twitter	Placebo	5.2 ± 1.6^{c}	8.3 ± 5.3^{b}	20.4 ± 7.3^{a}	**0.025**
	DAP	9.5 ± 8.5^{b}	6.5 ± 5.6^{c}	30.6 ± 14.0^{a}	**0.000**
p value treatments		***0.000***	***0.000***	***0.000***	
Social Twitter	Placebo	79.8 ± 34.4^{b}	209.0 ± 180.1^{a}	74.3 ± 24.3^{b}	**0.000**
	DAP	1.2 ± 17.7^{b}	25.5 ± 12.9^{b}	53.1 ± 27.9^{a}	**0.000**
p value treatments		***0.000***	***0.000***	***0.000***	

Begging cry	Placebo	9.3 ± 2.7^{c}	11.3 ± 4.6^{b}	20.3 ± 5.8^{a}	**0.016**
	DAP	8.3 ± 2.1^{b}	22.5 ± 11.9^{a}	8.8 ± 3.5^{b}	**0.000**
p value treatments		***0.038***	***0.000***	***0.000***	
Whimper	Placebo	10.4 ± 2.8^{b}	70.3 ± 46.0^{a}	10.3 ± 2.3^{b}	**0.000**
	DAP	11.2 ± 2.0^{b}	64.0 ± 28.5^{a}	8.8 ± 2.6^{b}	**0.000**
p value treatments		*0.700*	*0.774*	*0.332*	
Mob Twitter	Placebo	19.5 ± 9.4	10.0 ± 5.3	15.8 ± 6.3	0.756
	DAP	19.6 ± 5.9^{a}	5.6 ± 4.2^{c}	12.4 ± 4.5^{b}	0.213
p value treatments		*0.340*	*0.307*	*0.125*	
Fundamental frequency (kHz)					
Hoo call	Placebo	-	0.8 ± 0.0	-	-
	DAP	-	1.9 ± 0.0	-	-
p value treatments			***0.000***		
Moan	Placebo	2.1 ± 0.2	2.9 ± 0.1	2.8 ± 0.1	0.380
	DAP	2.5 ± 0.2	-	2.8 ± 0.2	0.303
p value treatments		*0.567*	*0.483*	*0.596*	
Growl	Placebo	0.6 ± 0.0^{c}	1.1 ± 0.1^{b}	1.7 ± 0.2^{a}	**0.000**
	DAP	1.0 ± 0.1	0.7 ± 0.0	1.7 ± 0.3	0.106
p value treatments		***0.001***	***0.021***	*0.782*	
Bark	Placebo	1.9 ± 0.2^{b}	1.2 ± 0.2^{b}	2.6 ± 0.3^{a}	**0.002**
	DAP	4.7 ± 0.3^{a}	-	2.0 ± 0.4^{b}	**0.001**
p value treatments		***0.006***	***0.005***	***0.006***	
Rumble	Placebo	1.6 ± 0.0^{a}	0.9 ± 0.1^{b}	0.7 ± 0.1^{b}	**0.000**
	DAP	0.8 ± 0.1^{c}	1.1 ± 0.1^{b}	2.2 ± 0.5^{a}	**0.000**
p value treatments		***0.007***	***0.011***	***0.024***	
Whine	Placebo	5.6 ± 0.5^{a}	2.6 ± 0.2^{b}	2.7 ± 0.2^{b}	**0.018**
	DAP	6.4 ± 2.2	9.2 ± 0.1	-	0.079
p value treatments		*0.444*	*0.309*	*0.444*	
Yelp/squeal	Placebo	5.3 ± 0.3^{a}	3.9 ± 0.3^{b}	3.3 ± 0.0^{c}	**0.000**
	DAP	3.7 ± 0.1^{b}	3.5 ± 0.2^{b}	4.7 ± 0.5^{a}	**0.027**
p value treatments		***0.006***	***0.003***	***0.015***	

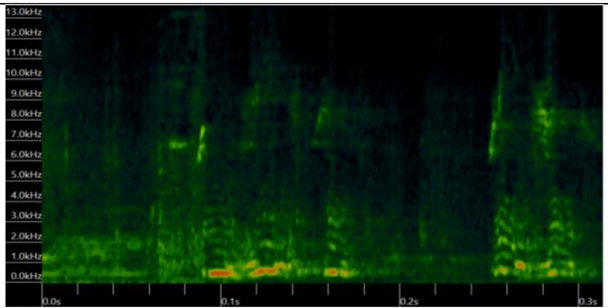
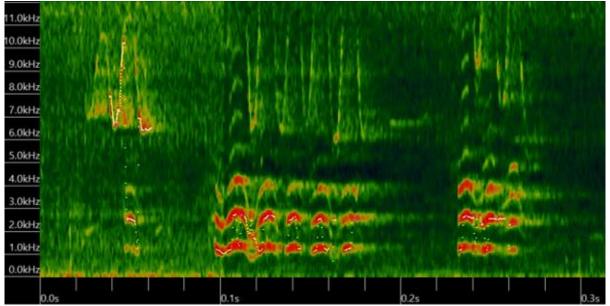
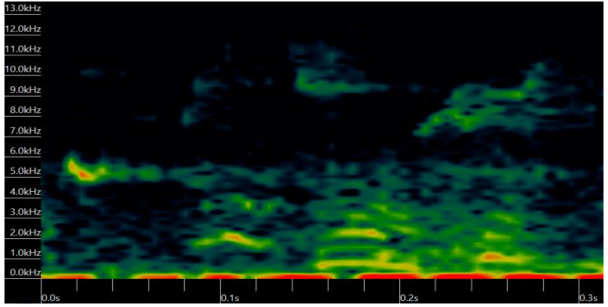
Spar Twitter	Placebo	7.0 ± 0.2	7.2 ± 0.3	6.5 ± 0.2	0.482
	DAP	6.6 ± 0.2^{b}	7.7 ± 0.5^{a}	7.4 ± 0.2^{a}	**0.002**
p value treatments		*0.060*	*0.061*	*0.575*	
Social Twitter	Placebo	14.8 ± 0.2	7.0 ± 0.3	6.2 ± 0.1	0.387
	DAP	6.8 ± 0.1	5.1 ± 0.1	6.4 ± 0.1	0.151
p value treatments		*0.099*	*0.725*	*0.074*	
Begging cry	Placebo	3.5 ± 0.1^{a}	2.9 ± 0.1^{b}	2.9 ± 0.1^{b}	**0.000**
	DAP	3.3 ± 0.1	2.6 ± 0.2	3.2 ± 0.1	0.368
p value treatments		*0.857*	***0.016***	*0.926*	
Whimper	Placebo	4.3 ± 0.2^{b}	5.5 ± 0.2^{a}	3.9 ± 0.3^{b}	**0.000**
	DAP	3.9 ± 0.1	6.3 ± 0.1	4.2 ± 0.2	0.446
p value treatments		*0.135*	*0.428*	***0.008***	
Mob Twitter	Placebo	5.7 ± 0.2	4.3 ± 0.7	6.4 ± 0.2	0.338
	DAP	6.9 ± 0.5^{a}	6.4 ± 0.2^{a}	5.8 ± 0.1^{b}	**0.002**
p value treatments		*0.813*	*0.353*	*0.306*	
			Syllable length (ms)		
Hoo call	Placebo	-	546.5 ± 46.0	-	-
	DAP	-	345.4 ± 27.7	-	-
p value treatments			*0.961*		
Moan	Placebo	436.4 ± 114.1	960.7 ± 52.8	898.8 ± 56.0	0.052
	DAP	481.8 ± 46.3	-	639.5 ± 89.3	0.202
p value treatments		*0.642*	*0.353*	*0.522*	
Growl	Placebo	395.0 ± 28.5^{b}	262.1 ± 52.6^{c}	819.8 ± 77.1^{a}	**0.003**
	DAP	365.2 ± 24.3	598.3 ± 30.8^{a}	517.1 ± 56.9	0.189
p value treatments		*0.500*	***0.000***	***0.000***	
Bark	Placebo	143.3 ± 24.2^{b}	761.9 ± 61.5^{a}	643.5 ± 99.1^{a}	**0.003**
	DAP	265.0 ± 20.1	-	259.0 ± 24.1	0.353
p value treatments		*0.246*	*0.239*	*0.246*	
Rumble	Placebo	633.0 ± 0.0	594.8 ± 242.8	410.5 ± 49.8	0.261
	DAP	414.0 ± 53.8	424.4 ± 46.7	581.3 ± 219.9	0.490
p value treatments		*0.284*	*0.273*	*0.703*	

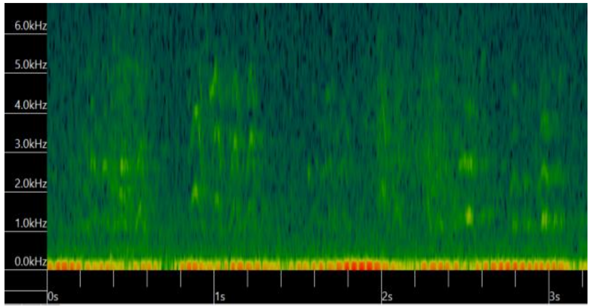
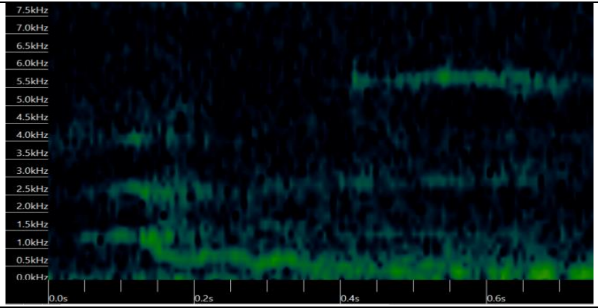
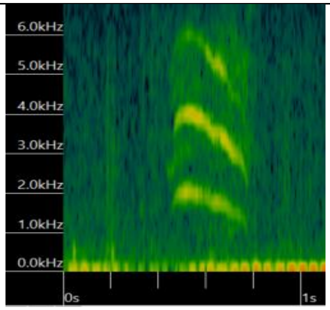
Whine	Placebo	79.1 ± 22.3^{c}	519.6 ± 103.7^{a}	225.2 ± 32.6^{b}	**0.005**
	DAP	305.5 ± 55.1	318.9 ± 13.3	-	0.126
p value treatments		*0.154*	*0.600*	*0.142*	
Yelp/squeal	Placebo	253.2 ± 37.5^{c}	474.8 ± 47.3^{b}	619.3 ± 25.7^{a}	**0.000**
	DAP	402.7 ± 19.8^{b}	463.0 ± 54.6^{a}	432.6 ± 57.3^{a}	**0.043**
p value treatments		*0.190*	*0.288*	*0.825*	
Spar Twitter	Placebo	218.5 ± 29.5	210.1 ± 33.9	277.9 ± 15.5	0.129
	DAP	206.7 ± 16.7^{b}	288.4 ± 76.2^{a}	203.5 ± 12.0^{b}	**0.039**
p value treatments		*0.164*	*0.484*	*0.565*	
Social Twitter	Placebo	454.5 ± 18.2^{a}	181.4 ± 30.4^{b}	130.1 ± 7.3^{c}	**0.000**
	DAP	154.4 ± 8.1^{b}	114.3 ± 32.5^{c}	183.3 ± 8.3^{a}	**0.038**
p value treatments		*0.652*	***0.017***	*0.870*	
Begging cry	Placebo	456.3 ± 33.7	644.9 ± 56.5	358.7 ± 23.0	0.158
	DAP	390.9 ± 27.1	508.0 ± 156.3	380.2 ± 20.1	0.397
p value treatments		*0.728*	*0.625*	*0.824*	
Whimper	Placebo	151.2 ± 10.2^{c}	181.2 ± 12.5^{b}	183.2 ± 11.8^{a}	**0.000**
	DAP	142.7 ± 9.9^{c}	295.1 ± 7.2^{a}	196.9 ± 8.2^{b}	**0.046**
p value treatments		***0.038***	*0.211*	*0.223*	
Mob Twitter	Placebo	229.9 ± 16.6	251.3 ± 64.7	270.1 ± 28.1	0.213
	DAP	237.8 ± 10.1^{b}	263.3 ± 23.2^{a,b}	273.1 ± 6.6^{a}	**0.036**
p value treatments		*0.589*	*0.865*	*0.777*	

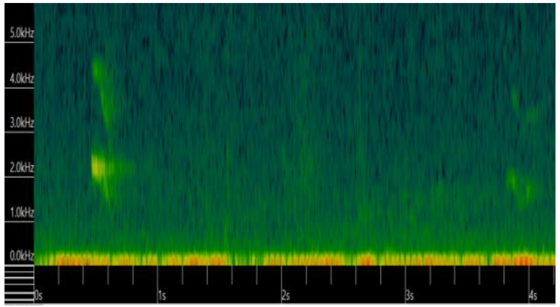
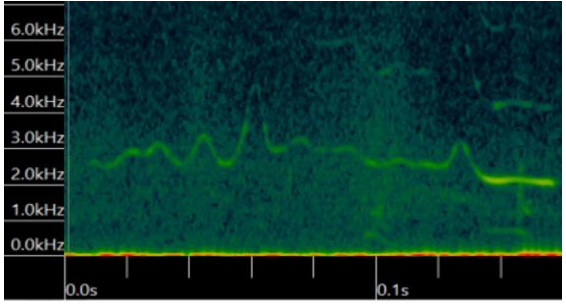
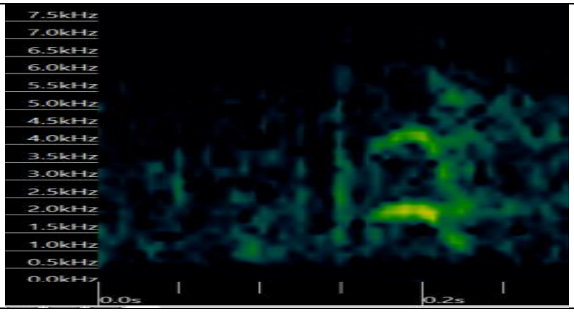
^{a,b,c} significant differences over time within a treatment

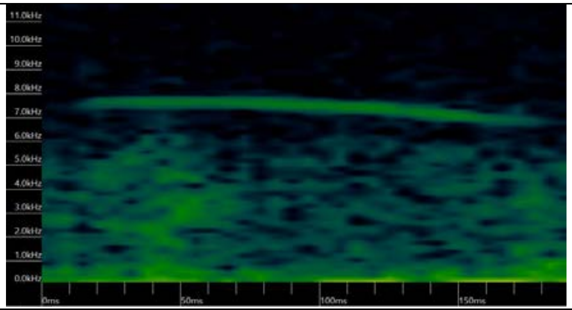
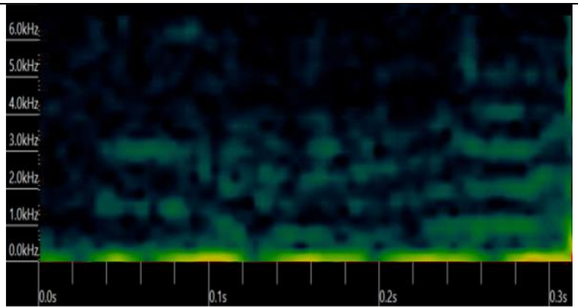
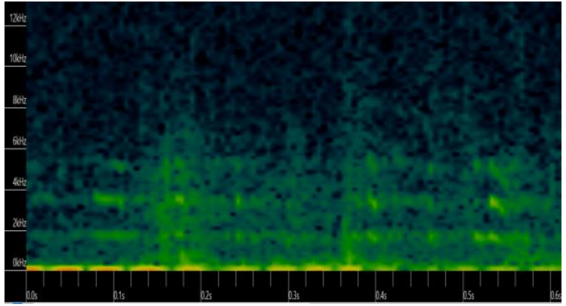
^{1,2} significant differences between treatments within a time point

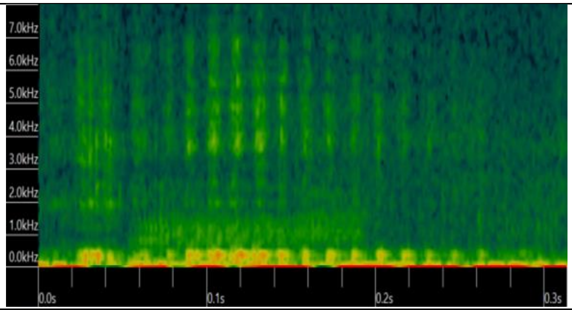
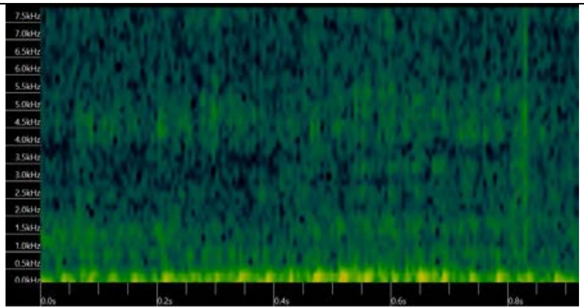
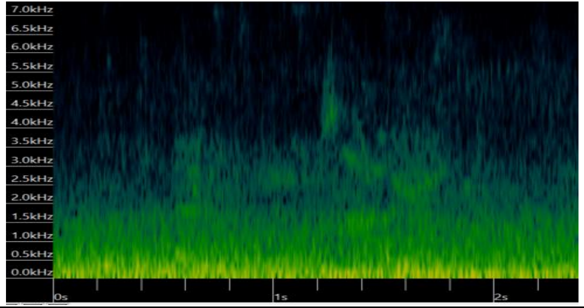
Appendix 6: Summary of African wild dog vocalisations as previously categorised and described by Robbins, 2000 and Walker et al., 2017 and their associated spectrogram**.**

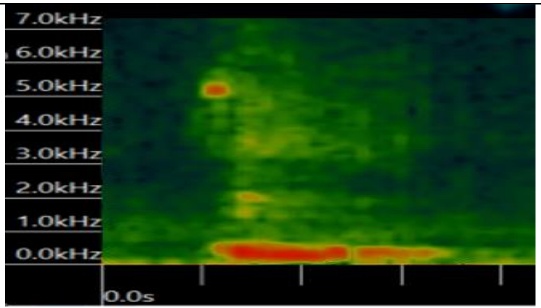
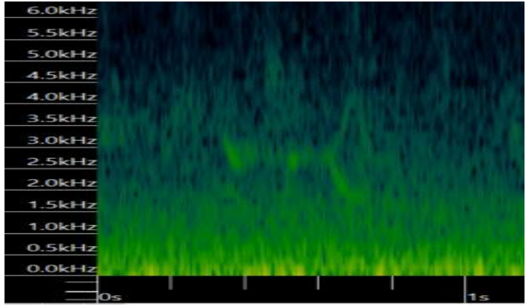
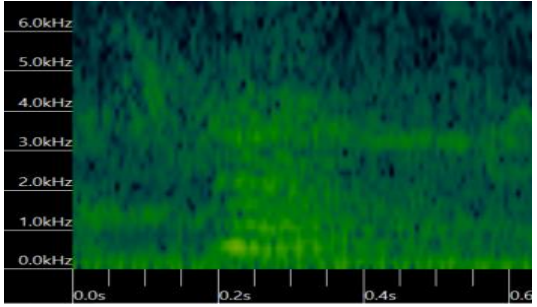
Call ID	**visualisation**	**Dominant Frequency Range (min-max; kHz)**	**Syllable lengths (s)**
Twitters			
Social Twitter		0.4 – 14.0	0.02 – 0.07
Spar Twitter		0.4 – 14.0	0.03 – 0.07
Mob Twitter		1.5 – 14.0	0.02 – 0.05

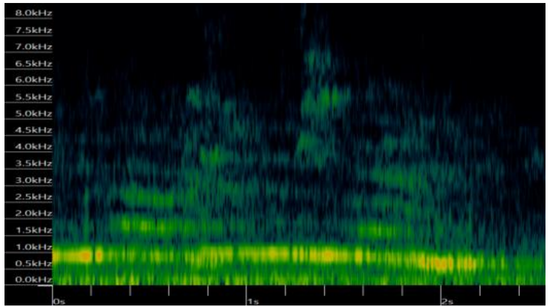
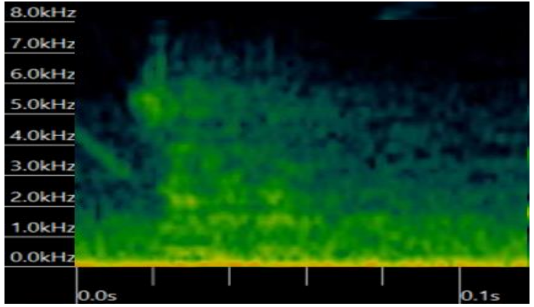
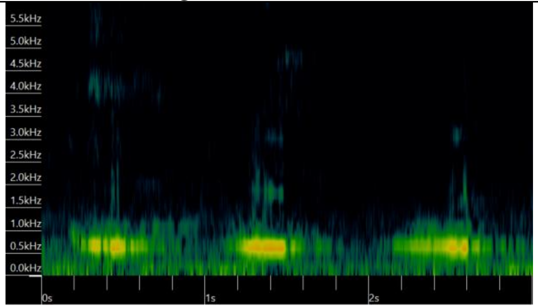
Attack twitter		0.2 – 12	0.02 – 0.06
Begging cries		0.3 – 5.5	0.03 – 7.0
Yelps/Squeals			
Yelps		0.7 – 7.0	0.1 – 0.3

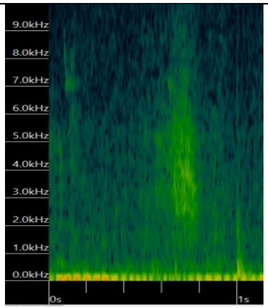
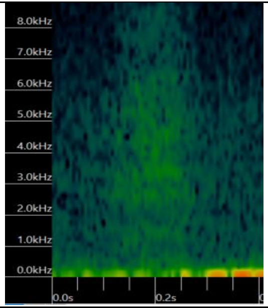
Whistle squeal		0.7 – 7.0	0.4 – 1.0
Begging squeals		1.0 – 7.0	0.5 – 1.0
Whimpers		0.3 – 8.0	0.1 – 0.4

Whines		0.4 – 5.0	0.2 – 2.7
Moans			
Full moan		0.1 – 1.2	0.2 – 1.2
Buzz moan		0.2 – 3.0	0.5 – 10

Rumbles		0.2 – 0.5	0.1 – 1.3
Growls			
Social growls		0.2 – 1.5	0.4 – 0.8
Alarm growls		0.3 – 2.0	0.2 – 0.4
Barks			

Alarm barks		0.2 – 4.5	0.1 – 0.7
Attack bark		0.3 – 4.0	0.04 – 0.1
Clear bark		0.3 – 4.0	0.1 – 0.4

Howl bark		0.5 – 3.0	0.7 – 1.2
Threat barks		0.5 – 4.0	0.2 – 0.3
Yelp Bark	No recordings made of this vocalisation	0.6 – 6.5	0.1 – 0.2
Hoo-call		0.6 – 0.8	0.1 – 0.8

Sneeze		1.0 – 7.0	0.05 – 0.1
Huff		1.0 – 6.0	0.1 – 0.2

References

Ader, R., Felton, D. L., & Cohen, N. (2001). *Psychoneuroimmunology (3rd ED)*. Academic Press.

Alexander, K. A., McNutt, J. W., Briggs, M. B., Standers, P. E., Funston, P., Hemson, G., . . . Van Vuuren, M. J. (2010). Multi-host pathogens and carnivore management in sourthern Africa. *Comparative Immunology, Microbiology and Infectious Diseases*, *33*(3), 249-265.

Almberg, E. S., Mech, L. D., Smith, D. W., Sheldon, J. W., & Crabtree, R. L. (2009). A serological survey of infectious disease in Yellowstone National Park's canid community. *PLoS one*, *4*(9), e7042.

Almberg, E. S., Cross, P. C., Mech, L. D., Smith, D. W., Sheldon, J. W., & Crabtree, R. L. (2011). Infectious Diseases in Yellowstone's Canid Community.

Anderson, N. (2018). *Linking in situ and ex situ management: Exploring vaccine immunogenicity and capture stress in the African Painted Dog (Lycaon pictus)* Flinders University]. South Australia.

Anderson, N., & Smith, I. (2019). Assessing the immunogenicity of an inactivated monovalent vaccine in the endangered African wild dog (Lycaon pictus). *Vaccine: X*, *1*, 100006.

Angeli, A., Masera, R. G., Sartori, M. L., Fortunati, N., Racca, S., Dovio, A., . . . Frairia, R. (1999). Modulation by Cytokines of Glucocorticoid Action. *Annals of the New York Academy of Sciences*, *876*(1), 210-220. https://doi.org/10.1111/j.1749-6632.1999.tb07641.x

Appel, M. J. G. (1987). *Virus infections of carnivores*. Elsevier Science.

Appleby, R., Mackie, J., Smith, B., Bernede, L., & Jones, D. (2018). Human–dingo interactions on Fraser Island: an analysis of serious incident reports. *Australian Mammalogy*, *40*(2), 146-156.

Apps, P., Mmualefe, L., & McNutt, J. W. (2012). Identification of volatiles from the secretions and excretions of African wild dogs (Lycaon pictus). *Journal of Chemical Ecology*, *38*(11), 1450-1461.

Apps, P. J. (2013). Are mammal olfactory signals hiding right under our noses? *Naturwissenschaften*, *100*(6), 487-506.

Apps, P. J., Weldon, P. J., & Kramer, M. (2015). Chemical signals in terrestrial vertebrates: search for design features. *Natural product reports*, *32*(7), 1131-1153.

Apps, P., Claase, M., van Mourik, E., Lostrom, S., Yexley, B., Webster, H., & McNutt, J. W. (2022). A description of a new discovery: African wild dog packs communicate with other packs by posting scent-mark messages at a shared marking site. *Behavioral Ecology and Sociobiology*, *76*(3).

Archie, E. A., Altmann, J., & Alberts, S. C. (2012). Social status predicts wound healing in wild baboons. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(23), 9017-9022.

Armstrong, D. P., & Seddon, P. J. (2008). Directions in reintroduction biology. *Trends in Ecology & Evolution*, *23*(1), 20-25.

Asa, C. S., & Valdespino, C. (1998). Canid reporductive biology: an integration of proximate mechanisims and ultimate causes. *American Zoologist*, *38*(1), 251-259.

Ausband, D. E., Mitchell, M. S., Stansbury, C. R., Stenglein, J. L., & Waits, L. P. (2017). Harvest and group effects on pup survival in a cooperative breeder. *Proceedings of the Royal Society B: Biological Sciences*, *284*(1855), 20170580.

Aviles-Rosa, E. O., Surowiec, K., & McGlone, J. (2020). Identification of Faecal Maternal Semiochemicals in Swine (Sus scrofa) and their Effects on Weaned Piglets. *Scientific reports*, *10*(1), 5349-5349.

Baer, G. M., & Walander, A. I. (1987). Rabies Virus. In M. J. Appel (Ed.), *Virus infections of carnivores*.

Baker, D. G. (2002). Combination therapy for footpad lesions in a captive Bengal tiger (Panthera tigris tigris). *Journal of Zoo and Wildlife Medicine*, 389-391.

Barja, I., Silván, G., & Illera, J. C. (2008). Relationships between sex and stress hormone levels in feces and marking behavior in a wild population of Iberian wolves (Canis lupus signatus). *Journal of Chemical Ecology*, *34*(6), 697-701.

Barnard, C. J., Behnke, J. M., & Sewell, J. (1996). Social Status and Resistance to Disease in House Mice (Mus musculus): Status-related Modulation of Hormonal Responses in Relation to Immunity Costs in Different Social and Physical Environments. *Ethology*, *102*(1), 63-84.

Bateup, H. S., Shirtcliff, E. A., Booth, A., & GRanger, D. A. (2002). Testosterone, cortisol, and women's competition. *Evolution and Human Behavior*, *23*(3), 181-192.

Batson, W. G., Gordon, I. J., Fletcher, D. B., & Manning, A. D. (2016). The effect of pre-release captivity on post-release performance in reintroduced eastern bettongs Bettongia gaimardi. *Oryx*, *50*(4), 664-673.

Baumgarth, N., Tung, J. W., & Herzenberg, L. A. (2005). Inherent specificities in natural antibodies: a key to immune defense against pathogen invasion. *Springer Seminars in Immunopathology*, *26*(4), 347-362.

Beineke, A., Baumgärtner, W., & Wohlsein, P. (2015). Cross-species transmission of canine distemper virus—an update. *One Health*, *1*(C), 49-59. https://doi.org/10.1016/j.onehlt.2015.09.002

Ben-Eliyahu, S., Shakhar, G., Page, G. G., Stefanski, V., & Shakhar, K. (2000). Suppression of NK Cell Activity and of Resistance to Metastasis by Stress: A Role for Adrenal Catecholamines and β-Adrenoceptors. *Neuroimmunomodulation*, *8*(3), 154-164.

Boehm, U., Zou, Z., & Buck, L. B. (2005). Feedback Loops Link Odor and Pheromone Signaling with Reproduction. *Cell*, *123*(4), 683-695. https://doi.org/10.1016/j.cell.2005.09.027

Borg, B. L., Brainerd, S. M., Meier, T. J., & Prugh, L. R. (2015). Impacts of breeder loss on social structure, reproduction and population growth in a social canid. *Journal of Animal Ecology*, *84*(1), 177-187.

Bos, P. A., Hechler, C., Beijers, R., Shinohara, K., Esposito, G., & de Weerth, C. (2018). Prenatal and postnatal cortisol and testosterone are related to parental caregiving quality in fathers, but not in mothers. *Psychoneuroendocrinology*, *97*, 94-103.

Bouissou, M.-F. (1983). Androgens, aggressive behaviour and social relationships in higher mammals. *Hormone Research in Paediatrics*, *18*(1-3), 43-61.

Bradley, E. H., Pletscher, D. H., Bangs, E. E., Kunkel, K. E., Smith, D. W., Mack, C. M., . . . Jimenez, M. D. (2005). Evaluating wolf translocation as a nonlethal method to reduce livestock conflicts in the northwestern United States. *Conservation biology*, *19*(5), 1498-1508.

Brahmachary, R., & Poddar-Sarkar, M. (2015). Fifty years of tiger pheromone research. *Current Science*, 2178-2185.

Briers-Louw, W. D., Verschueren, S., & Leslie, A. J. (2019). Big cats return to Majete Wildlife Reserve, Malawi: Evaluating reintroduction. *African journal of wildlife research*, *49*(1), 34-50.

Buchinger, T. J., & Li, W. (2020). The evolution of (non) species-specific pheromones. *EVOLUTIONARY ECOLOGY*.

Buettner, U. K., Du Toit, J. T., Davies-Mostert, H. T., & Mills, M. G. L. (2006). Factors affecting juvenile survival in African wild dogs (Lycaon pictus) in Kruger National Park, South Africa. *Journal of Zoology*, *272*(1), 10-19.

Buk, K. G., van der Merwe, V. C., Marnewick, K., & Funston, P. J. (2018). Conservation of severely fragmented populations: lessons from the transformation of uncoordinated reintroductions of cheetahs (Acinonyx jubatus) into a managed metapopulation with self-sustained growth. *Biodiversity and conservation*, *27*(13), 3393-3423.

Burroughs, R., Van Vuuren, M., Bingham, J., Stylianides, E., & Van Heerden, J. (2002). Clinical and serological response of wild dogs (Lycaon pictus) to vaccination against canine distemper, canine parvovirus infection and rabies. *Journal of the South African Veterinary Association*, *73*(1), 8-12.

Burrows, R. (1992). Rabies in wild dogs. *Nature*, *359*(6393), 277-277.

Burrows, R., Hofer, H., & East, M. L. (1994). Demography, Extinction and Intervention in a Small Population: the Case of the Serengeti Wild Dogs. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *256*(1347), 281-292.

Carbyn, L. N., Armbruster, H. J., & Mamo, C. (1994). The swift fox reintroduction program in Canada from 1983 to 1992. *Restoration of endangered species: conceptual issues, planning and implementation. Cambridge University Press, Cambridge, United Kingdom*, 247-271.

Chao, C. C., Peterson, P. K., Filice, G. A., Pomeroy, C., & Sharp, B. M. (1990). Effects of immobilization stress on the pathogenesis of acute murine toxoplasmosis. *Brain Behavior and Immunity*, *4*(2), 162-169.

Charlesworth, D., & Willis, J. H. (2009). The genetics of inbreeding depression. *Nature reviews. Genetics*, *10*(11), 783-796. https://doi.org/10.1038/nrg2664

Chase, I. D., Tovey, C., Spangler-Martin, D., & Manfredonia, M. (2002). Individual Differences versus Social Dynamics in the Formation of Animal Dominance Hierarchies. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5744-5749.

Ciribassi, J. J., & Ballantyne, K. (2014). Using clonidine and trazodone for anxiety-based behavior disorders in dogs. *Veterinary Medicine*, *109*(4), 131.

Clark, F. E., & Dunn, J. C. (2022). From Soundwave to Soundscape: A Guide to Acoustic Research in Captive Animal Environments. *Frontiers in veterinary science*, *9*, 889117-889117. https://doi.org/10.3389/fvets.2022.889117

Claase, M. J., Cherry, M. I., Apps, P. J., McNutt, J. W., Hansen, K. W., & Jordan, N. R. (2022). Interpack communication in African wild dogs at long-term shared marking sites. *Animal behaviour*, *192*, 27-38.

Coccaro, E. F., Lee, R., & Coussons-Read, M. (2014). Elevated Plasma Inflammatory Markers in Individuals With Intermittent Explosive Disorder and Correlation With Aggression in Humans. *JAMA Psychiatry*, *71*(2), 158-165.

Cock, H. R., Spiro, S., Stidworthy, M. F., Denk, D., & Guthrie, A. (2023). Retrospective study of morbidity and mortality of captive African wild dogs (*Lycaon pictus*) held within United Kingdom zoological institutions between 2000 and 2020. *Journal of Zoo and Wildlife Medicine*, *54*(3), 498-510.

Cohen, S., & Williamson, G. M. (1991a). Stress and infectious disease in humans. *Psychological bulletin*, *109*(1), 5-24.

Cohen, S., Tyrrell, D. A. J., & Smith, A. P. (1991b). Psychological Stress and Susceptibility to the Common Cold. *The New England Journal of Medicine*, *325*(9), 606-612.

Cohen, S., Line, S., Manuck, S. B., Rabin, B. S., Heise, E. R., & Kaplan, J. R. (1997). Chronic social stress, social status, and susceptibility to upper respiratory infections in nonhuman primates. *Psychosomatic medicine*, *59*(3), 213-221. 00001

Connolly, M., Thomas, P., Woodroffe, R., & 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*, *44*(4), 882-888.

Connolly, M., Thomas, P., Woodroffe, R., & Raphael, B. L. (2015). Single- versus double-dose rabies vaccination in captive African wild dogs (*Lycaon pictus*). *Journal of Zoo and Wildlife Medicine*, *46*(4), 691-698.

Courchamp, F., & Macdonald, D. W. (2001). Crucial importance of pack size in the African wild dog Lycaon pictus. *Animal conservation*, *4*(2), 169-174.

Cozzi, A., Monneret, P., Lafont-Lecuelle, C., Bougrat, L., Gaultier, E., & Pageat, P. (2010). The maternal cat appeasing pheromone: Exploratory study of the effects on aggressive and affiliative interactions in cats. *Journal of veterinary behavior*, *5*(1), 37-38.

Cozzi, G., Behr, D. M., Webster, H. S., Claase, M., Bryce, C. M., Modise, B., . . . Ozgul, A. (2020). African Wild Dog Dispersal and Implications for Management. *The Journal of wildlife management*, *84*(4), 614-621.

Creel, S., & Creel, N. M. (1995). Communal hunting and pack size in African wild dogs, Lycaon pictus. *Animal behaviour*, *50*(5), 1325-1339.

Creel, S., Creel, N. M., Mills, M. G. L., & Monfort, S. L. (1997a). Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. *Behavioral Ecology*, *8*(3), 298-306.

Creel, S., Creel, N. M., Munson, L., Sanderlin, D., & Appel, M. J. (1997b). Serosurvey for selected viral diseases and demography of African wild dogs in Tanzania. *Journal of wildlife diseases*, *33*(4), 823-832.

Creel, S., & Creel, N. M. (1998). Six ecological factors that may limit African wild dogs, Lycaon pictus. *Animal conservation*, *1*(1), 1-9.

Creel, S. (2001a). Four Factors Modifying the Effect of Competition on Carnivore Population Dynamics as Illustrated by African Wild Dogs. *Conservation biology*, *15*(1), 271-274. https://doi.org/10.1046/j.1523-1739.2001.99534.x

Creel, S. (2001b). Social dominance and stress hormones. *Trends in ecology & evolution*, *16*(9), 491-497.

Creel, S., & Creel, N. M. (2002). *The African wild dog: behaviour, ecology and evolution*. Princeton University Press.

Creel, S., Mills, M., & McNutt, J. (2004). Demography and Population Dynamics of African Wild Dogs in Three Critical Populations. *Biology and Conservation of Wild Canids*.

Creel, S. (2005). Dominance, aggression, and glucorticoid levels in social carnivores. *Journal of Mammalogy*, *86*(2), 255-264.

Cross, P. C., & Beissinger, S. R. (2001). Using Logistic Regression to Analyze the Sensitivity of PVA Models: A Comparison of Methods Based on African Wild Dog Models. *Conservation biology*, *15*(5), 1335-1346.

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

Crossey, B., Chimimba, C., Plessis, C. d., Hall, G., & Ganswindt, A. (2020). Using faecal glucocorticoid metabolite analyses to elucidate stressors of African wild dogs Lycaon pictus from South Africa. *Wildlife Biology*, *2020*(1).

Cunningham, A. A. (1996). Disease Risks of Wildlife Translocations. *Conservation biology*, *10*(2), 349-353.

Curno, O., Behnke, J. M., McElligott, A. G., Reader, T., & Barnard, C. J. (2009). Mothers produce less aggressive sons with altered immunity when there is a threat of disease during pregnancy. *Proceedings of the Royal Society B: Biological Sciences*, *276*(1659), 1047-1054.

D'Onise, K., Hazel, S., & Caraguel, C. (2017). Mandatory desexing of dogs: one step in the right direction to reduce the risk of dog bite? A systematic review. *Injury Prevention*, *23*(3), 212-218.

Darnell, A. M., Graf, J. A., Somers, M. J., Slotow, R., & Szykman Gunther, M. (2014). Space use of African wild dogs in relation to other large carnivores. *PLoS one*, *9*(6), e98846.

Das, S., Deuri, S. K., Sarmah, A., Pathak, K., Baruah, A., Sengupta, S., . . . Hazarika, J. (2016). Aggression as an independent entity even in psychosis- the role of inflammatory cytokines. *Journal of Neuroimmunology*, *292*, 45-51. https://doi.org/10.1016/j.jneuroim.2016.01.012

Davies, H. T., & du Toit, J. T. (2004). Anthropogenic factors affecting wild dog Lycaon pictus reintroductions: a case study in Zimbabwe. *Oryx*, *38*(1), 32-39.

Davies-Mostert, H. T., Mills, M. G. L., & Macdonald, D. W. (2009). A Critical Assessment of South Africa's Managed Metapopulation Recovery Strategy for African Wild Dogs. In (pp. 10-42). Wiley-Blackwell.

Davies-Mostert, H. T., Mills, M. G., & Macdonald, D. W. (2015). The demography and dynamics of an expanding, managed African wild dog metapopulation. *African journal of wildlife research*, *45*(2), 258-273.

Decaro, N., Pratelli, A., Tinelli, A., Martella, V., Camero, M., Buonavoglia, D., . . . Buonavoglia, C. (2004). Fecal Immunoglobulin A Antibodies in Dogs Infected or Vaccinated with Canine Coronavirus. *Clinical and Diagnostic Laboratory Immunology*, *11*(1), 102-105.

de Kloet, E. R., Vreugdenhil, E., Oitzl, M. S., & Joëls, M. (1998). Brain corticosteroid receptor balance in health and disease. *Endocrine Reviews*, *19*(3), 269-301.

De Villiers, M. S., Meltzer, D. G. A., Van Heerden, J. V., Mills, M. G. L., Richardson, P. R. K., & Van Jaarsveld, A. S. (1995). Handling-Induced Stress and Mortalities in African Wild Dogs (Lycaon pictus). *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *262*(1364), 215-220.

De Villiers, M. S., van Jaarsveld, A. S., Meltzer, D. G. A., & Richardson, P. R. K. (1997). Social Dynamics and the Cortisol Response to Immobilization Stress of the African Wild Dog,Lycaon pictus. *Hormones and Behavior*, *31*(1), 3-14.

Déaux, É. C., & Clarke, J. A. (2013). Dingo (Canis lupus dingo) acoustic repertoire: form and contexts. *Behaviour*, *150*(1), 75-101.

Déaux, E. C., Allen, A. P., Clarke, J. A., & Charrier, I. (2016). Concatenation of 'alert'and 'identity'segments in dingoes' alarm calls. *Scientific reports*, *6*(1), 30556.

Dehnhard, M. (2011). Mammal semiochemicals: understanding pheromones and signature mixtures for better zoo-animal husbandry and conservation. *International Zoo Yearbook*, *45*, 55-79. https://doi.org/10.1111/j.1748-1090.2010.00131.x

DePorter, T. L., Bledsoe, D. L., Beck, A., & Ollivier, E. (2019). Evaluation of the efficacy of an appeasing pheromone diffuser product vs placebo for management of feline aggression in multi-cat households: a pilot study. *Journal of feline medicine and surgery*, *21*(4), 293-305.

Dhabhar, F. S., & McEwen, B. S. (1997). Acute Stress Enhances while Chronic Stress Suppresses Cell-Mediated Immunity in Vivo: A Potential Role for Leukocyte Trafficking. *Brain, Behavior, and Immunity*, *11*(4), 286-306.

Dhabhar, F. S., & McEwen, B. S. (1999). Enhancing versus Suppressive Effects of Stress Hormones on Skin Immune Function. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(3), 1059-1064.

Dhabhar, F. S. (2000). Acute Stress Enhances While Chronic Stress Suppresses Skin Immunity: The Role of Stress Hormones and Leukocyte Trafficking. *Annals of the New York Academy of Sciences*, *917*(1), 876-893.

Dickens, M. J., Delehanty, D. J., & Michael Romero, L. (2010). Stress: An inevitable component of animal translocation. *Biological Conservation*, *143*(6), 1329-1341.

Dloniak, S. M., French, J. A., & Holekamp, K. E. (2006). Rank-related maternal effects of androgens on behaviour in wild spotted hyaenas. *Nature*, *440*(7088), 1190-1193.

Dodman, N., Donnelly, R., Shuster, L., Mertens, P., Rand, W., & Miczek, K. (1996). Use of fluoxetine to treat dominance aggression in dogs. *Journal of the American Veterinary Medical Association*, *209*(9), 1585-1587.

Døving, K. B., & Trotier, D. (1998). Structure and function of the vomeronasal organ. *Journal of Experimental Biology*, *201*(21), 2913-2925.

Dugovich, B. S., Peel, M. J., Palmer, A. L., Zielke, R. A., Sikora, A. E., Beechler, B. R., . . . Dolan, B. P. (2017). Detection of bacterial-reactive natural IgM antibodies in desert bighorn sheep populations. *PLoS one*, *12*(6), e0180415.

Durairaj, R., Bienboire-Frosini, C., Cozzi, A., & Pageat, P. (2019). Sequence analysis and multi-template threading of cat vomeronasal type-1 receptor with an evaluation of feline semiochemical interactions using virtual screening.

Edwards, E. A., & Dean, L. M. (1977). Effects of crowding of mice on humoral antibody formation and protection to lethal antigenic challenge. *Psychosomatic Medicine*, *39*(1), 19-24.

Eisenegger, C., Naef, M., Snozzi, R., Heinrichs, M., & Fehr, E. (2010). Prejudice and truth about the effect of testosterone on human bargaining behaviour. *Nature*, *463*(7279), 356-359.

Engelhard, N. v., Kappeler, P. M., & Heistermann, M. (2000). Androgen levels and female social dominance in Lemur catta. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *267*(1452), 1533-1539.

Ennis, M., Hamilton, K. A., & Hayar, A. (2007). Neurochemistry of the Main Olfactory System. In (pp. 137-204). Boston, MA: Springer US.

Falewee, C., Gaultier, E., Lafont, C., Bougrat, L., & Pageat, P. (2006). Effect of a synthetic equine maternal pheromone during a controlled fear-eliciting situation. *Applied Animal Behaviour Science*, *101*(1), 144-153.

Fanshawe, J. H., & Fitzgibbon, C. D. (1993). Factors influencing the hunting success of an African wild dog pack. *Animal behaviour*, *45*(3), 479-490.

Faragó, T., Pongrácz, P., Range, F., Virányi, Z., & Miklósi, Á. (2010). 'The bone is mine': affective and referential aspects of dog growls. *Animal behaviour*, *79*(4), 917-925.

Farris, Z. J., Golden, C. D., Karpanty, S., Murphy, A., Stauffer, D., Ratelolahy, F., . . . Kelly, M. J. (2015). Hunting, Exotic Carnivores, and Habitat Loss: Anthropogenic Effects on a Native Carnivore Community, Madagascar. *PLoS one*, *10*(9), e0136456-e0136456.

Fischer, J., & Lindenmayer, D. B. (2000). An assessment of the published results of animal relocations. In (Vol. 96, pp. 1-11): Elsevier Ltd.

Flacke, G., Becker, P., Cooper, D., Szykman Gunther, M., Robertson, I., Holyoake, C., . . . Warren, K. (2013). An Infectious Disease and Mortality Survey in a Population of Free-roaming African Wild Dogs and Sympatric Domestic Dogs. *International Journal of Biodiversity*, *2013*, 1-9.

Fleischer, J., Schwarzenbacher, K., & Breer, H. (2007). Expression of Trace Amine-Associated Receptors in the Grueneberg Ganglion. *Chemical Senses*, *32*(6), 623-631.

Frankham, R. (1996). Relationship of Genetic Variation to Population Size in Wildlife. *Conservation biology*, *10*(6), 1500-1508.

Franklin, A. D., Waddell, W. T., Behrns, S., & Goodrowe, K. L. (2020). Estrous cyclicity and reproductive success are unaffected by translocation for the formation of new reproductive pairs in captive red wolves (Canis rufus). *Zoo Biology*.

Frantzen, M. A. J., Ferguson, J. W. H., & de Villiers, M. S. (2001). The conservation role of captive African wild dogs (Lycaon pictus). *Biological Conservation*, *100*(2), 253-260.

Fredrickson, R. J., Siminski, P., Woolf, M., & Hedrick, P. W. (2007). Genetic rescue and inbreeding depression in Mexican wolves. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1623), 2365-2371.

Friard, O., Gamba, M., & Fitzjohn, R. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. *Methods in ecology and evolution*, *7*(11), 1325-1330.

Fritts, S. H., Paul, W. J., & Mech, L. D. (1985). Can relocated wolves survive? *Wildlife Society Bulletin (1973-2006)*, *13*(4), 459-463.

Fritts, S. H., Bangs, E. E., Fontaine, J. A., Johnson, M. R., Phillips, M. K., Koch, E. D., & Gunson, J. R. (1997). Planning and Implementing a Reintroduction of Wolves to Yellowstone National Park and Central Idaho. *Restoration Ecology*, *5*(1), 7-27.

Fuller, T. K., Kat, P. W., Bulger, J. B., Maddock, A. H., Ginsberg, J. R., Burrows, R., . . . Mills, M. G. L. (1992). Population Dynamics of African Wild Dogs. In D. R. McCullough & R. H. Barrett (Eds.), *Wildlife 2001: Populations* (pp. 1125-1139). Springer Netherlands.

Gandia Estellés, M., & Mills, D. S. (2006). Signs of travel-related problems in dogs and their response to treatment with dog appeasing pheromone. *Veterinary record*, *159*(5), 143-148.

Gascoyne, S., King, A., Laurenson, M., Borner, M., Schildger, B., & Barrat, J. (1993a). Aspects of rabies infection and control in the conservation of the African wild dog (Lycaon pictus) in the Serengeti region, Tanzania.

Gasgoyne, S., Laurenson, M.., Lelo, S., & Borner, M. (1993b). Rabies in African wild dogs (Lycaon pictus) in the Serengeti region, Tanzania. *Journal of wildlife diseases*, *29*(3), 396-402.

Gaultier, E., Bonnafous, L., Bougrat, L., Lafont, C., & Pageat, P. (2005). Comparison of the efficacy of a synthetic dog-appeasing pheromone with clomipramine for the treatment of separation-related disorders in dogs. *Veterinary record*, *156*(17), 533-538.

Gebremedhin, B., Ficetola, G. F., Naderi, S., Rezaei, H. R., Maudet, C., Rioux, D., . . . Taberlet, P. (2009). Combining genetic and ecological data to assess the conservation status of the endangered Ethiopian walia ibex. *Animal conservation*, *12*(2), 89-100.

Gettler, L. T., McDade, T. W., Feranil, A. B., & Kuzawa, C. W. (2011). Longitudinal evidence that fatherhood decreases testosterone in human males. *Proceedings of the National Academy of Sciences*, *108*(39), 16194-16199.

Gilbert-Gregory, S. E., Stull, J. W., Rice, M. R., & Herron, M. E. (2016). Effects of trazodone on behavioral signs of stress in hospitalized dogs. *Journal of the American Veterinary Medical Association*, *249*(11), 1281-1291.

Ginsberg, J. R., Mace, G. M., & Albon, S. (1995). Local Extinction in a Small and Declining Population: Wild Dogs in the Serengeti. *Proceedings of the Royal Society of London. Series B: Biological Sciences*, *262*(1364), 221-228.

Girman, D. J., Mills, M. G. L., Geffen, E., & 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*, *40*(3), 187-198.

Girman, D. J., Vilà, C., Geffen, E., Creel, S., Mills, M. G. L., McNutt, J. W., . . . Wayne, R. K. (2001). Patterns of population subdivision, gene flow and genetic variability in the African wild dog (Lycaon pictus). *Molecular Ecology*, *10*(7), 1703-1723.

Glaser, R., Kiecolt-Glaser, J. K., Bonneau, R. H., Malarkey, W., Kennedy, S., & Hughes, J. (1992). Stress-induced modulation of the immune response to recombinant hepatitis B vaccine. *Psychosomatic Medicine*, *54*(1), 22-29.

Glickman, S., Frank, L., Pavgi, S., & Licht, P. (1992). Hormonal correlates of 'masculinization' in female spotted hyaenas (Crocuta crocuta). 1. Infancy to sexual maturity. *Reproduction*, *95*(2), 451-462.

Goller, K. V., Fyumagwa, R. D., Nikolin, V., East, M. L., Kilewo, M., Speck, S., . . . Wibbelt, G. (2010). Fatal canine distemper infection in a pack of African wild dogs in the Serengeti ecosystem, Tanzania. *Veterinary microbiology*, *146*(3), 245-252.

Gordon, C. H., Banyard, A. C., Hussein, A., Laurenson, M. K., Malcolm, J. R., Marino, J., . . . Sillero-Zubiri, C. (2015). Canine distemper in endangered Ethiopian wolves. *Emerging infectious diseases*, *21*(5), 824.

Gosling, L. M., & Sutherland, W. J. (2000). *Behaviour and Conservation. 438 pp.* . Cambridge University Press.

Goymann, W., East, M. L., & Hofer, H. (2001). Androgens and the Role of Female "Hyperaggressiveness" in Spotted Hyenas (Crocuta crocuta). *Hormones and Behavior*, *39*(1), 83-92.

Group, W. D. A. (2023, 25 October 2023). Update on the Wild Dogs of Southern Africa. the Wild Dog Advisory Group (WAG) meeting, the Endangered Wildlife Trust (EWT) Conservation Campus and online.

Grus, W. E., & Zhang, J. (2008). Distinct Evolutionary Patterns between Chemoreceptors of 2 Vertebrate Olfactory Systems and the Differential Tuning Hypothesis. *Molecular biology and evolution*, *25*(8), 1593-1601.

Gusset, M., Slotow, R., & Somers, M. J. (2006). Divided we fail: the importance of social integration for the re-introduction of endangered African wild dogs (Lycaon pictus). *Journal of Zoology*, *270*(3), 502-511.

Gusset, M., Gusset, M., Maddock, A. H., Maddock, A. H., Gunther, G. J., Gunther, G. J., . . . Somers, M. J. (2008). Conflicting human interests over the re-introduction of endangered wild dogs in South Africa. *Biodiversity and conservation*, *17*(1), 83-101.

Gusset, M., Stewart, G. B., Bowler, D. E., & 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*, *18*(3), 230-234.

Hallberg, K. I. (2007). *Information in a long-distance vocal signal: chorus howling in the coyote (Canis latrans)* The Ohio State University].

Hampson, K., Dushoff, J., Cleaveland, S., Haydon, D. T., Kaare, M., Packer, C., & Dobson, A. (2009). Transmission dynamics and prospects for the elimination of canine rabies. *PLoS biology*, *7*(3).

Hartwig, S. (2005). Individual acoustic identification as a non-invasive CONSERVATION tool: An approach to the conservation of te African wild dog Lycaon pictus (TEMMINCK, 1820). *Bioacoustics (Berkhamsted)*, *15*(1), 35-50.

Haydon, D., Laurenson, M., & 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*, *16*(5), 1372-1385.

Hayward, M. W., Adendorff, J., O'Brien, J., Sholto-Douglas, A., Bissett, C., Moolman, L. C., . . . Kerley, G. I. H. (2007). The reintroduction of large carnivores to the Eastern Cape Province, South Africa: An Assessment. *Oryx*, *41*, 205-214.

Hedrick, P. W., Lee, R. N., & Parker, K. M. (2000). Major histocompatibility complex (MHC) variation in the endangered Mexican wolf and related canids. *Heredity*, *85*(6), 617-624.

Heller, R., Lorenzen, E. D., Okello, J. B. A., Masembe, C., & Siegismund, H. R. (2008). Mid-Holocene decline in African buffalos inferred from Bayesian coalescent-based analyses of microsatellites and mitochondrial DNA. *Molecular Ecology*, *17*(22), 4845-4858.

Hermiston, C., Montrose, V. T., & Taylor, S. (2018). The effects of dog-appeasing pheromone spray upon canine vocalizations and stress-related behaviors in a rescue shelter. *Journal of Veterinary Behavior*, *26*, 11-16.

Hofmyer, M., Bingham, J., Lane, E. P., Ide, A., & Nel, L. (2000). Rabies in African wild dogs (*Lycaon pictus*) in the Madikwe Game Reserve, South Africa. *Veterinary Record*, *146*(2), 50-52.

Hofmeyr, M., Hofmeyr, D., Nel, L., & Bingham, J. (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*, *7*(2), 193-198.

Hueletl-Soto, M. E., Carro-Juárez, M., & Rodríguez-Manzo, G. (2012). Fluoxetine chronic treatment inhibits male rat sexual behavior by affecting both copulatory behavior and the genital motor pattern of ejaculation. *The journal of sexual medicine*, *9*(4), 1015-1026.

Isogai, Y., Si, S., Pont-Lezica, L., Tan, T., Kapoor, V., Murthy, V. N., & Dulac, C. (2011). Molecular organization of vomeronasal chemoreception. *Nature*, *478*(7368), 241-245.

IUCN. (1998). *Guidelines for Re-introductions*. Gland, Switxerland and Cambridge, UK.

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

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

IUCN/SSC. (2009). *Regional Conservation Strategy for the Cheetah and Wild Dog in Southern Africa*.

Jackson, C. R., McNutt, J. W., & Apps, P. J. (2012). Managing the ranging behaviour of African wild dogs (Lycaon pictus) using translocated scent marks. *Wildlife Research*, *39*(1), 31-34.

Jackson, C. R., John Power, R., Groom, R. J., Masenga, E. H., Mjingo, E. E., Fyumagwa, R. D., . . . Davies-Mostert, H. (2014). Heading for the hills: Risk avoidance drives den site selection in African wild dogs. *PLoS one*, *9*(6), e99686.

Jackson, C. R., Groom, R. J., Jordan, N. R., & McNutt, J. W. (2017). The effect of relatedness and pack size on territory overlap in African wild dogs. *Movement Ecology*, *5*(1), 10-10.

Jeong, D.-H., Yang, J.-J., & Yeon, S.-C. (2019). Fluoxitine therapy to decrease stereotypic behaviour in the asiatic black bear (*Ursus Thibetanus*). *Journal of Zoo and Wildlife Medicine*, *50*(3), 718.

Johnson, W. E., & Franklin, W. L. (1994). Spatial resource partitioning by sympatric grey fox (Dusicyon griseus) and culpeo fox (*Dusicyon culpaeus*) in southern Chile. *Canadian journal of zoology*, *72*(10), 1788-1793.

Johnston, S. D., Ward, D., Lemon, J., Gunn, I., MacCallum, C. A., Keeley, T., & Blyde, D. (2007). Studies of male reproduction in captive African wild dogs (Lycaon pictus). *Animal reproduction science*, *100*(3), 338-355.

Jones, B. L., Oswald, M., Tufano, S., Baird, M., Mulsow, J., & Ridgway, S. H. (2021). A System for Monitoring Acoustics to Supplement an Animal Welfare Plan for Bottlenose Dolphins. *Journal of zoological and botanical gardens*, *2*(2), 222-233. https://doi.org/10.3390/jzbg2020015.

Jongeling, T. B., & Koetsier, T. (2014). predicament of the African wild dog, Lycaon pictus, is less precarious than claimed. *African journal of ecology*, *52*(4), 466-470.

Jordan, N. R., Buse, C., Wilson, A. M., Golabek, K. A., Apps, P. J., Lowe, J. C., . . . McNutt, J. W. (2017). Dynamics of direct inter-pack encounters in endangered African wild dogs. *Behavioral Ecology and Sociobiology*, *71*(8), 1-12.

Jordan, N. R., Golabek, K. A., Behr, D. M., Walker, R. H., Plimpton, L., Lostrom, S., . . . McNutt, J. W. (2022). Priority of access to food and its influence on social dynamics of an endangered carnivore. *Behavioral Ecology and Sociobiology*, *76*(1).

Jordan, N. R., Golabek, K. A., Marneweck, C. J., Marneweck, D. G., Mbizah, M. M., Ngatia, D., . . . Srinivasan, M. (2023). Hunting Behavior and Social Ecology of African Wild Dogs. In (pp. 177-227). Springer International Publishing AG.

Joslyn, W. D. (1973). Androgen-induced social dominance in infant female Rhesus monkeys. *Journal of Child Psychology and Psychiatry*, *14*(2), 137-145.

Jule, K. R., Leaver, L. A., & Lea, S. E. G. (2008). The effects of captive experience on reintroduction survival in carnivores: A review and analysis. *Biological Conservation*, *141*(2), 355-363.

Kalantaridou, S., Zoumakis, E., Makrigiannakis, A., Lavasidis, L., Vrekoussis, T., & Chrousos, G. (2010). Corticotropin-releasing hormone, stress and human reproduction: an update. *Journal of reproductive immunology*, *85*(1), 33-39.

Karlson, P., & Lüscher, M. (1959). 'Pheromones': a New Term for a Class of Biologically Active Substances. *Nature*, *183*(4653), 55-56.

Kat, P. W., Alexander, K. A., Smith, J. S., & Munson, L. (1995). Rabies and African wild dogs in Kenya. *Poceedings of the Royal Society B*, *262*, 229-233.

Kennedy, L., Angles, J., Barnes, A., Carter, S., Francino, O., Gerlach, J., . . . Wagner, J. (2001). Nomenclature for factors of the dog major histocompatibility system (DLA), 2000: second report of the ISAG DLA Nomenclature Committee. *Animal genetics*, *32*(4), 193-199.

Kennedy, L. J., Angles, J. M., Barnes, A., Carmichael, L. E., Radford, A. D., Ollier, W. E., & Happ, G. M. (2007). DLA-DRB1, DQA1, and DQB1 alleles and haplotypes in North American gray wolves. *Journal of Heredity*, *98*(5), 491-499.

Kennedy, L., Randall, D., Knobel, D., Brown, J., Fooks, A., Argaw, K., . . . Macdonald, D. (2011). Major histocompatibility complex diversity in the endangered Ethiopian wolf (*Canis simensis*). *Tissue antigens*, *77*(2), 118-125.

Kershenbaum, A., Root-Gutteridge, H., Habib, B., Koler-Matznick, J., Mitchell, B., Palacios, V., & Waller, S. (2016). Disentangling canid howls across multiple species and subspecies: Structure in a complex communication channel. *Behavioural processes*, *124*, 149-157.

Kiecolt-Glaser, J. K., Glaser, R., Gravenstein, S., Malarkey, W. B., & Sheridan, J. (1996). Chronic Stress Alters the Immune Response to Influenza Virus Vaccine in Older Adults. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(7), 3043-3047.

Kiecolt-Glaser, J. K., Loving, T. J., Stowell, J. R., Malarkey, W. B., Lemeshow, S., Dickinson, S. L., & Glaser, R. (2005). Hostile Marital Interactions, Proinflammatory Cytokine Production, and Wound Healing. *Archives of General Psychiatry*, *62*(12), 1377-1384.

Kirschbaum, C., & Hellhammer, D. H. (1994). Salivary cortisol in psychoneuroendocrine research: recent developments and applications. *Psychoneuroendocrinology*, *19*, 313-333.

Kissui, B. M. (2008). Livestock predation by lions, leopards, spotted hyenas, and their vulnerability to retaliatory killing in the Maasai steppe, Tanzania. *Animal conservation*, *11*(5), 422-432.

Koolhaas, J., Korte, S., De Boer, S., Van Der Vegt, B., Van Reenen, C., Hopster, H., . . . Blokhuis, H. (1999). Coping styles in animals: current status in behavior and stress-physiology. *Neuroscience & Biobehavioral Reviews*, *23*(7), 925-935.

Kreeger, T. J., Seal, U. S., & Plotka, E. D. (1992). Influence of hypothalamic-pituitary-adrenocortical hormones on reproductive hormones in gray wolves (*Canis lupus*). *Journal of Experimental Zoology*, *264*(1), 32-41.

Laikre, L., Olsson, F., Jansson, E., Hössjer, O., & Ryman, N. (2016). Metapopulation effective size and conservation genetic goals for the Fennoscandian wolf (*Canis lupus*) population. *Heredity*, *117*(4), 279-289.

Laikre, L., & Ryman, N. (1991). Inbreeding Depression in a Captive Wolf (*Canis lupus*) Population. *Conservation biology*, *5*(1), 33-40. https://doi.org/10.1111/j.1523-1739.1991.tb00385.x

Landa, A., Flagstad, Ø., Areskoug, V., Linnell, J. D., Strand, O., Ulvund, K. R., . . . Eide, N. E. (2017). The endangered Arctic fox in Norway—the failure and success of captive breeding and reintroduction. *Polar Research*, *36*(sup1), 9.

Landsberg, G. M., Beck, A., Lopez, A., Deniaud, M., Araujo, J. A., & Milgram, N. W. (2015). Dog-appeasing pheromone collars reduce sound-induced fear and anxiety in beagle dogs: a placebo-controlled study. *Veterinary record*, *177*(10), 260-260.

Leigh, K. A., Zenger, K. R., Tammen, I., & Raadsma, H. W. (2012). Loss of genetic diversity in an outbreeding species: small population effects in the African wild dog (*Lycaon pictus*). *Conservation Genetics*, *13*(3), 767-777.

Letty, J., Aubineau, J., Marchandeau, S., & Clobert, J. (2003). Effect of translocation on survival in wild rabbit (*Oryctolagus cuniculus*). *Mammalian Biology*, *68*(4), 250-255.

Lew, W., Oppenheim, J. J., & Matsushima, K. (1988). Analysis of the suppression of IL-1 alpha and IL-1 beta production in human peripheral blood mononuclear adherent cells by a glucocorticoid hormone. *The Journal of Immunology*, *140*(6), 1895.

Liberg, O., Andrén, H., Pedersen, H.-C., Sand, H., Sejberg, D., Wabakken, P., . . . Bensch, S. (2005). Severe inbreeding depression in a wild wolf (*Canis lupus*) population. *Biology letters*, *1*(1), 17-20.

Liberles, S. D. (2014). Mammalian pheromones. *Annual review of physiology*, *76*(1), 151-175.

Lindsey, P. A., Du Toit, J. T., & Mills, M. G. L. (2004). Area and prey requirements of African wild dogs under varying habitat conditions: implications for reintroductions. *South African Journal of Wildlife Research*, *34*, 77-86.

Lindsey, P. A., & Davies-Mostert, H. T. (2009). *South African action plan for conservation of cheetahs and wild dogs. In: Report from a National conservation Action Planning Workshop* (Report from a National conservation Action Planning Workshop, Issue.

Line, S. W., Kaplan, J. R., Heise, E. R., Hilliard, J. K., Cohen, S., Rabin, B. S., & Manuck, S. B. (1996). Effects of social reorganization on cellular immunity in male cynomolgus monkeys. *American Journal of Primatology*, *39*(4), 235-249.

Liu, J., Tian, Z., & Zhang, Y. (2016). Structure-based discovery of potentially active semiochemicals for Cydia pomonella (L.). *Scientific reports*, *6*(1), 34600-34600.

Lovejoy, M. C., Graczyk, P. A., O'Hare, E., & Neuman, G. (2000). Maternal depression and parenting behavior: A meta-analytic review. *Clinical psychology review*, *20*(5), 561-592.

Macdonald, D. W., Davies-Mostert, H. T., & Mills, M. G. L. (2015). The demography and dynamics of an expanding, managed African wild dog metapopulation. *African journal of wildlife research*, *45*(2), 258-273.

Maestripieri, D., & Carroll, K. A. (1998a). Behavioral and environmental correlates of infant abuse in group-living pigtail macaques. *Infant Behavior and Development*, *21*(4), 603-612.

Maestripieri, D., & Carroll, K. A. (1998b). Child abuse and neglect: Usefulness of the animal data. *Psychological Bulletin*, *123*(3), 211.

Maier, S. F., & Watkins, L. R. (1998). Cytokines for Psychologists: Implications of Bidirectional Immune-to-Brain Communication for Understanding Behavior, Mood, and Cognition. *Psychological Review*, *105*(1), 83-107.

Malcolm, J. R., & Marten, K. (1982). Natural Selection and the Communal Rearing of Pups in African Wild Dogs (*Lycaon pictus*). *Behavioral Ecology and Sociobiology*, *10*(1), 1-13.

Marceau, K., Dorn, L. D., & Susman, E. J. (2012). Stress and puberty-related hormone reactivity, negative emotionality, and parent–adolescent relationships. *Psychoneuroendocrinology*, *37*(8), 1286-1298.

Marino, J., Sillero-Zubiri, C., Gottelli, D., Johnson, P., & Macdonald, D. (2013). The fall and rise of E thiopian wolves: lessons for conservation of long-lived, social predators. *Animal conservation*, *16*(6), 621-632.

Marneweck, C. J., Becker, P. A., Beverley, G., Davies-Mostert, H. T., du Plessis, C., Forssman, K., . . . Parker, D. M. (2019a). Factors affecting the success of artificial pack formation in an endangered, social carnivore: the African wild dog. *Animal conservation*, *22*(5), 493-502.

Marneweck, C. J., Marchal, A. F. J., Marneweck, D. G., Beverley, G., Davies-Mostert, H. T., & Parker, D. M. (2019b). A Novel Technique for Artificial Pack Formation in African Wild Dogs Using Odour Familiarity. *African journal of wildlife research*, *49*(1).

Marneweck, D. G., Druce, D. J., Cromsigt, J. P. G. M., le Roux, E., & Somers, M. J. (2022). The relative role of intrinsic and extrinsic drivers in regulating population change and survival of African wild dogs (*Lycaon pictus*). *Mammalian biology : Zeitschrift für Säugetierkunde*, *102*(4), 1215-1229.

Marsden, C. D., Mable, B. K., Woodroffe, R., Rasmussen, G. S. A., Cleaveland, S., McNutt, J. W., . . . Kennedy, L. J. (2009). Highly endangered african wild dogs (*Lycaon pictus*) lack variation at the major histocompatibility complex. *Journal of Heredity*, *100*(Supplement 1), S54-S65.

Marsden, C. D. (2010). *Evolutionary & ecological genetics of African wild dogs* University of Glasgow.

Marsden, C. D., Woodroffe, R., Mills, M. G. L., McNutt, J. W., Creel, S., Groom, R., . . . Mable, B. K. (2012). Spatial and temporal patterns of neutral and adaptive genetic variation in the endangered African wild dog (*Lycaon pictus*): Spatial and temporal diversity in wild dogs. *Molecular Ecology*, *21*(6), 1379-1393.

Marsland, A. L., Prather, A. A., Petersen, K. L., Cohen, S., & Manuck, S. B. (2007). Antagonistic characteristics are positively associated with inflammatory markers independently of trait negative emotionality. *Brain, Behavior, and Immunity*, *22*(5), 753-761.

Marucha, P. T., Kiecolt-Glaser, J. K., & Favagehi, M. (1998). Mucosal wound healing is impaired by examination stress. *Psychosomatic Medicine*, *60*(3), 362-365.

Marx, A., Lenkei, R., Pérez Fraga, P., Bakos, V., Kubinyi, E., & Faragó, T. (2021). Occurrences of non-linear phenomena and vocal harshness in dog whines as indicators of stress and ageing. *Scientific reports*, *11*(1), 4468-4468.

Marten, K. L. (1981). Ecological sources of natural selection on animal vocalizations, with special reference to the African wild dog (*Lycaon pictus)*

Mazzini, F., Townsend, S. W., Virányi, Z., & Range, F. (2013). Wolf howling is mediated by relationship quality rather than underlying emotional stress. *Current Biology*, *23*(17), 1677-1680.

McCanne, T. R., & Hagstrom, A. H. (1996). Physiological hyperreactivity to stressors in physical child abusers and individuals at risk for being physically abusive. *Aggression and violent behavior*, *1*(4), 345-358.

McCreery, E. K. (2000). Spatial relationships as an indicator of successful pack formation in free-roaming African wild dogs. *Behaviour*, *137*(5), 579-590.

McCreery, E. K., & Robbins, R. (2001). Proximate explainations for failed pack formation in *Lycaon pictus*. *Behaviour*, *138*(11-12), 1467-1479. https://doi.org/10.1163/156853901317367708

McGlone, J. J., & Anderson, D. L. (2002). Synthetic maternal pheromone stimulates feeding behavior and weight gain in weaned pigs. *Journal of animal science*, *80*(12), 3179-3183.

McLellan, S. R., & Rabon Jr, D. R. (2006). Translocating red wolves using a modified soft-release technique. *Canid News*, *9*, 1-10.

McNutt, J. W. (1996). Sex-biased dispersal in African wild dogs, Lycaon pictus. *Animal behaviour*, *52*(6), 1067-1077.

Mech, L. D., & Goyal, S. M. (1993). Canine parvovirus effect on wolf population change and pup survival. *Journal of wildlife diseases*, *29*(2), 330-333.

Medzhitov, R., & Janeway Jr, C. A. (1998). Innate immune recognition and control of adaptive immune responses. *Seminars in immunology*, *10*(5), 351-353.

Mehta, P. H., Jones, A. C., & Josephs, R. A. (2008). The social endocrinology of dominance: Basal testosterone predicts cortisol changes and behavior following victory and defeat. *Journal of Personality and Social Psychology*, *94*(6), 1078-1093.

Meisami, E., & Bhatnagar, K. P. (1998). Structure and diversity in mammalian accessory olfactory bulb. *Microscopy research and technique*, *43*(6), 476-499.

Meredith, M., & O'connell, R. (1979). Efferent control of stimulus access to the hamster vomeronasal organ. *The Journal of physiology*, *286*(1), 301-316.

Meredith, M., Marques, D. M., O'connell, R., & Stern, F. L. (1980). Vomeronasal pump: significance for male hamster sexual behavior. *Science*, *207*(4436), 1224-1226.

Meredith, M. (1994). Chronic recording of vomeronasal pump activation in awake behaving hamsters. *Physiology & behavior*, *56*(2), 345-354.

Meredith, M. (1998). Vomeronasal function. *Chemical senses*, *23*(4), 463-466.

Miller, B., Ralls, K., Reading, R. P., Scott, J. M., & Estes, J. (1999). Biological and technical considerations of carnivore translocation: a review. *Animal conservation*, *2*(1), 59-68.

Mills, M., G. L., & Gorman, M. L. (1997). Factors Affecting the Density and Distribution of Wild Dogs in the Kruger National Park. *Conservation biology*, *11*(6), 1397-1406.

Mills, M. G. L., Ellis, S., Woodroffe, R., Maddock, A., Stander, P., Rasmussen, G., . . . Seal, U. (1998). Population and Habitat Viability Assessment for the African Wild Dog *(Lycaon pictus)* in Southern Africa. *Final workshop Report*.

Mills, D. S., & Hargrave, C. (2004). Dog appeasing pheromone reduces the anxiety of aggressive dogs in the veterinary practice. *American Veterinary Society of Animal Behaviour Proc Philadelphia*, 6-7.

Mills, D. S., Ramos, D., Estelles, M. G., & Hargrave, C. (2006). A triple blind placebo-controlled investigation into the assessment of the effect of Dog Appeasing Pheromone (DAP) on anxiety related behaviour of problem dogs in the veterinary clinic. *Applied Animal Behaviour Science*, *98*(1), 114-126.

Mkonyi, F. J., Estes, A. B., Msuha, M. J., Lichtenfeld, L. L., & Durant, S. M. (2017). Local Attitudes and Perceptions Toward Large Carnivores in a Human-Dominated Landscape of Northern Tanzania. *Human Dimensions of Wildlife*, *22*(4), 314-330.

Moehrenschlager, A., & Somers, M. (2004). Canid reintroductions and metapopulation management. *Canids: Foxes, Wolves, Jackals and Dogs. Status Survey and Conservation Action Plan*, 289-297.

Mogensen, T. H. (2009). Pathogen Recognition and Inflammatory Signaling in Innate Immune Defenses. *Clinical microbiology reviews*, *22*(2), 240-273.

Mombaerts, P., Wang, F., Dulac, C., Chao, S. K., Nemes, A., Mendelsohn, M., . . . Axel, R. (1996). Visualizing an olfactory sensory map. *Cell*, *87*(4), 675-686.

Monfort, S. L., Wasser, S. K., Mashburn, K. L., Burke, M., Brewer, B. A., & Creel, S. R. (1997). Steroid metabolism and validation of noninvasive endocrine monitoring in the African wild dog (*Lycaon pictus*). *Zoo Biology*, *16*(6), 533-548.

Montoya, E. R., Montoya, E. R., Terburg, D., Terburg, D., Bos, P. A., Bos, P. A., . . . van Honk, J. (2012). Testosterone, cortisol, and serotonin as key regulators of social aggression: A review and theoretical perspective. *Motivation and Emotion*, *36*(1), 65-73.

Moueix, C. (2007). Genetic verification of multiple paternity in two free-roaming isolated populations of African wild dogs (*Lycaon pictus*). University of Pretoria, South Africa.

Mulreany, L. M., Cushing, A. C., & Ramsay, E. C. (2021). Canine Distemper and Parvovirus vaccination with Recombitek C3 in African wild dogs (*Lycaon pictus*). *Journal of Zoo and Wildlife Medicine*, *52*(4), 1229-1233.

Nel, L., Sabeta, C., Von Teichman, B., Jaftha, J., Rupprecht, C., & Bingham, J. (2005). Mongoose rabies in southern Africa: a re-evaluation based on molecular epidemiology. *Virus research*, *109*(2), 165-173.

Newell-Fugate, A. E., O. Nöthling, J., & J. Bertschinger, H. (2012). Seasonal changes in steroid hormone profiles, body weight, semen quality, and the reproductive tract in captive African wild dogs (*Lycaon pictus*) in South Africa. *General and Comparative Endocrinology*, *178*(2), 272-281.

Nicholson, S. K., Marneweck, D. G., Lindsey, P. A., Marnewick, K., & Davies-Mostert, H. T. (2020). A 20-Year Review of the Status and Distribution of African Wild Dogs (*Lycaon pictus*) in South Africa. *African journal of wildlife research*, *50*(1), 8-19.

Niimura, Y., Matsui, A., & Touhara, K. (2014). Extreme expansion of the olfactory receptor gene repertoire in African elephants and evolutionary dynamics of orthologous gene groups in 13 placental mammals. *Genome research*, *24*(9), 1485-1496.

O'Neal, D. M., Reichard, D. G., Pavilis, K., & Ketterson, E. D. (2008). Experimentally-elevated testosterone, female parental care, and reproductive success in a songbird, the Dark-eyed Junco (*Junco hyemalis*). *Hormones and Behavior*, *54*(4), 571-578.

O'Riain, M. J., Davies-Mostert, H. T., & Potgieter, K. R. (2015). Behavioural cues can be used to predict the outcome of artificial pack formation in African wild dogs (*Lycaon pictus*). *African journal of wildlife research*, *45*(2), 215-222.

Okello, J. B. A., Wittemyer, G., Rasmussen, H. B., Arctander, P., Nyakaana, S., Douglas-Hamilton, I., & Siegismund, H. R. (2008). Effective population size dynamics reveal impacts of historic climatic events and recent anthropogenic pressure in African elephants. *Molecular Ecology*, *17*(17), 3788-3799.

Olds, J. E. (2017). Use of oral fluoxetine for the treatment of abnormal aggression in two red-necked wallabies (*macropus rufogriseus*). *Journal of Zoo and Wildlife Medicine*, *48*(3), 922-924.

Ortega, A. C., Dwinnell, S. P., Lasharr, T. N., Jakopak, R. P., Denryter, K., Huggler, K. S., . . . Monteith, K. L. (2020). Effectiveness of Partial Sedation to Reduce Stress in Captured Mule Deer. *The Journal of wildlife management*, *84*(8), 1445-1456.

Ortiz-Leal, I., Torres, M. V., Barreiro-Vázquez, J.-D., López-Beceiro, A., Fidalgo, L., Shin, T., & Sanchez-Quinteiro, P. (2024). The vomeronasal system of the wolf (*Canis lupus signatus*): The singularities of a wild canid. *Journal of anatomy*.

Packer, C., Sindimwo, A., Goodall, J., & Collins, D. A. (1995). Reproductive constraints on aggressive competition in female baboons. *Nature*, *373*(6509), 60-63.

Pageat, P. (2002). Animal appeasing pheromones. In: Google Patents.

Pageat, P., & Gaultier, E. (2003). Current research in canine and feline pheromones. *The Veterinary clinics of North America. Small animal practice*, *33*(2), 187-211.

Pageat, P., Mengoli, M., & Cozzi, A. (2014). Using Feline pheromones: from pet cats to wild Felids. In: September.

Palomares, F., Caro, T. M., Associate Editors: John, A. B., & Holt, R. D. (1999). Interspecific Killing among Mammalian Carnivores. *The American Naturalist*, *153*(5), 492-508.

Paris, D. B. B. P., Riddell, P., Joone, C., de la Rey, M., Ganswindt, A., & Paris, M. C. J. (2024). Cold Dogs: sperm freezing, artificial insemination & non-invasive monitoring tools to facilitate a hybrid conservation management approach for endangered African wild dogs. *Theriogenology Wild*, 100073.

Pasquali, R. (2012). The hypothalamic–pituitary–adrenal axis and sex hormones in chronic stress and obesity: pathophysiological and clinical aspects. *Annals of the New York Academy of Sciences*, *1264*(1), 20-35.

Peterson, R. O., Thomas, N. J., Thurber, J. M., Vucetich, J. A., & Waite, T. A. (1998). Population Limitation and the Wolves of Isle Royale. *Journal of Mammalogy*, *79*(3), 828-841.

Phillips, M. (1995). Conserving the red wolf. *Canid News*, *3*, 13-17.

Plant, T. M., & Zeleznik, A. J. (2014). *Knobil and Neill's physiology of reproduction*. Academic Press.

Pomilia, M. A., McNutt, J. W., & Jordan, N. R. (2015). Ecological Predictors of African Wild Dog Ranging Patterns in Northern Botswana. *Journal of Mammalogy*, *96*(6), 1214-1223.

Pongrácz, P., Lenkei, R., Marx, A., & Faragó, T. (2017). Should I whine or should I bark? Qualitative and quantitative differences between the vocalizations of dogs with and without separation-related symptoms. *Applied Animal Behaviour Science*, *196*, 61-68.

Potgieter, K. R., O'Riain, M. J., & Davies-Mostert, H. T. (2015). Behavioural cues can be used to predict the outcome of artificial pack formation in African wild dogs (*Lycaon pictus*). *African journal of wildlife research*, *45*(2), 215-222.

Poulsen, E. M., Honeyman, V., Valentine, P., & Teskey, G. (1996). Use of fluoxetine for the treatment of stereotypical pacing behavior in a captive polar bear. *Journal-American Veterinary Medical Association*, *209*, 1470-1474.

Prager, K., Mazet, J. A., Dubovi, E. J., Frank, L. G., Munson, L., Wagner, A. P., & Woodroffe, R. (2012). Rabies virus and canine distemper virus in wild and domestic carnivores in Northern Kenya: Are domestic dogs the reservoir? *EcoHealth*, *9*(4), 483-498.

Puglisi, I., Masucci, M., Cozzi, A., Teruel, E., Navarra, M., Cirmi, S., . . . Siracusa, C. (2022). Effects of a Novel Gel Formulation of Dog Appeasing Pheromone (DAP) on Behavioral and Physiological Stress Responses in Dogs Undergoing Clinical Examination. *Animals (Basel)*, *12*(18), 2472.

Putman, P., Putman, P., Antypa, N., Antypa, N., Crysovergi, P., Crysovergi, P., . . . van der Does, W. A. J. (2010). Exogenous cortisol acutely influences motivated decision making in healthy young men. *Psychopharmacology*, *208*(2), 257-263.

Quan, N., Avitsur, R., Stark, J. L., He, L., Shah, M., Caligiuri, M., . . . Sheridan, J. F. (2001). Social stress increases the susceptibility to endotoxic shock. *Journal of Neuroimmunology*, *115*(1), 36-45.

Quervel-Chaumette, M., Faerber, V., Faragó, T., Marshall-Pescini, S., & Range, F. (2016). Investigating Empathy-Like Responding to Conspecifics' Distress in Pet Dogs. *PLoS one*, *11*(4), e0152920-e0152920.

Raath, J. P., Mills, M. G., Gorman, M. L., & Speakman, J. R. (1998). High hunting costs make African wild dogs vulnerable to kleptoparasitism by hyaenas. *Nature*, *391*(6666), 479-481.

Randall, D. A., Williams, S. D., Kuzmin, I. V., Rupprecht, C. E., Tallents, L. A., Tefera, Z., . . . Sillero-Zubiri, C. (2004). Rabies in endangered Ethiopian wolves. *Emerging infectious diseases*, *10*(12), 2214.

Randall, D., Marino, J., Haydon, D., Sillero-Zubiri, C., Knobel, D., Tallents, L., . . . Laurenson, M. (2006). An integrated disease management strategy for the control of rabies in Ethiopian wolves. *Biological Conservation*, *131*(2), 151-162.

Ressler, K. J., Sullivan, S. L., & Buck, L. B. (1993). A zonal organization of odorant receptor gene expression in the olfactory epithelium. *Cell*, *73*(3), 597-609.

Riddell, P., Paris, M. C. J., Joonè, C. J., Pageat, P., & Paris, D. B. B. P. (2021). Appeasing Pheromones for the Management of Stress and Aggression during Conservation of Wild Canids: Could the Solution Be Right under Our Nose? *Animals*, *11*(6), 1574.

Robbins, R. (2000). Vocal communication in free-roaming African wild dogs (*Lycaon pictus*). *Behaviour*, *137*(10), 1271-1298.

Robbins, R., & McCreery, E. K. (2003). African wild dog pup vocalizations with special reference to Morton's model. *Behaviour*, *140*(3), 333-351.

Robles, T. F., Glaser, R., & Kiecolt-Glaser, J. K. (2005). Out of Balance: A New Look at Chronic Stress, Depression, and Immunity. *Current Directions in Psychological Science*, *14*(2), 111-115.

Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology & Evolution*, *19*(5), 249-255. https://doi.org/10.1016/j.tree.2004.03.008

Rosado, B., García-Belenguer, S., León, M., Chacón, G., Villegas, A., & Palacio, J. (2011). Effect of fluoxetine on blood concentrations of serotonin, cortisol and dehydroepiandrosterone in canine aggression. *Journal of veterinary pharmacology and therapeutics*, *34*(5), 430-436.

Salazar, I., Sánchez-Quinteiro, P., Alemañ, N., & Prieto, D. (2008). Anatomical, immnunohistochemical and physiological characteristics of the vomeronasal vessels in cows and their possible role in vomeronasal reception. *Journal of anatomy*, *212*(5), 686-696.

Saltzman, W., & Abbott, D. H. (2009). Effects of elevated circulating cortisol concentrations on maternal behavior in common marmoset monkeys (*Callithrix jacchus*). *Psychoneuroendocrinology*, *34*(8), 1222-1234.

Santos, N. R., Beck, A., Blondel, T., Maenhoudt, C., & Fontbonne, A. (2020). Influence of dog-appeasing pheromone on canine maternal behaviour during the peripartum and neonatal periods. *Veterinary record*, *186*(14), 449-449.

Sapolsky, R. M. (1992). *Stress, the aging brain, and the mechanisms of neuron death*. The MIT Press.

Sapolsky, R. M., Romero, L. M., & Munck, A. U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocrine reviews*, *21*(1), 55-89.

Scheepers, J. L., & Venzke, K. A. E. (1995). Attempts to re-introduce African wild dogs *Lycaon pictus* into Entosha National Park, Namibia. *South African Journal of Wildlife Research*, *25*, 138-140.

Seddon, J. M., & Ellegren, H. (2002). MHC class II genes in European wolves: a comparison with dogs. *Immunogenetics*, *54*(7), 490-500.

Seddon, P. J., Armstrong, D. P., & Maloney, R. F. (2007). Developing the Science of Reintroduction Biology. *Conservation biology*, *21*(2), 303-312.

Sergestrom, S. C., & Miller, G. E. (2004). Psychological stress and the human immune system: meta-analytic study of 30 years of inquiry. *Psychological Bulletin*, *130*(4), 601-630.

Seyfarth, R. M., Cheney, D. L., & Marler, P. (1980). Monkey responses to three different alarm calls: evidence of predator classification and semantic communication. *Science*, *210*(4471), 801-803.

Sheriff, M. J., Dantzer, B., Delehanty, B., Palme, R., & Boonstra, R. (2011). Measuring stress in wildlife: techniques for quantifying glucocorticoids. *Oecologia*, *166*(4), 869-887.

Shier, D. M. (2006). Effect of Family Support on the Success of Translocated Black-Tailed Prairie Dogs. *Conservation biology*, *20*(6), 1780-1790.

Shine, R., Reed, R. N., Shetty, S., Lemaster, M., & Mason, R. T. (2002). Reproductive isolating mechanisms between two sympatric sibling species of sea snakes. *Evolution*, *56*(8), 1655-1662.

Sillero-Zubiri, C., King, A. A., & Macdonald, D. W. (1996). Rabies and mortality in Ethopian wolves (*Canis simensis*). *Journal of wildlife diseases*, *32*(1), 80-86.

Sillero-Zubiri, C., Hoffmann, M., & Macdonald, D. W. (2004). *Canids: foxes, wolves, jackals, and dogs: status survey and conservation action plan*. IUCN Gland, Switzerland.

Silverman, A. (1988). The gonadotropin-releasing hormone (GnRH) neuronal systems: immunocytochemistry. *The physiology of reproduction*.

Simonis, J. L., Harrison, R. B., Long, S. T., Rabon Jr, D. R., Waddell, W. T., & Faust, L. J. (2018). Managed movement increases metapopulation viability of the endangered red wolf. *The Journal of Wildlife Management*, *82*(3), 573-582.

Simpson, B. S., & Papich, M. G. (2003). Pharmacologic management in veterinary behavioral medicine. *The Veterinary clinics of North America. Small animal practice*, *33*(2), 365-404.

Siracusa, C., Manteca, X., Cuenca, R., del Mar Alcalá, M., Alba, A., Lavín, S., & Pastor, J. (2010). Effect of a synthetic appeasing pheromone on behavioral, neuroendocrine, immune, and acute-phase perioperative stress responses in dogs. *Journal of the American Veterinary Medical Association*, *237*(6), 673-681.

Sorensen, P. W., & Baker, C. (2015). Species-specific pheromones and their roles in shoaling, migration, and reproduction: a critical review and synthesis. *Fish pheromones and related cues*, *2015*, 11-32.

Speakman, J. R., Gorman, M. L., Mills, M. G. L., & Raath, J. P. (2016). Wild dogs and kleptoparasitism: some misunderstandings. *African journal of ecology*, *54*(1), 125-127.

Spehr, M., Hatt, H., & Wetzel, C. H. (2002). Arachidonic acid plays a role in rat vomeronasal signal transduction. *Journal of neuroscience*, *22*(19), 8429-8437.

Spehr, M., Kelliher, K. R., Li, X.-H., Boehm, T., Leinders-Zufall, T., & Zufall, F. (2006). Essential Role of the Main Olfactory System in Social Recognition of Major Histocompatibility Complex Peptide Ligands. *Journal of Neuroscience*, *26*(7), 1961-1970.

Spiering, P. A., Somers, M. J., Maldonado, J. E., Wildt, D. E., & Gunther, M. S. (2010). Reproductive Sharing and Proximate Factors Mediating Cooperative Breeding in the African Wild Dog (*Lycaon pictus*). *Behavioral Ecology and Sociobiology*, *64*(4), 583-592.

Spiering, P. A., Spiering, P. A., Szykman Gunther, M., Szykman Gunther, M., Somers, M. J., Somers, M. J., . . . Maldonado, J. E. (2011). Inbreeding, heterozygosity and fitness in a reintroduced population of endangered African wild dogs (*Lycaon pictus*). *Conservation Genetics*, *12*(2), 401-412.

Stammen, R. L., Cohen, J. K., Meeker, T. L., Crane, M. M., Amara, R. R., Hicks, S. L., . . . Ethun, K. F. (2018a). Effect of Chronic Social Stress on Prenatal Transfer of Antitetanus Immunity in Captive Breeding Rhesus Macaques (*Macaca mulatta*). *Journal of the American Association for Laboratory Animal Science : JAALAS*, *57*(4), 357-367.

Stammen, R. L., Cohen, J. K., Meeker, T. L., Crane, M. M., Amara, R. R., Hicks, S. L., . . . Ethun, K. F. (2018b). Effect of chronic social stress on prenatal transfer of antitetanus immunity in captive breeding rhesus macaques (macaca mulatta). *Journal of the American Association for Laboratory Animal Science*, *57*(4), 357-367.

Stern, K., & McClintock, M. K. (1998). Regulation of ovulation by human pheromones. *Nature*, *392*(6672), 177-179.

Stewart, A. M., Roy, S., Wong, K., Gaikwad, S., Chung, K. M., & Kalueff, A. V. (2015). Cytokine and endocrine parameters in mouse chronic social defeat: Implications for translational 'cross-domain' modeling of stress-related brain disorders. *Behavioural Brain Research*, *276*, 84-91.

Stowell, D. (2022). Computational bioacoustics with deep learning: a review and roadmap. *PeerJ (San Francisco, CA)*, *10*, e13152-e13152. https://doi.org/10.7717/peerj.13152

Sundqvist, C., Lukola, A., & Valtonen, M. (1984). Relationship between serum testosterone concentrations and fertility in male mink (*Mustela vison*). *Reproduction*, *70*(2), 409-412.

Takahashi, A., Flanigan, M. E., McEwen, B. S., & Russo, S. J. (2018). Aggression, social stress, and the immune system in humans and animal models. *Frontiers in Behavioral Neuroscience*, *12*, 56.

Takeuchi, Y., Houpt, K. A., & Scarlett, J. M. (2000). Evaluation of treatments for separation anxiety in dogs. *Journal of the American Veterinary Medical Association*, *217*(3), 342-345.

Taniguchi, M., Wang, D., & Halpern, M. (2000). Chemosensitive conductance and inositol 1, 4, 5-trisphosphate-induced conductance in snake vomeronasal receptor neurons. *Chemical senses*, *25*(1), 67-76.

Taylor, K., & Mills, D. S. (2007). A placebo-controlled study to investigate the effect of Dog Appeasing Pheromone and other environmental and management factors on the reports of disturbance and house soiling during the night in recently adopted puppies (*Canis familiaris*). *Applied Animal Behaviour Science*, *105*(4), 358-368.

Teixeira, C. P., de Azevedo, C. S., Mendl, M., Cipreste, C. F., & Young, R. J. (2007). Revisiting translocation and reintroduction programmes: the importance of considering stress. *Animal behaviour*, *73*(1), 1-13.

Templeton, A. R., Shaw, K., Routman, E., & Davis, S. K. (1990). The genetic consequences of habitat fragmentation. *Annals of the Missouri Botanical Garden*, 13-27.

Tensen, L., Groom, R. J., van Belkom, J., Davies-Mostert, H. T., Marnewick, K., & Jansen van Vuuren, B. (2016). Genetic diversity and spatial genetic structure of African wild dogs (*Lycaon pictus*) in the Greater Limpopo transfrontier conservation area. *Conservation Genetics*, *17*(4), 785-794.

Tensen, L., Currat, M., Davies-Mostert, H., Plessis, C. d., & Fischer, K. (2024). Genetic surfing during the range expansion of an endangered large carnivore. *Biodiversity and conservation*, *33*(1), 361-378.

Thompson, W. G. (2013). *Pheromones and intermones change heart rate and behaviour of anxious dogs* Texas Tech University.

Tirindelli, R., Dibattista, M., Pifferi, S., & Menini, A. (2009). From Pheromones to Behavior. *Physiological Reviews*, *89*(3), 921-956.

Torres-Calleja, J., Gonzalez-Unzaga, M., DeCelis-Carrillo, R., Calzada-Sanchez, L., & Pedron, N. (2001). Effect of androgenic anabolic steroids on sperm quality and serum hormone levels in adult male bodybuilders. *Life sciences*, *68*(15), 1769-1774.

Trinkel, M., Ferguson, N., Reid, A., Reid, C., Somers, M., Turelli, L., . . . Slotow, R. (2008). Translocating lions into an inbred lion population in the Hluhluwe-iMfolozi Park, South Africa. *Animal conservation*, *11*(2), 138-143.

Troisi, A., & D'Amato, F. R. (1994). Mechanisms of primate infant abuse: the maternal anxiety hypothesis. In S. Parmigiani & F. vom Saal (Eds.), *Infanticide and parental care* (pp. 199-210). Routledge.

Uchida, Y., Dodman, N., & DeGhetto, D. (1998). Animal Behavior Case of the Month. A Captive Bear Was Observed to Exhibit Signs of Separation Anxiety, Decreased Fear of Human Beings, and Stereotypical Activity. *Journal of the American Veterinary Medical Association*, *212*(3), 354-355.

Van den Berghe, F., Paris, D. B. B. P., Van Soom, A., Rijsselaere, T., Van der Weyde, L., Bertschinger, H. J., & Paris, M. C. J. (2012). Reproduction in the endangered African wild dog: Basic physiology, reproductive suppression and possible benefits of artificial insemination. *Animal Reproduction Science*, *133*(1–2), 1-9.

Van den Berghe, F., Paris, M. C. J., Sarnyai, Z., Briggs, M. B., Millar, R. P., Ganswindt, A., & Paris, D. B. B. P. (2018a). Social Dominance does not Affect Semen Quality in African Wild Dogs (Lycaon pictus). *Reproduction, Fertility and Development*, *30*(1), 188. https://doi.org/10.1071/RDv30n1Ab97

Van den Berghe, F., James Cook University. College of Public Health, M., & Veterinary, S. (2018b). *A conservation management toolkit: developing assisted breeding and behavioural management tools for the African wild dog (Lycaon pictus)* (Publication Number Dissertation/Thesis)

Van den Berghe, F., Paris, M. C. J., Sarnyai, Z., Briggs, M. B., Millar, R. P., Ganswindt, A., & Paris, D. B. B. P. (2019a). Social rank does not affect sperm quality in male African wild dogs (Lycaon pictus). *Reproduction, fertility, and development*, *31*(5), 875.

Van den Berghe, F., Paris, M. C. J., Sarnyai, Z., Vlamings, B., Millar, R. P., Ganswindt, A., . . . Paris, D. B. B. P. (2019b). Dog appeasing pheromone prevents the androgen surge and may reduce contact dominance and active submission after stressful interventions in African wild dogs (Lycaon pictus). *PLoS one*, *14*(3), e0212551.

Van den Berghe, F., Paris, M. C. J., Sarnyai, Z., Ganswindt, A., & Paris, D. B. B. P. (2023). Periovulatory changes in behavior and fecal hormone metabolite concentrations but not vaginal cytology or vaginoscopy are indicative for the fertile period in female African wild dogs (Lycaon pictus). *Theriogenology Wild (Online)*, *2*, 100032.

Van der Weyde, L. K. (2013). *Reproductive Biology of the Endangered African Wild Dog (Lycaon pictus) in Captivity and Free-roaming Populations: an Endocrine, Behavioural and Demographic Approach* University of Western Australia].

Van der Weyde, L. K., Martin, G. B., & Paris, M. C. J. (2015). Monitoring stress in captive and free-roaming African wild dogs (Lycaon pictus) using faecal glucocorticoid metabolites. *General and Comparative Endocrinology*, *226*, 50-55.

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

van Kesteren, F., Sillero-Zubiri, C., Millar, R., Argaw, K., Macdonald, D. W., & Paris, M. (2012). Sex, stress and social status: patterns in fecal testosterone and glucocorticoid metabolites in male Ethiopian wolves. *General and comparative endocrinology*, *179*(1), 30-37.

Vassar, R., Ngai, J., & Axel, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell*, *74*(2), 309-318.

Viau, V. (2002). Functional cross-talk between the hypothalamic-pituitary-gonadal and -adrenal axies. *Journal of Neuroendocrinology*, *14*, 506-513.

Vlamings, B. H. A. C. (2011). *Dog appeasing pheromone®: A useful tool to minimize stress and aggression of African wild dogs (Lycaon picuts)?* University of Utrecht]. the Netherlands.

Von Campenhausen, H., & Mori, K. (2000). Convergence of segregated pheromonal pathways from the accessory olfactory bulb to the cortex in the mouse. *European Journal of Neuroscience*, *12*(1), 33-46.

Vrekoussis, T., Kalantaridou, S. N., Mastorakos, G., Zoumakis, E., Makrigiannakis, A., Syrrou, M., . . . Chrousos, G. P. (2010). The role of stress in female reproduction and pregnancy: an update. *Annals of the New York Academy of Sciences*, *1205*(1), 69-75.

Vucetich, J. A., & Creel, S. (1999). Ecological interactions, social organisation and extinction risk in African wild dogs. *Conservation biology*, *13*, 1172-1182.

Walker, R. H., King, A. J., McNutt, J. W., & Jordan, N. R. (2017). Sneeze to leave: African wild dogs (Lycaon pictus) use variable quorum thresholds facilitated by sneezes in collective decisions. *Proceedings of the Royal Society. B, Biological sciences*, *284*(1862), 20170347-20170347.

Wang, Z., Balet Sindreu, C., Li, V., Nudelman, A., Chan, G. C. K., & Storm, D. R. (2006). Pheromone Detection in Male Mice Depends on Signaling through the Type 3 Adenylyl Cyclase in the Main Olfactory Epithelium. *Journal of Neuroscience*, *26*(28), 7375-7379.

Waters, S. (2010). Swift fox Vulpes velox reintroductions: a review of release protocols. *International Zoo Yearbook*, *44*(1), 173-182.

Wayne, R. K., Geffen, E., Girman, D. J., Koepfli, K. P., Lau, L. M., & Marshall, C. R. (1997). Molecular Systematics of the Canidae. *Systematic Biology*, *46*(4), 622-653.

Webster, H. S. (2009). Vocal communication and cognitive abilities in a fugitive species: the African wild dog. Sussex University.

Webster, H., McNutt, J. W., & McComb, K. (2012). African Wild Dogs as a Fugitive Species: Playback Experiments Investigate How Wild Dogs Respond to their Major Competitors: African Wild Dogs as a Fugitive Species. *Ethology*, *118*(2), 147-156.

Weise, T. F. (1975). *An experimental translocation of the eastern timber wolf*. National Audubon Society.

Weiss, I., Hofferberth, J., Ruther, J., & Stökl, J. (2015). Varying importance of cuticular hydrocarbons and iridoids in the species-specific mate recognition pheromones of three closely related Leptopilina species. *Frontiers in ecology and evolution*, *3*, 19.

Wells, J. V., & Richmond, M. E. (1995). Populations, metapopulations, and species populations: what are they and who should care? *Wildlife Society Bulletin (1973-2006)*, *23*(3), 458-462.

Whittington-Jones, B. M., Parker, D. M., Bernard, R. T. F., & Davies-Mostert, H. T. (2014). Habitat Selection by Transient African Wild Dogs (Lycaon pictus) in Northern KwaZulu-Natal, South Africa: Implications for Range Expansion. *South African Journal of Wildlife Research*, *44*(2), 135-147.

Wild Dog Advisory Group (2023, 25 October 2023). Update on the Wild Dogs of Southern Africa. the Wild Dog Advisory Group (WAG) meeting, the Endangered Wildlife Trust (EWT) Conservation Campus and online.

Williamson, S. R. (2013). *North American regional studbook: African wild dog (Lycaon pictus)*.

Wilson, D. R., & Hare, J. F. (2006). The adaptive utility of Richardsons ground squirrel (Spermophilus richardsonii) short-range ultrasonic alarm signals. *Canadian journal of zoology*, *84*(9), 1322-1330.

Windham, A. M., Rosenberg, L., Fuddy, L., McFarlane, E., Sia, C., & Duggan, A. K. (2004). Risk of mother-reported child abuse in the first 3 years of life. *Child abuse & neglect*, *28*(6), 645-667.

Wingfield, J. C., & Ramenofsky, M. (1999). *Hormones and the behavioural ecology of stress (P. H. M. Baum ed.)*. CRC Press

Wingfield, J. C., & Sapolsky, R. M. (2003). Reproduction and Resistance to Stress: When and How. *Journal of Neuroendocrinology*, *15*(8), 711-724.

Wolf, C. M., Griffith, B., Reed, C., & Temple, S. A. (1996). Avian and mammalian translocations: update and reanalysis of 1987 survey data. *Conservation biology*, *10*(4), 1142-1154.

Woodford, M. H., & Rossiter, P. B. (1994). Disease risks associated with wildlife translocation projects. In P. J. S. Olney, G. M. Mace, & A. T. C. Feistner (Eds.), *Creative Conservation: Interactive management of wild and captive animals* (pp. 178-200). Springer Netherlands.

Woodroffe, R., Ginsberg, J., & Macdonald, D. (1997). *The African Wild Dog*. IUCN.

Woodroffe, R., & Ginsberg, J. R. (1998). Edge Effects and the Extinction of Populations Inside Protected Areas. *Science*, *280*(5372), 2126-2128.

Woodroffe, R., & Ginsberg, J. R. (1999). Conserving the African wild dog Lycaon pictus. II. Is there a role for reintroduction? *Oryx*, *33*(2), 143-151.

Woodroffe, R. (2001). Assessing the risks of intervention: immobilization, radio-collaring and vaccination of African wild dogs. *Oryx*, *35*(3), 234-244.

Woodroffe, R., Davies-Mostert, H., Ginsberg, J., Graf, J., Leigh, K., McCreery, K., . . . Szykman, M. (2007). Rates and causes of mortality in Endangered African wild dogs Lycaon pictus: lessons for management and monitoring. *Oryx*, *41*(2), 215-223.

Woodroffe, R., Prager, K. C., Munson, L., Conrad, P. A., Dubovi, E. J., & Mazet, J. A. K. (2012). Contact with domestic dogs increases pathogen exposure in endangered African wild dogs (Lycaon pictus). *PLoS one*, *7*(1), e30099.

Woodroffe, R., & Sillero-Zubric, C. (2012). *Lycaon pictu. The IUCN Red List of Threatened Species 2012: e.T12436A16711116. The IUCN Red List of Threatened Species 2012.*

Woodroffe, R., & Sillero-Zubiri, C. (2020). *Lycaon pictus (amended version of 2012 assessment). The IUCN Red List of Threatened Species: e.T12436A166502262*. IUCN. Retrieved 14 April from https://www.iucnredlist.org/species/12436/16711116

Woodroffe, R. (2021). Modified live distemper vaccines carry low mortality risk for captive African wild dogs, Lycaon pictus. *Journal of Zoo and Wildlife Medicine*, *52*(1), 176-184.

Woodroffe, R. (2022, 14 February). 20 years of wild dog research in Kenya. African wild dogs United, Online.

Wyatt, T. D. (2017). Pheromones. *Current Biology*, *27*(15), R739-R743.

Yalcin, E., & Aytug, N. (2007). Use of fluoxetine to treat stereotypical pacing behavior in a brown bear (Ursus arctos). *Journal of Veterinary Behavior*, *2*(3), 73-76.

Yin, S., & McCowan, B. (2004). Barking in domestic dogs: context specificity and individual identification. *Animal behaviour*, *68*(2), 343-355.

Yohe, L. R., Suzuki, H., & Lucas, L. R. (2012). Aggression is suppressed by acute stress but induced by chronic stress: Immobilization effects on aggression, hormones, and cortical 5-HT1B/ striatal dopamine D2 receptor density. *Cognitive, Affective, & Behavioral Neuroscience*, *12*(3), 446-459.

Yonezawa, T., Koori, M., Kikusui, T., & Mori, Y. (2009). Appeasing Pheromone Inhibits Cortisol Augmentation and Agonistic Behaviors During Social Stress in Adult Miniature Pigs. *Zoological Science*, *26*(11), 739-744.

Yoon, H., Enquist, L. W., & Dulac, C. (2005). Olfactory Inputs to Hypothalamic Neurons Controlling Reproduction and Fertility. *Cell*, *123*(4), 669-682. https://doi.org/10.1016/j.cell.2005.08.039

Yordy, J., & Mossotti, R. H. (2016). Kinship, maternal effects, and management: Juvenile mortality and survival in captive African painted dogs, Lycaon pictus. *Zoo Biology*, *35*(5), 367-377.

Zancanaro, C., Caretta, C. M., Bolner, A., Sbarbati, A., Nordera, G. P., & Osculati, F. (1997). Biogenic amines in the vomeronasal organ. *Chemical senses*, *22*(4), 439-445.

Zuur, A. F. (2009). *Mixed effects models and extensions in ecology with R* (1st ed. 2009. ed.). Springer.