UNIVERSIDADE DE LISBOA

Faculdade de Medicina Veterinária

VECTOR-BORNE PATHOGENS FOUND IN CARNIVORES IN WILD NAMIBIA

MARIA CAROLINA REGATEIRO MACHADO E COSTA

CONSTITUIÇÃO DO JÚRI:

Doutor Virgílio da Silva Almeida

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ORIENTADOR

Doctor Georg von Samson-Himmelstjerna

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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To H, who first introduced me to the magic and wonders of animals

Acknowledgements

I would like to thank first and foremost to Doctor Jürgen Krücken, my closest supervisor at the Institut für Parasitologie und Tropenveterinärmedizin in Berlin for all the patience and explanations and all the help he provided me with. His wisdom and guidance were crucial to this dissertation and my knowledge has grown immensely whilst under his wing.

To my supervisor Professor Doctor Georg von Samson-Himmelstjerna I am deeply grateful, for welcoming me to his incredible laboratory, giving me a chance to work alongside some incredible scientists, even though I didn't even speak German. Thank you for investing in me.

I would also like to express my wholehearted appreciation for Professor Doctor Luís Madeira de Carvalho, for always believing in me and in my abilities. Thank you so much for all the support and friendship.

I also want to thank Flávia and Mariana, my Portuguese speaking "partners in crime" in Berlin, without whom my experience would definitely not have been the same. Flávia for not only mentoring me, but also for teaching me persistence and how to see the good things in life, but most importantly for all the special memories in this brand new really cold city. And Mariana, for bringing joy and light and always laughing with me, even when we were way out of our depth and didn't know what we were doing. I am also really grateful for everyone at the Institute, especially Sabrina and all the PhD students, with their good disposition and patience for all of my questions and doubts and for teaching me and giving me some of their time without a second thought. Monika and Catarina as well, for being a part of this project and doing some of the work. And lastly I would like to thank Bettina Wachter, Gabor Czirják, Marion East and Ortwin Aschenborn, without them this project would have never existed.

To my girls from FMV, Bina, Tina, Lenka, Mer e Bea for putting up with me. I have grown, changed and been through a lot during this past few years but you have made everything better and easier, and I could not have asked for better company and friends. Words cannot express how lucky I am to have you in my life and to have shared this journey with you, forever as minhas linduxas.

To my incredible friends Rita, Bea, Mary, Ana, Mafaldas, Herberto, Miguel, Monty e Bernardo, for all the love and support and for accepting me exactly as I am, weirdness and all. And to everyone else who has crossed my life and contributed somehow to the person I am today, I am forever grateful.

And lastly to my family, for your unconditional love. Thank you Avó, for not understanding what I was doing, but still supporting me through it. Thank you Isinha, Nena and Lu for becoming a part of my family, even though you were not in it initially. Thank you Pai for your constant support, interest and for showing me that constantly wanting to know more is perfectly normal. Thank you Henrique for shining a light on me from wherever you are. Thank you Maggie for being the best sister I could have asked for, and for being weird and random with me. And lastly, thank you Mãe, my biggest rock, for raising me and giving everything you had to your children, for being the most incredible person I know and one of my major inspirations. Love every single one of you with all my heart.

This study was a partnership between the Institute for Parasitology and Tropical Veterinary Medicine of Freie Universität in Berlin, the Leibniz Institute for Zoo and Wildlife Research and the Research Training Group 2046 "Parasite Infections: From Experimental Models to Natural Systems"

Leibniz Institute for Zoo and Wildlife Research
IN THE FORSCHUNGSVERBUND BERLIN E.V.

Abstract

Vector-borne pathogens found in carnivores in wild Namibia

This dissertation aimed to identify and molecularly characterize vector-borne pathogens from several parasite families, all possessing stages found in peripheral blood, from a wide variety of free-ranging carnivores living in Namibia, in the southern part of Africa.

Blood samples collected from 9 bat-eared foxes (*Otocyon megalotis*), 17 brown hyenas (*Parahyaena brunnea*), 19 spotted hyenas (*Crocuta crocuta*) and 85 cheetahs (*Acinonyx jubatus*) were screened by Polymerase Chain Reactions (PCRs) and tested for pathogens of the Onchocercidae family, the order Piroplasmida, bacteria from the Anaplasmataceae and the Rickettsiaceae families and, lastly, the Hepatozoidae family. The PCRs targeted both the ITS-2 and 12S, 18S, 16S, 18S and 18S rRNA genes respectively and were followed by nucleotide sequencing.

In total, sampled animals showed a 43.1% rate of Onchocercidae infection, 67.7% of Piroplasmida, 60% of them were positive for Anaplasmataceae, 10% for Rickettsiaceae and Hepatozoidae were detected in 47.7% of them.

Obtained filaroid sequences showed high homologies with both *Acanthocheilonema reconditum* and *Acanthocheilonema dracunculoides* and further phylogenetic analysis were performed in both brown and spotted hyenas, with the construction of a phylogenetic tree. Piroplasmida results were not studied any further. For Anaplasmataceae, subsequent sequencing results indicated high similarity with both *Anaplasma phagocytophilum* and *Anaplasma platys* and varied PCR protocols were conducted in order to differentiate between these organisms, but no conclusions were reached. The Rickettsiaceae found displayed high homologies with *Rickettsia raoultii.* And finally, the Hepatozoidae infection showed to be a mixed one with both *Hepatozoon canis* and *Hepatozoon felis*.

These results are important not only on a conservation level for the infected host species, but are also relevant for domestic animals coexisting in the surrounding areas, as well as humans, especially since a few of the parasites found may have zoonotic potential. Future studies should focus on understanding vectors, transmission routes, infection dynamics and host specificity in order to better evaluate the possible danger these infections may withhold.

Key-words: Bat-eared fox; Brown hyena; Spotted hyena; Cheetah; Parasitology; Wildlife; Africa; Namibia; *Acanthocheilonema; Anaplasma; Rickettsia; Hepatozoon.*

Resumo

Agentes patogénicos transmitidos por vetores presentes em carnívoros na Namíbia

Esta dissertação teve como principal objetivo identificar e caracterizar molecularmente agentes patogénicos transmitidos por vetores de várias famílias parasitárias, com o aspeto em comum de todas possuírem fases do desenvolvimento encontradas no sangue, de espécies variadas de carnívoros selvagens que habitam na Namíbia, no Sul de África.

Foram testadas amostras sanguíneas de 9 raposas-orelhas-de-morcego (*Otocyon megalotis*), 17 hienas-castanhas (*Parahyaena brunnea*), 19 hienas-malhadas (*Crocuta crocuta*) e 85 chitas (*Acinonyx jubatus*) por PCR e analisadas para pesquisa de parasitas da família Onchocercidae, da ordem Piroplasmida, bactérias das famílias Anaplasmataceae e Rickettsiaceae e, finalmente, da família Hepatozoidae. Os PCRs foram direcionados aos genes do rRNA ITS-2 e 12S, 18S, 16S, 18S e 18S respetivamente e foram seguidos de sequenciação de nucleótidos.

Na totalidade, os animais testados mostraram uma taxa de infeção de 43.1% por Onchocercidae, de 67.7% de Piroplasmida, 60% deles tiveram resultados positivos para Anaplasmataceae, 10% para Rickettsiaceae e Hepatozoidae foram detetados em 47.7% da população.

As sequências obtidas de filarídeos, mostraram possuir elevadas homologias com *Acanthocheilonema reconditum* e *Acanthocheilonema dracunculoides,* e estudos filogenéticos mais intensivos foram realizados, nomeadamente uma árvore filogenética que inclui ambas as espécies de hienas. Os resultados relativos a Piroplasmida não foram aprofundados. Para as Anaplasmataceae, as sequenciações subsequentes indicaram elevada similaridade com *Anaplasma phagocytophilum* e *Anaplasma platys* e múltiplos protocolos de PCRs foram efetuados, com o intuito de diferenciar entre estas duas espécies, mas não foram retiradas quaisquer conclusões. As Rickettsiaceae presentes evidenciaram fortes semelhanças com *Rickettsia raoultii.* E finalmente, as infeções por Hepatozoidae mostraram ser uma infeção mista por ambos *Hepatozoon canis* e *Hepatozoon felis*.

A importância destes resultados não se limita apenas à conservação das espécies animais em causa, mas são também relevantes em termos dos animais domésticos coabitantes na mesma região, assim como humanos, especialmente tendo em conta o possível potencial zoonótico de algumas espécies parasitárias. Estudos futuros devem ter como principais objetivos o estudo dos vetores respetivos, tipo de transmissão, dinâmica da infeção e especificidade parasitária, para melhor avaliar os possíveis perigos que podem advir da presença destes parasitas.

Palavras-chave: Raposa-orelhas-de-morcego; Hiena-castanha; Hiena-malhada; Chita; Parasitologia; Vida selvagem; África; Namíbia; *Acanthocheilonema; Anaplasma; Rickettsia; Hepatozoon*.

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List of Abbreviations

µl – Microliter
µM – Micromole
BLAST – Basic Local Alignment Search Tool
BLASTn – Nucleotide-nucleotide Basic Local Alignment Search Tool
Bp – Base pair
CIs – Confidence intervals
CITES – Convention on International Trade in Endangered Species of Wild Fauna and Flora
DNA – Deoxyribonucleic nucleic acid
dsDNA – Double strand DNA
ssDNA – Single strand DNA
dNTP – Deoxyribonucleotide triphosphate
IUCN – International Union for Conservation of Nature
$MgCl_2$ – Magnesium Chloride
mM – Milimole
PCR – Polymerase Chain Reaction
RNA – Ribonucleic acid
rRNA – Ribosomal RNA
Sp. – Species
Spp. – Species plural
U/µl – Units per microlitre

1 – Internship Activities

This study resulted from the curricular internship that took place between the 18th September 2017 and the 27rd of March of 2018, at the Institut für Parasitologie und Tropenveterinärmedizin of Freie Universität, Berlin, Germany, under the direct supervision of Dr. Jürgen Krücken and Professor Georg von Samson-Himmelstjerna.

During the period mentioned above, the student performed a study of parasite infection in wild animals, based on PCR amplifications of specific gene fragments on blood samples from Bat-Eared Foxes, Brown and Spotted Hyenas and Cheetahs and directly supervised screenings performed on Cheetah samples. Then, she proceeded to clone the DNA obtained within several positive samples and sequenced them and with that information, she was able to analyse the different existent homologies and disparities.

Besides this project, the author still had the chance to learn other techniques being used by PhD colleagues at the Laboratory and actively participated on occurring events, such as the 28th Annual Meeting of the German Society for Parasitology, between the 21st and the 24th of March 2018, in Berlin.

2 – Introduction

Parasites are extremely prevalent worldwide (Kofer, Hofer, & Hartmann, 2017) and, since the pioneering work of Anderson & May (1978) in the late 1970's, there has been an increasing interest on the impact of diseases and parasites of wild animals at a population level (McCallum & Dobson, 1995; Thompson & Polley, 2015). However, the focus has been primarily on emerging diseases with zoonotic potential or those affecting domestic animals, generally of viral or bacterial origin (McCallum & Dobson, 1995; Holmes, 1996; Jones et al., 2008; Taylor, Latham, & Woolhouse, 2001; Thompson, Lymbery, & Smith, 2010; Rhyan & Spraker, 2010; Robertson, Utaaker, Goyal, & Sehgal, 2014), neglecting the biodiversity and ecology of wildlife parasites.

This means we have yet to gain a broader understanding of the normal parasite fauna of wildlife populations and how these emergent pathogens interact with the assemblage of cohabiting organisms in the ecosystem, since very little is known about their life cycles, transmission routes, host specificity, or pathogenic potential (Thompson et al., 2010; MacPhee & Greenwood, 2013; Kelly et al., 2014). Also, due to these close linkages between hosts, parasites, and ecosystem structure and function, host-parasite associations can be useful in the recognition and assessment of ecosystem and environmental disruption and instability, and this knowledge may help with optimizing global wildlife and overall planet Earth's health (Thompson et al., 2010; Polley & Thompson, 2015). Without improved and ongoing surveillance of wildlife hosts, not only will we always lag behind in terms of predicting the possibility of reservoirs being established and/or outbreaks occurring, but also at a disadvantage in preventing declines of native fauna resulting from infectious disease, situations which justify the increasingly importance of understanding the impact and transmission of parasites in wildlife populations (Polley, 2005; MacPhee & Greenwood, 2013; Watson, 2013; Seguel & Gottdenker, 2017).

Additionally, vector-borne pathogens of carnivores can be responsible for severe diseases in domestic animals, such as babesiosis (Matijatko, Torti, & Schetters, 2012; Solano-Gallego, Sainz, Roura, Estrada-Peña, & Miró, 2016) and heartworm disease (Dantas-Torres & Otranto, 2013; Hoch & Strickland, 2014). And whilst canine vector-borne diseases have received a lot of attention these past decades, felines are not very frequently investigated, are overall neglected (Otranto et al., 2009a, 2009b; Day, 2011; Otranto, 2018) and wild carnivores' diseases are only addressed if there is the possibility of vector interaction with either domestic animals or humans. Hypervirulent canine babesiosis in sub-Saharan Africa caused by *Babesia rossi*, of which wild canids such as side-striped jackals and wild dogs are natural reservoirs, is an example of the former (Penzhorn, 2011).

While we have a relatively good notion regarding the current impact of wildlife in highly industrialised areas, such as Europe or the U.S.A, the knowledge regarding vector-borne pathogens of wild carnivores in tropical and subtropical areas and its impact on the surrounding humans and domestic animals is quite limited, as well as their effects on the health and fitness of endangered species.

Increasing contact between domestic animals and wildlife is always expanding (Junker, Horak, & Penzhorn, 2015; Espinaze, Hellard, Horak, & Cumming, 2018), especially due to decreasing habitats for wildlife, anthropogenic factors and changes in the environment. This can also be dangerous for threatened wildlife species, since domestic animals might serve as reservoirs and amplifiers for vector-borne pathogens transmitted to wildlife (Daszak, Cunningham, & Hyatt, 2001; Czupryna et al., 2016; Espinaze et al., 2018). Furthermore, these novel pathogens can then become responsible for high mortality, decline and even local extinctions (Cleaveland, Laurenson, & Taylor, 2001; Aguirre, 2016; Van der Weyde, Mbisana, & Klein, 2018).

3 – Goals

The main goal of the current study was the identification and molecular characterization of several vector-borne pathogens of the family Onchocercidae, the order Piroplasmida, the family Hepatozoidae and bacteria from the Rickettsiales order and to further study their phylogenetic relationship with other related organisms.

4 – Bibliographic Review

4.1 – Bat-eared Fox

4.1.1 – Taxonomy and Distribution

Bat-eared Fox (*Otocyon megalotis*)
(Desmarest, 1822)
Kingdom – Animalia
Phylum – Chordata
Class – Mammalia
Order – Carnivora
Family – Canidae
Subfamily – Caninae
Genus – *Otocyon* (Müller, 1835)

Figure 1 - Bat-eared fox by Yathin Krishnappa. Image downloaded from https://en.wikipedia.org/wiki/Bat-eared_fox

Bat-eared foxes are the only species in the *Otocyon* genus, being set apart from the rest of the Canidae family by their different morphological characteristics, more specifically their dentition.
These animals are currently divided between two discrete subpopulations in eastern and southern Africa (representing each of the known subspecies) and they are both widespread and common in conservation areas (Dalerum, Roux, Vries, & Kamler, 2016). However, they are becoming uncommon in arid areas and on farms in South Africa where they are occasionally persecuted (Hoffmann, 2014). Subspecies *O. m. virgatus* ranges from southern Sudan, Ethiopia and Somalia down through Uganda and Kenya to southwestern Tanzania, whilst *O. m. megalotis* occurs from Angola through Namibia and Botswana to Mozambique and South Africa (Nel, J. A. J. and Maas, B. 2013; Hoffmann, 2014; Dalerum *et al.*, 2016). They generally dwell on semi-arid and arid habitats (Nel & Mackie, 1990; Thomson & Meredith, 1993), especially open grasslands, open scrub, *Acacia* savanna and shrublands (Kuntzsch and Nel, 1992; Nel, J. A. J. and Maas, B. 2013; Dalerum *et al.*, 2016).
According to the 2014 International Union for Conservation of Nature (IUCN) Red List of Threatened Species (http: //www.iucnredlist.org), they are regarded as “Least Concern” (Hoffmann, 2014) since they are occasionally persecuted, but there seem to be no main threats that can result in any major range-wide declines, affecting the population trend overall (Dalerum et al., 2016). Their current population is considered as “stable” and within a circumscribed habitat, numbers can fluctuate from abundant to rare depending on rainfall, food, breeding stage and disease (Hoffmann, 2014; Dalerum et al., 2016).

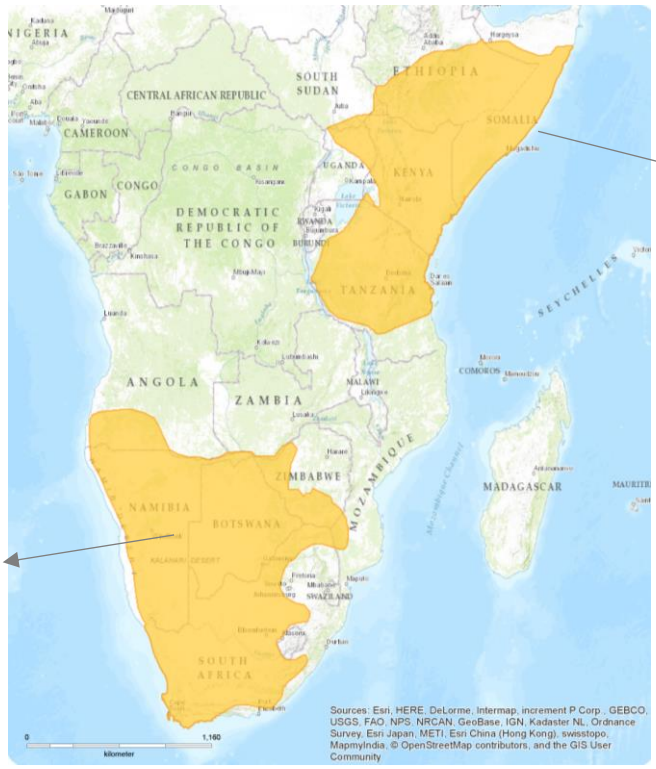


Figure 2 – Distribution map of the two bat-eared fox subspecies. *Otocyon megalotis*. The IUCN RED List of Threatened Species

3.1.2 – Group size, Diet, Ranging and Social Behaviour

Generally considered as group living species, bat-eared foxes are regarded as the most social of canids (Nel & Mackie, 1990). They generally form monogamous pairs (Wright, 2006; Nel, J. A. J. and Maas, B. 2013) with or without cubs, with possible extra-pair paternity (Nel, Mills and Vanaarde, 1984; Pauw, 2000), and were also found in family groups consisting of one male and up to three related females (Maas, 1993). The size of the groups and the way they geographically distribute themselves are not socially fixed parameters, and it might change according to various reasons, such as litter size, mortality and the time of year (Nel, Mills, & Vanaarde, 1984) and their foraging habits increase during the dry season due to changes in food resources (Dalerum et al., 2016).

Also, they have been consistently described as not very territorial animals, different group ranges seemingly overlapping without generating any further conflict (Kamler, Gray, Oh, & Macdonald, 2013). However, in the Serengeti the majority of intergroup encounters were hostile, possibly due to the fact that these areas were lower in food availability (Maas, 1993).

Bat-eared foxes are considered the only truly insectivorous member of the canid family (Kuntzsch & Nel, 1992), their meals consisting primarily of harvester termites, as well as other insects, with the rare appearance of small vertebrates (Nel, J. A. J. and Maas, B. 2013) and sometimes berries (Kuntzsch & Nel, 1992; Nel & Mackie, 1990).

Due to their unorthodox diet, these animals encounter different constraints when compared to other carnivores, especially because insect eaters rely on foraging time to collect a sufficient amount of food (Maas, 1993). In order to contradict this fact, male bat-eared foxes play a very important role in raising their cubs, taking over parental duties when they are about 2 weeks old and later teaching them how to forage (Nel, 1993; Wright, 2006), in this way also maximizing their own foraging time (Nel, J. A. J. and Maas, B. 2013).

4.2 – Brown Hyena

4.2.1 – Taxonomy and Distribution

Brown Hyena (*Hyaena brunnea*, formerly *Parahyaena brunnea*) (Thunberg, 1820)
Kingdom – Animalia
Phylum – Chordata
Class – Mammalia
Order – Carnivora
Family – Hyaenidae
Genus: *Hyaena*

Figure 3 - Brown Hyena by Tambako the Jaguar, donwloaded from http://animalia.bio/brown-hyena

The 2015 IUCN Red List of Threatened Species lists this species as “Near Threatened”, almost qualifying as “threatened” under criterion C1, being the rarest of all hyena species (Westbury et al., 2018). This is due to the low mean global population size (estimated to be below 10,000 mature individuals) and to the deliberate and incidental persecution of these animals, which number may come close to meeting a continuing decline of 10% over the next three generations (Wiesel, 2015). They are also listed as Class B under the African Convention on the Conservation of Nature and Natural Resources. Moreover, old studies (Rohland et al., 2005) have hinted towards very low genetic diversity within the species, and more recently (Westbury et al., 2018) proved it, but it is still unknown the true influence this may have on the survivability of the brown hyena. However, knowledge of the evolutionary processes affecting a species is critical to inform conservation plans aimed at the long-term management of its evolutionary potential, which justifies potential further investigations concerning these animals (Westbury et al., 2018).
This species is endemic to southern Africa and is widely spread throughout the south-western arid region including most of Botswana, Namibia, southern Angola, southern Zimbabwe, South Africa, Swaziland and southern Mozambique (Furstenburg, 2012; Wiesel, 2015). In Namibia, they are mainly found along the coast, in Etosha National Park and more sporadically over the rest of the

country (Hofer & Mills 1998a). Even though the range of the brown hyena has shrunk significantly since the end of the 18th century (Hofer and Mills 1998), they remain widespread in southern Africa, and in more recent years their distribution has been found larger than previously believed, particularly due to re-introductions (Slater & Muller, 2014) and range expansions (Thorn, Green, Bateman, Waite, & Scott, 2011). The total population size on the continent, has been estimated as a minimum of 5,000 to 8,000 individuals (Wiesel, 2015), with Botswana having the largest population (Hofer and Mills 1998; Kent and Hill, 2013) and Namibia the apparent second (Stein, Fuller, & Marker, 2013; Wiesel, 2015), even though Maude (2005) estimates that the numbers in Botswana may be a little higher than the ones stated before.

A significant proportion of the global population is now inhabiting non-protected areas (Thorn, Green, Bateman, et al., 2011), which may suggest the importance of these zones, when it comes to species conservation (Kent & Hill, 2013; Lindsey et al., 2013; Stein et al., 2013), but the hyenas still persist in areas of commercial farmland in a bigger number than previously thought (Thorn, Green, Keith, et al., 2011).

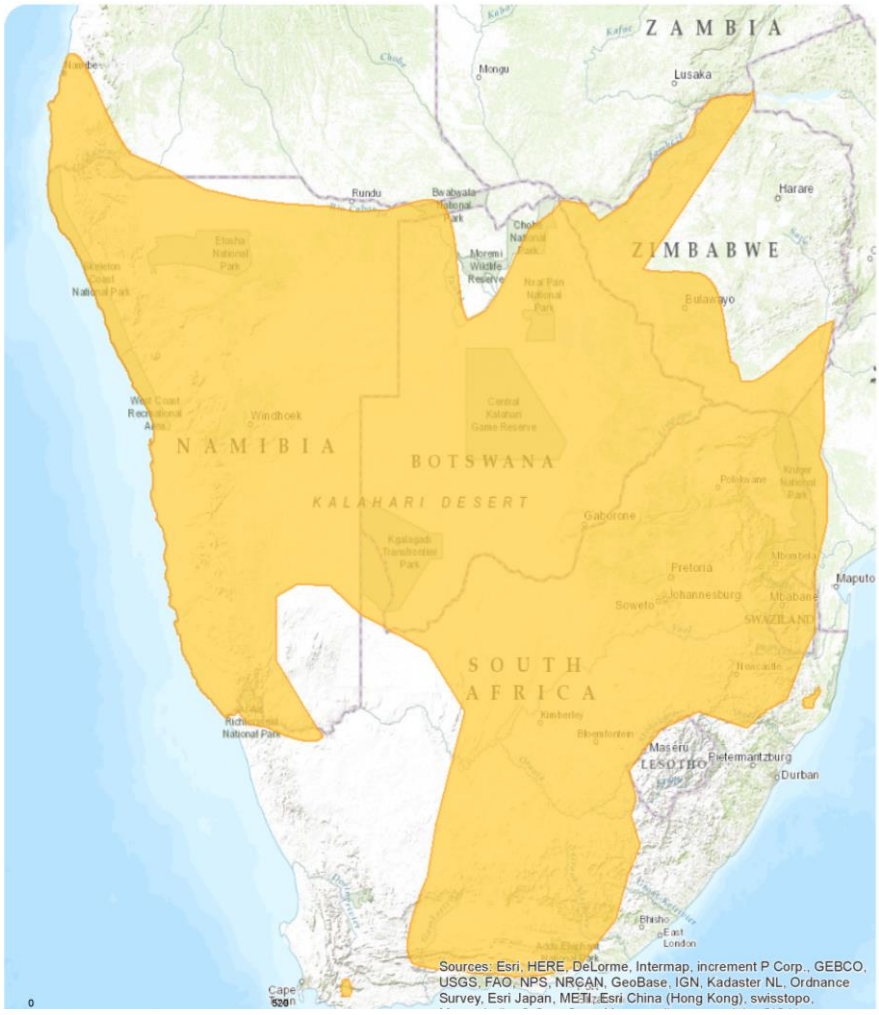


Figure 4 – Brown hyena distribution map.

Parahyaena brunnea. The IUCN RED List of Threatened Species

3.2.2 – Group size, Diet, Ranging and Social Behaviour

This species shows an ability to survive close to urban areas and it is predominantly nocturnal in its activity (Mills, M.G.L. 2013), being most commonly found in deserts like the Namib (Mills, 1998) and other dry areas, particularly along the coast, and also semi-desert, open scrub and open woodland savanna (Hulsman *et al.*, 2010; Mills, M.G.L. 2013). Although they are not dependent on the existence of drinking water (Furstenburg, 2012), they do require a covered space to lie on during the day, favouring rocky mountainous areas with bushes (Wiesel, 2015).

They are primarily scavengers of a wide range of vertebrate remains, scavenging larger carnivore kills, like leopard's on Namibian farmlands (Stein et al., 2013), but their diet can be supplemented by wild fruit or other plants, insects, bird's eggs (Owens & Owens, 1978; Siegfried, 1984; Stuart & Shaughnwssy, 1984; Burgener & Gusset, 2003; Kuhn, Skinner, & Wiesel, 2008; Maude & Mills, 2005; Furstenburg, 2012; Slater & Muller, 2014) and sometimes small animals, like the Cape Fur Seal pups they are able to hunt or scavenge along the Namib Desert (Siegfried, 1984; Stuart & Shaughnwssy, 1984; Kuhn et al., 2008; Wiesel, 2010), even though hunting is presumably opportunistic and largely unsuccessful (Furstenburg, 2012; Mills, M.G.L. 2013). The presence of other carnivores may be beneficial for this species due to the bigger amount of scavenging opportunities (Mills, M.G.L. 2013), scavenging being defined as feeding from prey that was killed and abandoned by another predator or that had died of a reason unrelated to predation (Höner, Wachter, East, & Hofer, 2002).

Although hunting comprises a relatively small proportion of their foraging behaviour, only sometimes killing sheep, goats, calves or poultry (Kent & Hill, 2013), when living in areas occupied by pastoralist herders, they are often persecuted, since the livestock owners believe them to be responsible for their losses (G. Mills & Hofer, 1998; Kent & Hill, 2013; Lindsey et al., 2013).

The dietary benefit derived from the presence of subsistence pastoralists and the availability of livestock carcasses may be the primary reason that brown hyena populations are viable in cattle areas (Maude and Mills 2005), with the added bonus of lower levels of competition, which allows the brown hyenas to feed undisturbed (Kent & Hill, 2013). Increased efforts to educate farmers and pastoralists about the fact that brown hyenas pose very little risk to livestock may even have beneficial effects, as far as disease prevention and is thought to enhance conservation of these animals (Kent & Hill, 2013; Wiesel, 2015).

Although sightings of brown hyena give the idea of a solitary social structure, they are, actually a gregarious, socialized species (Furstenburg, 2012) that lives in clans ranging in size from a single female and her cubs to extended families, that include a female, her adult offspring of both sexes and an immigrant male (Owens & Owens, 1978; Westbury et al., 2018), however, they are strict solitary foragers (Skinner & van Aarde, 1981; Mills, M.G.L. 2013). Several individuals may come together at a large food source (Owens & Owens, 1978) and whilst the type of food determines clan

size, the way in which the food resources are distributed determines territory size (Furstenburg, 2012; Mills, M.G.L. 2013). They are not usually territorial animals, with each individual having a home range to which it generally adheres in its movements, even though there is great overlap between members of the group (Owens & Owens, 1978).

4.3 – Spotted Hyena

4.3.1 – Taxonomy and Distribution

Spotted Hyena (*Crocuta crocuta)*
(Erxleben, 1777)
Kingdom - Animalia
Phylum - Chordata
Class - Mammalia
Order - Carnivora
Suborder - Feliformia
Family - Hyaenidae
Genus – *Crocuta* (Kaup, 1828)

Figure 5 - Spotted Hyena by Bettina Watcher, downloaded from https://hyena-project.com/hyenas/

Considered by the 2015 IUCN Red List of Threatened Species as “Least Concern” as the species remains widespread in Africa, though with a continuing decline in populations outside, and even within protected areas, due to persecution and habitat loss. This, however, is not sufficient to warrant listing in a threatened category, with the total world population well exceeding 10,000 mature individuals (Kay E. Holekamp & Dloniak, 2010; Bohm & Höner, 2015) and their behavioural and ecological plasticity contributes majorly to this fact (J. M. Kolowski & Holekamp, 2009).

Spotted hyenas are relatively widely distributed in the south of the Sahara region of Africa (Bohm & Höner, 2015), with the largest known population occurring in the Serengeti ecosystem in Tanzania and Kenya and in South Africa at Kruger National Park (Hofer and Mills, 1998). They are, however, uncommon in South West Africa/ Namibia (Gasaway, Mossestad, & Stander, 1989). And even though the current population trend is decreasing, there may be recent evidence that a few populations have increased during the past years, more precisely in Eritrea (Bohm & Höner, 2015) and in Chad (Olléová and Dogringar 2013).

Spotted hyenas occupy an extraordinarily diverse array of habitats, including savannah, deserts, swamps, open woodlands, and montane forest (Kay E. Holekamp & Dloniak, 2010) and lower densities can be found in arid and semi-arid desert areas (Kay E. Holekamp & Dloniak, 2010; Bohm & Höner, 2015).

Some of the reasons for its decline in population number include their use by locals for tourism in Ethiopia and Nigeria, their persecution outside protected areas and even within the boundaries of conservation ones and the decline in densities of wildlife species on which these animals feed on (Bohm & Höner, 2015).

Figure 6 – Spotted hyena distribution map

Crocuta crocuta. The IUCN RED List of Threatened Species

3.3.2 – Group size, Diet, Ranging and Social Behaviour

Spotted hyenas are more commonly active at night and around dawn and dusk (Joseph M. Kolowski, Katan, Theis, & Holekamp, 2007; Matt W. Hayward & Hayward, 2007) and they can obtain food either by hunting live animals, or by scavenging on carrion, but they are very effective predators and mostly hunt, especially medium to large ungulates (Eloff, 1964; K. E. Holekamp, Smale, Berg, & Cooper, 1997; Kruuk, 1972; J. D. Skinner & van Aarde, 1981). However, they feed on a wide variety of animals, from insects to large herbivores (Höner et al., 2002), mostly on locally abundant prey species (S. M. Cooper, Holekamp, & Smale, 1999; Breuer, 2005), showcasing a remarkable plasticity when it comes to their prey preferences, which overall allows them to thrive in a large array of habitats (Kay E. Holekamp & Dloniak, 2010). Also, they are somewhat dependent on water when selecting an area to live and they commonly exist in areas within close contact with humans (Bohm & Höner, 2015).

They mainly form social groups called clans, generally female-dominated (Trinkel & Kastberger, 2005; Höner et al., 2010), that consist of societies in which individual members travel, rest and forage in subgroups (Kruuk, 1972; Smith, Kolowski, Graham, Dawes, & Holekamp, 2008) and they always include several closely related adult females and their offspring (Kay E. Holekamp & Dloniak, 2010) and one to several resident immigrant adult males (Boydston, Morelli, & Holekamp, 2001). These clans fluctuate a lot in size, ranging from small in the deserts of southern Africa (Gasaway et al., 1989; Tilson & Henschel, 1986) to the large groups found in eastern Africa (Kruuk, 1972), according to prey availability and stability. Under conditions where these are abundant, hyenas usually associate in large territorial clans, whilst in places where big fluctuations in prey number occurs, hyenas form smaller groups to hunt (Tilson & Henschel, 1986), needing to be more flexible with their range size in response to these fluctuations of migratory prey abundance (Trinkel & Kastberger, 2005)

The territorial behaviour is quite different among study populations, varying from territorial in high density locations (Kruuk, 1972; Boydston et al., 2001) to places with very low hyena density where both clan wars and border patrols tend to be rare, or are not observed at all (Tilson & Henschel, 1986).

Overall, a large variation has been documented throughout the range of spotted hyenas concerning their temporal patterning of activity, clan size, diet, territorial defence, patterns of space use and intrusion pressure. Nevertheless, like most other African carnivores, this species is facing encroachment, habitat loss and direct persecution from humans, as well as an increasingly uncertain future due to the potential effects of climate change (Kay E. Holekamp & Dloniak, 2010).

4.4 – Cheetah

4.4.1 – Taxonomy and Distribution

Cheetah (*Acinonyx jubatus*)
(Schreber, 1775)
Kingdom - Animalia
Phylum - Chordata
Class - Mammalia
Order - Carnivora
Suborder - Feliformia
Family - Felidae
Genus – *Acinonyx*

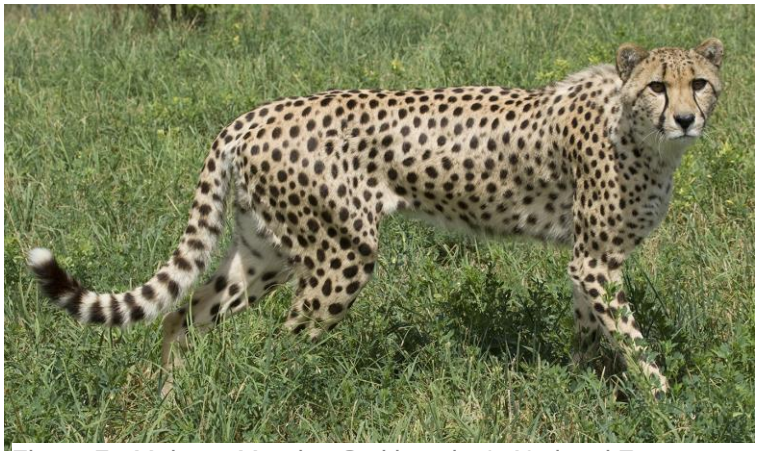


Figure 7 - Mehgan Murphy, Smithsonian's National Zoo, downloaded from https://nationalzoo.si.edu/animals/cheetah

Currently, 5 subspecies with different habitats are considered: *A. j. jubatus jubatus* (Southern Africa), *A. j. hecki* (Northwest Africa), *A.j. raineyii (*East Africa), *A. j. soemmerringi* (Central Africa) and *A. j. venaticus* (Iran). Out of these 5, only *A. j. jubatus* and *A. j. raineyii* have been genetically compared, and even though they were found to be extremely similar, the subspecies distinction was still maintained (S. J. O'Brien et al., 1987; Menotti-Raymond & O'Brien, 1993).

Cheetahs are listed on Appendix I of Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), as Class A under the African Convention on the Conservation of Nature and Natural Resources and considered as "Vulnerable" by the 2015 IUCN Red List of Threatened Species, predominantly due to the quite small world population that exists in present days (approximately 6,700 adult and adolescent animals distributed across 29 subpopulations). They are also protected under national legislation throughout most of its extant and some of its former range (IUCN SSC 2007a, b, 2012) and are considered as Africa's most endangered large felid (Laurie L Marker, Muntifering, Dickman, Mills, & Macdonald, 2003).

Over the years, cheetahs have disappeared from vast tracts of their range. Ray, Hunter, & Zigouris (2005) estimated that the Cheetah has disappeared from at least 76% of its historical range in Africa and IUCN (2012) actually believes them to persist in only 10% of their historic range in Africa, whilst their distribution in Asia is limited to the central deserts of Iran (S. Durant, Mitchell, Ipavec, & Groom, 2015). Most of these animals currently reside in the northern part of Tanzania, Kenya, almost the entire southern boundary of Ethiopia, South Sudan and Uganda and in the eastern and southern part of Africa most of the animals residing belong to a single population that stretches across Namibia, Botswana, south-western Angola, northern South Africa, south-western Mozambique and southern Zambia (IUCN SSC 2007b). The entire species is considered as "Critically Endangered" in the region of North and West Africa (S. Durant et al., 2015).

Furthermore, there are two cheetah subspecies currently listed as “Critically Endangered” in all its habitat – *A. j. hecki*, that can be found in northwest Africa and is currently largely confined to desert environments (Belbachir, 2008), having been extirpated from nearly all its range (S. Durant et al., 2015) and which is thought to number less than 250 individuals (IUCN, 2012; Belbachir, Pettorelli, Wacher, Belbachir-Bazi, & Durant, 2015), and the Asiatic cheetah – *A. j. venaticus,* which is known to reside only in Iran (Charruau et al., 2011; M. S. Farhadinia et al., 2017), with the possibility of a few individuals existing in Pakistan (B. M. S. Farhadinia, 2004) and Afghanistan (Manati & Nogge, 2008).

Their decline over time is mainly a result of habitat loss and fragmentation, killing and capture as a result of livestock depredation, conflict and loss of prey (Gros, 2002; L. Marker, 2005; Mallon, 2007; L. L. Marker, Dickman, Mills, Jeo, & Macdonald, 2008; S. Durant et al., 2015; M. S. Farhadinia et al., 2017), and in Iran there was also the habit of capturing live cheetahs that were then trained to hunt deer and gazelle as sport for the aristocracy (Mallon, 2007), even though in this country the key factor affecting cheetah numbers is the disappearance of prey (L. Hunter et al., 2007).

Overall, the current population trend is decreasing, since these animals are not doing well in protected wildlife reserves due to increased competition from other large predators such as lions and hyenas. Therefore, a large percentage of the remaining free-ranging cheetah populations are outside of protected reserves or conservation areas (L. Marker, 2005). Also, they are considered a genetically depauperate species (S. J. O’Brien et al., 1987; May R. M, 1995; Stephen J. O’Brien & Johnson, 2005), with their populations being extremely fragmented, which means conservation requires large scale land management planning as most existing protected areas are not large enough to ensure their long term survival (S. Durant et al., 2015).

In Africa, nearly all range states are actively involved with the Range Wild e Conservation Program for Cheetah and African Wild Dogs, developing regional strategies and national conservation action plans using the IUCN strategic planning process (IUCN, 2008). Apart from that, there are still other projects and/or non-governmental organizations established in southern and eastern Africa that are working towards the conservation of cheetahs specifically or of general large carnivores (S. Durant et al., 2015).

In Iran, the Asiatic Cheetah is completely protected since the United Nations Development Programme have established a program of work to support its conservation since 2008 (S. Durant et al., 2015).

3.4.2 – Group size, Diet, Ranging and Social Behaviour

Cheetahs are the fastest land mammals and they take advantage of that fact, especially when it comes to catching their prey, which, although it may vary, generally consists of the most available medium sized prey present (M. W. Hayward, Hofmeyr, O’Brien, & Kerley, 2006). It can range from

ground-dwelling birds, to small mammals, (M. G. L. Mills, 1984), medium sized ungulates and large herbivores (Purchase & du Toit, 2000; M. G. L. Mills, Broomhall, & Du Toit, 2004; M. W. Hayward et al., 2006; A. B. Cooper, Pettorelli, & Durant, 2007; L. Hunter et al., 2007). Also, contrary to many other African predators, they rarely scavenge (Sarah M. Durant, Bashir, Maddox, & Laurenson, 2007) and they also rarely prey on domestic stock, with apparent selection towards common, indigenous game species (Laurie L Marker et al., 2003). Even so, the cheetah has long been regarded as a significant threat to the interest of farmers of both game and livestock (Laurie L Marker et al., 2003).

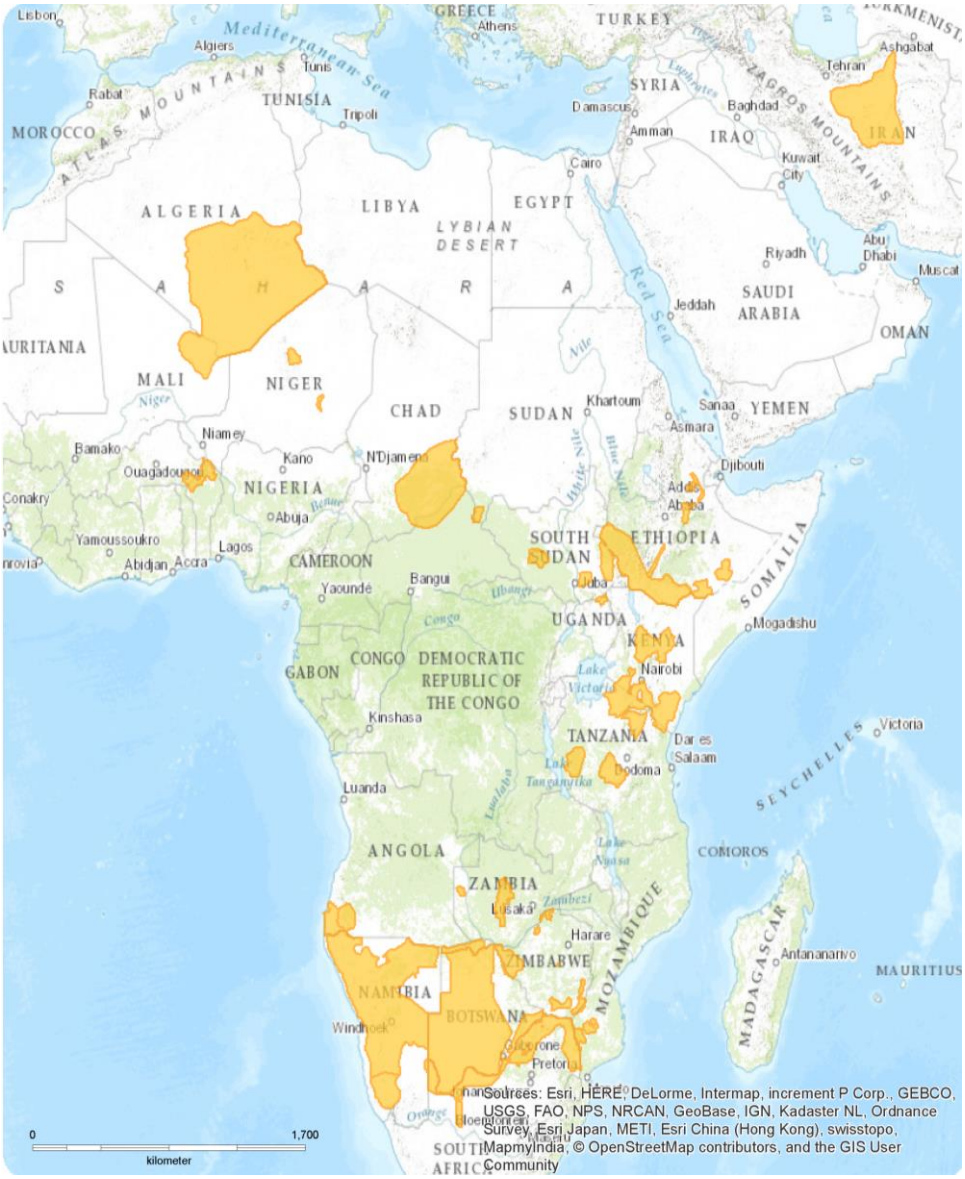


Figure 8 - Cheetahs' distribution map

Acinonyx jubatus. The IUCN RED List of Threatened Species

They tend to be active primarily during the day to minimize the competition (Sarah M. Durant, 1998; S. Durant et al., 2015) since they can lose up to 10% of their kills to lions and spotted hyenas, especially in areas where these live in higher densities (J. S. Hunter, Durant, & Caro, 2007a) and they also don't usually stay with their kills for long, abandoning the carcasses as soon as they have eaten (J. S. Hunter, Durant, & Caro, 2007b). On the other hand, in areas on which prey is more abundant, cheetahs are mainly nocturnal (Belbachir et al., 2015), but this can also be a result of increased human activity in these zones.

Unique among felids (Gottelli, Wang, Bashir, & Durant, 2007), cheetahs' social organization is generally comprised by solitary females, that can be accompanied by their offspring when these are still dependent (Caro & Collins, 1987 cited by Sarah M. Durant, Bashir, Maddox, & Laurenson, 2007), and these groups usually follow the herds in case of migratory prey. The males on the other hand can either live alone or in stable coalitions of two or three members (Caro & Collins, 1987 cited by Sarah M. Durant, Bashir, Maddox, & Laurenson, 2007), generally siblings (Broomhall, Mills, & du Toit, 2003), and they chose to establish on small areas attractive to females. In the cases of non-migratory prey both females and males may share their range (Broomhall et al., 2003), which can be quite extended.

Older (Sarah M. Durant, 1998, 2000) and more recent studies (Broekhuis, Cozzi, Valeix, Mcnutt, & Macdonald, 2013; Rostro-García, Kamler, & Hunter, 2015), defend that the natural evolution of cheetahs towards these different social systems and ranging patterns has its premise on risk avoidance by the animals, as this strategy to remain mobile in the presence of larger and stronger competitors enables them to avoid direct spatio-temporal competition.

Cheetahs appear to show relatively low habitat selectivity in comparison with other carnivores (Sarah M. Durant et al., 2010). In Africa, this species can be found in a wide array of habitats, such as woodland savannahs (J. Skinner & Smithers, 1990) as dry forest, thick scrub, open grassland plains (M. G. L. Mills et al., 2004) and even arid deserts like the Sahara (S. M. Durant et al., 2014) and in Iran their habitats consists of deserts.

4.5 – Vector-borne Parasites

Parasitism can be defined as an ecological association between species in which one organism, the parasite, lives on or in the body of another organism, the host. The parasite may spend the majority of its life in association with one or more host species, or alternatively, it may spend only short periods, adopting a free-living mode for the major part of its developmental cycle. But during the parasitic phase of its life cycle, the organism depends upon its host for the synthesis of one or more nutrients essential for its own metabolism. The relationship is usually regarded as obligatory for the parasite and harmful or damaging for the host. To classify an animal species as parasitic, therefore, three conditions must be satisfied: utilization of the host as a habitat, nutritional dependence, and

causing harm to its host (Anderson & May, 1978). Basically, a parasite is any small organism living at the expenses of another, by feeding, inhabiting on or in a bigger organism, the host, and exploiting its biological, ecological and metabolic patterns (Bowman, D., 2014; Otranto, 2018).

According to where they reside in the host's body, parasites can then be divided into endoparasites, living within the body of hosts, and ectoparasites, inhabiting on the external surface of the host or in its skin. Parasites can also be vectors, if they transmit other parasites directly from host to host and the term vector-borne disease refers to any of a broad array of infectious diseases caused by pathogens that are transmitted by arthropods or other invertebrate as biologic intermediaries (Bowman, D., 2014).

Vector-borne pathogens have developed a close relationship with blood feeding arthropod ectoparasites, such as mosquitoes, ticks, phlebotomine sand flies, black flies, fleas, kissing bugs and lice (Otranto, 2018). And these parasitic arthropods are highly efficient vectors of several bacteria, viruses, protozoa and helminths affecting livestock, domestic and wild animals and even humans worldwide (Jongejan & Uilenberg, 2004; Otranto, Dantas-Torres, & Breitschwerdt, 2009; Colwell, Dantas-Torres, & Otranto, 2011; Kofer et al., 2017). Along the way, the life cycles of these pathogens turned into a long evolved balance with the respective arthropod biology, ecology and blood feeding habits, having taken advantage of the biology of blood feeders to ensure their transmission and distribution to receptive hosts (Otranto, 2018). Transmission of vector-borne pathogens usually occurs during blood feeding by an infected insect or acarine, but it can also happen when a vertebrate host ingests a vector, or on wound contaminations by infectious organisms in the faeces of the arthropod (Bowman, D., 2014).

Vector-borne pathogenic zoonoses are part of the constantly changing world and they are constantly adapting to their new circumstance, changing their vectors, hosts, distribution and also their virulence. Furthermore, they are also quite difficult to diagnose, posing a high amount of constraints, and also hard to control and to prevent, due to their complex transmission among host compartments, trophic levels, and the environment (Dantas-Torres, Chomel, & Otranto, 2012). Climate change, for example, can alter the geographic distribution of arthropod vectors, enhancing the risk of infectious disease transmission in wild species and the incidence of zoonoses in humans (Cumming & Van Vuuren, 2006). So, due to the lack of data on vectors for specific parasites, studies are needed to identify vectors as well as determine transmission routes, infection dynamics, and host specificity (Williams et al., 2014).

The incidence of tick-borne diseases, for example, is rapidly increasing worldwide (Piesman & Eisen, 2002; Dantas-Torres, 2007; Nicholson, Allen, McQuiston, Breitschwerdt, & Little, 2010; Estrada-Peña, Ayllón, & de la Fuente, 2012).

4.5.1 – Onchocercidae

In humans and domestic animals, nematodes and their deleterious effects are well documented at an individual and population level, being one of the most significant, though neglected, tropical parasite responsible for diseases in humans (Bartsch et al., 2016). However, these parasites are rarely studied in wild animals and despite the serious impact they can have in their hosts, there is no currently available summary on the number of nematodes described and the significance of their infection in free-ranging wild mammals (Seguel & Gottdenker, 2017).

Concerning their life cycle, sexually mature females found in the definitive host are viviparous and sexual reproduction occurs, with the production of microfilariae that are released onto the bloodstream. Then, microfilariae are ingested during the blood feeding of the intermediate host and they go through a series of transformations until reaching the infective stage and eventually accumulating in the mouthparts of the respective vector involved. Final hosts become infected during a new blood feeding (E. V Schwan & Schroter, 2006).

As for wildlife findings, two spotted hyenas in Kenya were reported to be positive for *Acanthocheilonema dracuncoloides* (Lightner & Reardon, 1983) and this parasite was also found in two dogs in the same area, as well as in dogs in Namibia (E. V Schwan & Schroter, 2006). However, parasites in both studies were either only identified by morphometry of microfilaria (Lightner & Reardon, 1983) or by acid phosphatase staining (E. V Schwan & Schroter, 2006) and therefore the exact species identification should be considered as doubtful in the absence of any morphological data on adult parasites or DNA sequence data.

4.5.2 – Piroplasmida

Piroplasmosis are among the most prevalent arthropod transmitted diseases of animals and in the last few years there has been a dramatic increase in the number of studies reporting infection with piroplasmids in wildlife (Yabsley & Shock, 2013; Alvarado-Rybak, Solano-Gallego, & Millán, 2016).

Alvarado-Rybak et al. (2016) presented abundant evidence of piroplasmid infections in wild carnivores worldwide and whilst some of these species serve as reservoirs for piroplasmids, others are potential vectors, allowing these parasites to maintain endemic sylvatic lifecycles in their geographical distribution area. For example, wildlife species are known reservoirs for several *Babesia* spp. even though the vectors of many species are still unknown. Babesiosis is currently considered a worldwide emerging zoonosis (Yabsley & Shock, 2013; Zanet et al., 2014), with *Babesia* spp. being considered the second most commonly found parasite in the blood of mammals after trypanosomes (Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012).

Their importance spreads farther than just veterinary medicine, also demonstrating a great economic and medical impact worldwide (Anna Mari Bosman, Oosthuizen, Peirce, Venter, & Penzhorn, 2010; Giadinis et al., 2012; Schnittger et al., 2012).

Piroplasmosis are diseases caused by hemoprotozoan parasites of the phylum Apicomplexa belonging to four related genera: *Babesia, Theileria, Cytauxzoon* and *Rangelia*, some of which can occasionally cause severe disease in domestic animals, humans (Yabsley & Shock, 2013) and wild animals, even though piroplasmid infections in these last ones are typically subclinical (Banie L. Penzhorn, 2006; Williams et al., 2014). Their main vectors appear to be ticks (Chauvin, Moreau, Bonnet, Plantard, & Malandrin, 2009; Alvarado-Rybak et al., 2016).

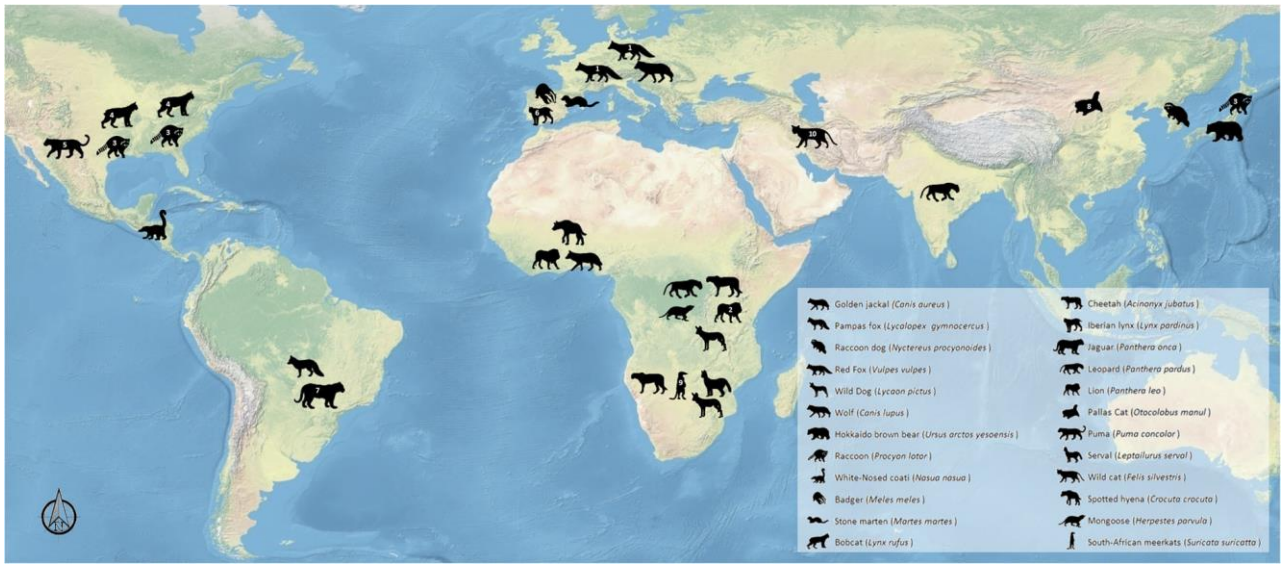


Figure 9 - Distribution map of piroplasmid infection in wild carnivores worldwide from Alvarado-Rybak et al. (2016)

As for their life cycle, the sexual phase of reproduction occurs in the tick when the gametes fuse to form a zygote, followed by an asexual form of reproduction, sporogony, also in the tick. The resultant forms, ookinetes, invade either the salivary gland or the ovary of the tick, where they participate in transtadial and transovarial (only *Babesia* spp.) transmissions, respectively (Banie L. Penzhorn, 2006; Chauvin et al., 2009; Schnittger et al., 2012). In the case of transtadial transmission, the sporozoites are released from the tick's salivary glands while it's feeding and enter the blood stream of the vertebrate host. Then, once sporozoites are in the erythrocytes or leukocytes of the host, they undergo asexual reproduction and merogony, and the daughter cells can infect new cells (Banie L. Penzhorn, 2006; Chauvin et al., 2009).

Several *Babesia* sp. have been identified in wild carnivores, but the more relevant to this particular study were the findings of a novel species, *Babesia lengau*, thought to exist exclusively in cheetahs (Anna Mari Bosman et al., 2010). However, more recently, Williams et al., 2014 identified *Babesia* sp. in spotted hyenas that were very similar to *Babesia lengau* and Burroughs et al. (2017) confirmed this fact, extending it to brown hyenas as well.

4.5.3 – Bacteria Rickettsiales (*Anaplasma, Rickettsia*)

Members of the Rickettsiales order are obligate intracellular gram-negative bacteria and the survival of these organisms and transmission between animals are dependent on invertebrate vectors, ticks being by far the most common one (Bowman, D., 2014).

They are transmitted through the bite of an infected nymphal or adult tick vector that had been previously infected in the larval or nymphal stage while feeding on an infected animal (usually a wild animal species) which are known as being reservoir hosts (Nicholson et al., 2010).

So far, to our knowledge, there is no report of *Anaplasma* sp. infecting wild animal species, and for *Rickettsia* sp. there is a single old serological study describing that one of three investigated spotted hyaenas was positive for antibodies against *Rickettsia akari* (Heisch et al., 1962).

4.5.4 – Adeleorina (*Hepatozoon*)

Hepatozoon sp. constitute a group of apicomplexan parasites that primarily infect leukocytes of mammals and erythrocytes of amphibians, reptiles and birds (Gad Baneth, Samish, Alekseev, Aroch, & Shkap, 2001), involving arthropods such as ticks, mites, fleas and lice as intermediate hosts (McCully, Basson, Bigalke, De Vos, & Young, 1975).

Their life cycle typically involves gametogenesis, fertilization, and sporogony in a hematophagus invertebrate, and merogony followed by gametogony in a vertebrate intermediate host. Infection of the vertebrate host normally occurs by ingestion of an infected invertebrate host such as a tick (East et al., 2008).

They have been observed in a wide variety of wild carnivores, including hyenas, jackals, lions, leopards and cheetahs (McCully et al., 1975; Averbeck, Bjork, Packer, & Herbst, 1990; Lopez-Rebollar, Penzhorn, de Waal, & Lewis, 1999). Generally *Hepatozoon* infections in domestic and wild carnivore species have been attributed to *Hepatozoon canis* or closely related undetermined species (Brocklesby & Vidler, 1965; Conceição-Silva, Abranches, Silva-Pereira, & Janz, 1988).

Spotted hyenas have long been known to be infected with *Hepatozoon* sp. (McCully et al., 1975) and in the Serengeti it has been shown that, these parasites are highly similar or identical to *Hepatozoon felis* (East et al., 2008). In Zambia, however, spotted hyenas were found to be positive for both, *Hepatozoon canis* and *H. felis* (Williams et al., 2014).

Although generally regarded as being non-pathogenic in wild animals (Kocan et al., 2000; Rishniw et al., 2006; Williams et al., 2014), there is a report of *Hepatozoon* infection perhaps contributing to the death of spotted hyenas in Tanzania (East et al., 2008).

4.6 – PCR in parasitology

Molecular diagnostic assays, primarily those based on particular amplification by polymerase chain reaction (PCR) allow detection and diagnosis of pathogens with enhanced specificity and sensitivity compared to the traditional methods of microscopy and serology (Bosman, Venter, & Penzhorn, 2007; Piron et al., 2007; Abdul-Ghani, Al-Mekhlafi, & Karanis, 2012).

The deoxyribonucleic acid (DNA) molecule consists of two intertwined and complimentary strains, the 3' end and the 5' end. The bases of opposite strands pair up with one another, and are held together by hydrogen bonds and hydrophobic interactions and can be dissociated to produce two single strands, either by chemical means or by heating to a temperature of at least 94°C. Once denaturation has occurred, fragments or portions of DNA can be specificaIIy bound to complementary regions, in a process called hybridisation (Singh, 1997).

By using Taq DNA polymerase, a thermostable enzyme isolated from the thermophilic bacterium *Thermophilus aquaticus*, the problem of low sensitivity of DNA hybridisation assays, or low amounts of target DNA was solved, because it became possible to amplify original target DNA a million-fold with a method employing concurrent cycles DNA duplication, called polymerase chain reaction (Singh, 1997).

During PCR, specific regions of DNA are amplified enzymatically through successive cycles, each consisting of three steps:

1. Denaturation: double-strand DNA (dsDNA) is denatured to produce two single-strand DNA (ssDNA) strands;
2. Annealing: two different oligonucleotides (referred to as primers) hybridise to complementary DNA sequences on each of the target ssDNA strands;
3. Extension: the enzyme DNA polymerase catalyses the addition of deoxynucleotide triphosphates to the two oligonucleotides in a 5' to 3' direction.

Each cycle of PCR doubles the amount of specific DNA resulting in a million-fold amplification of the target sequence after a minimum of 30 cycles. Each of the three steps is undertaken at different temperatures: usually the denaturation step occurs at 94°C, extension occurs at 72°C and the annealing temperature depends on the length and the nucleotide content of the oligonucleotide used in the PCR. These temperatures and the duration of each step are attained using a thermocycling machine (Singh, 1997).

To put this into practice in parasite detection, it involves processing the specimen to produce parasite DNA template, several different protocols having been described for this step according to the parasite in question, identification of target DNA sequence, design of primers, optimisation of PCR parameters and detection of product. The next step involves the removal of inhibitors of PCR, like heparin for example, which is used in the collection of blood to purify the DNA and then, once the PCR product is produced following amplification, it can be detected by various means. The

commonest method is to analyse the product using agarose gel electrophoresis, in which case the DNA products are separated according to their respective sizes (Singh, 1997).

The epidemiology of parasitic diseases includes the study of host-parasite interactions, and for parasites transmitted by vectors it involves host-vector and vector-parasite interactions, and these DNA-based methods are also applicable for the detection of parasites in vectors (Singh, 1997).

BLAST (Basic Local Alignment Search Tool) is a method that directly approximates alignments that optimize a measure of local similarity, the maximal segment pair (MSP) score, based on well-defined mutation scores. It allows the detection of weak, but biologically significant sequence similarities, and as DNA and amino acid sequence databases continue to grow in size, they become increasingly useful in the analysis of newly sequenced genes and proteins because of the greater chance of finding homologies (Altschul, Gish, Miller, Myers, & Lipman, 1990).

4.7 – Phylogenetics

Taxonomic characterization of organisms was originally based on morphological observations and certain general phenotypic characteristics, which enabled many parasites to be categorized into a particular genus. However, application of molecular genetic techniques, such as the PCR for gene amplification and DNA sequencing have revealed gross inconsistencies in the assignation of some parasite genetic variants.

Furthermore, recently gene sequencing has become more easily available and less costly, which means molecular phylogeny is emerging as a major tool, especially since it can be applied in a wide variety of areas. These include the analysis of gene or genome duplication events (Pfeil, Schlueter, Shoemaker, & Doyle, 2005), recombination (Chare & Holmes, 2006), horizontal gene transfer (Philippe & Douady, 2003), variation of selective pressures and adaptive evolution (Nielsen et al., 2005), divergence times between species (Ramírez, Gravendeel, Singer, Marshall, & Pierce, 2007), elucidate the origin of epidemics (Taubenberger, 2006), and host-parasite cospeciation events (Pompei, Loreto, & Tria, 2012). They can also be used as complementary tools for taxonomy (Hajibabaei, Singer, Hebert, & Hickey, 2007), have contributed to the formulation of strategies in conservation biology and have also been employed outside of the realm of biological sciences, in areas such as linguistics (Gray & Atkinson, 2003).

Phylogenetics can be described as the study of the evolutionary history and relationships among individuals or groups of organisms and it is used to classify sequences of unknown origin based on their evolutionary relationships to other considered sequences (Lemey, Salemi & Vandamme, 2009; Medlar, Aivelo, & Löytynoja, 2014). In the case of molecular phylogeny, it is based on the comparison of DNA or amino acid sequences (Baldauf, 2003; Whelan, Liò, & Goldman, 2001), and phylogenetic methods consider the similarity among the genes, since taxonomic comparisons show that the genes of closely related species usually only differ by a limited number of point mutations (Lemey, Salemi & Vandamme, 2009).

The idea of representing these evolutionary relationship hypotheses as trees probably dates back to Darwin and his theory of evolution (Darwin, 1809-1882), but their application to molecular data is relatively recent (Zuckerkandl & Pauling, 1965). A phylogenetic tree can be defined as a diagram that describes evolutionary relationships (Holder & Lewis, 2003) and it is composed of branches and nodes, being nodes the point at which two or more branches diverge, connecting these last ones. There are rooted and unrooted trees, and the latter position the individual taxa relative to each other without indicating the direction of the evolutionary process (Lemey, Salemi & Vandamme, 2009).

The simplest test of a phylogenetic tree's accuracy is the bootstrap that shows how strongly the dataset supports each of the relationships depicted in the tree (Baldauf, 2003; Holder & Lewis, 2003). This is done by taking random subsamples of the dataset, building trees from each of these and calculating the frequency with which the various parts of your tree are reproduced in each of these random subsamples (Baldauf, 2003; Holder & Lewis, 2003; Lemey, Salemi & Vandamme, 2009). Also, molecular phylogenetic trees are usually drawn with proportional branch lengths, which means the lengths of the branches roughly correspond to the amount of evolution between the two nodes they connect. Thus, the longer the branches, the more relatively divergent are the sequences attached to them (Baldauf, 2003; Whelan et al., 2001).

The use of molecular data for inferring phylogenetic trees has gained considerable interest (Lemey, Salemi & Vandamme, 2009), and a few genes have become reference markers. The small subunit ribosomal ribonucleic acid (rRNA) gene has been proven extremely useful for classification, because these genes are under tight structural and functional constraint, substitution rates are low and there is no evidence of lateral gene transfer across lineages (Allsopp & Allsopp, 2006), which means it holds a considerable degree of conservation across all organisms (Delsuc, Brinkmann, & Philippe, 2005). Therefore, it is the gene for which most sequence information is available for phylogenetic analysis, and its comparison has then become a powerful tool for deducing phylogenetic and evolutionary relationships among bacteria, archaebacteria, and eukaryotic organisms (Weisburg, Barns, Pelletier, & Lane, 1991).

Most eukaryotes possess hundreds of tandem copies of this gene, each consisting of the 18S, 5.8S, and 28S rRNA genes, two external transcribed spacers (ETS1 and ETS2), two internal transcribed spacers (ITS1 and ITS2), and an intergenic spacer (IGS) (Nei & Rooney, 2005), like it is shown in Figure 6.

Amongst these, the 18S rRNA gene has several features that have led it to being widely used for the assignation of organisms to a particular genus. It has both conserved and variable regions, the former allowing unequivocal sequence alignment and the latter providing phylogenetic discrimination, even though it remains difficult to establish how much gene sequence variation must exist for the source organism to be considered a different species or to be considered merely a variant and/or genotype of a species. Moreover, the 18S rRNA gene, consisting of both conserved and variable regions, has the practical advantage of allowing the design of primers for PCR

amplification of near full-length genes in the presence of mammalian DNA, making it a suitable marker for detection and genetic characterization of blood parasites (Allsopp & Allsopp, 2006).

ITS-2 sequence comparisons are also really common, perhaps representing the most common source of phylogenetic reconstructions at the species, genus and family level among all eukaryotes (Álvarez & Wendel, 2003; Coleman, 2003, 2009; Keller et al., 2010; Song et al., 2012). And they can be the most informative for discrimination at the species and subspecies levels, due to their conserved secondary structures that can be used to facilitate alignments of higher taxonomic categories (from genus to order) because of its function in rRNA processing (Nei & Rooney, 2005).

Figure 10 - Molecular structures of rRNA gene repeats (Álvarez & Wendel, 2003)

5 – Material and Methods

5.1 – Study area, sample collection and DNA extraction

Whole blood samples collected during prior studies were used. These samples were collected from a wide variety of free-ranging Namibian carnivores, in different regions of the country, mainly in Central Namibia, but a few samples also originated from other areas, such as Etosha Natural Park and the Skeleton Coast, in the northern part of Namibia.

In this study, samples from 9 bat-eared foxes, 17 brown and 19 spotted hyenas and 85 cheetahs were included. Samples were stored at -20°C after being collected. Then, DNA was extracted using the Maxwell® 16 LEV Blood DNA Kit and the Maxwell® 16 instrument (Promega) still in Namibia. After, all DNA samples were stored at -20°C until further use, and were then shipped to Berlin, in Germany, where their analysis took place.

5.2 – PCR amplifications and sequencing

The total of 130 samples were screened and tested for pathogens of the Onchocercidae family, the order Piroplasmida, bacteria from the Anaplasmataceae and the Rickettsiaceae families and, lastly, the Hepatozoidae family.

PCR protocols were chosen according to previous published bibliography, and the reactions were either performed using Phusion Hot Start II High Fidelity DNA Polymerase or Maxima Hot Start Taq DNA Polymerase (both Thermo Scientific). All reactions with Phusion enzyme were performed in a 20 microliters (µl) reaction volume consisting of 0.2 milimoles (mM) of deoxyribonucleotide triphosphates (dNTPs), 0.25 micromoles (µM) of each primer, 0.02 units per microliter (U/µl) of Phusion Hot Start II High Fidelity DNA Polymerase and 2 µl of template DNA in 1 × Phusion High Fidelity Buffer. Alternatively, reactions were performed in a 25 µl (*Hepatozoon*) reaction volume, consisting of 0.2 mM dNTPs, 0.3 µM of each primer, 0.04 U/µl Maxima Hot Start Taq DNA Polymerase, 2.5 mM $MgCl_2$ and 2 µl of template DNA in 1 × Maxima Hot Start PCR Buffer and in a 20 µl (Onchocercidae 12s rRNA) reaction volume consisting of 0.2 mM dNTPs, 0.4 µM of each primer, 0.02 U/µl Maxima Hot Start Taq DNA Polymerase, 2 mM $MgCl_2$ and 2 µl of template DNA in 1 × Maxima Hot Start PCR Buffer. All PCR reaction specific denaturation and annealing temperatures, as well as times used for the different PCR steps, are all provided in Table 1. The number of PCR cycles was 40, except for the PCR to detect Anaplasmataceae, where 50 cycles were conducted. For negative controls, nuclease free water was used instead of template DNA in all PCRs and as positive control, plasmid DNA containing the respective amplicon were used. Primers used for pathogen-specific PCRs were derived from previous publications (Casiraghi et al., 2004; Gubbels et al., 1999; Inokuma, Okuda, Ohno, Shimoda, & Onishi, 2002; Krücken et al., 2013; P. T. Matjila, Penzhorn, Bekker, Nijhof, & Jongejan, 2004; Rishniw et al., 2006; Roux, Rydkina, Eremeeva, & RaoultT, 1997; Schreiber et al., 2014; Tabar et al., 2008) and are provided in Table 2.

Organism	Thermal Profile											PCR mix concentrations						
	ID		Step 1		Step 2		Step 3		FE		N	Buffer	Polymerase	Primers	$MgCl_2$	dNTPs	Template DNA	Total volume
	T	D	T	D	T	D	T	D	T	D		-	(U/µl)	(µM)	(mM)	(mM)	(µl)	(µl)
Onchocercidae	98	30	98	10	60	30	72	30	72	5	40	1	0.02	0.25	-	0.2	2	20
Onchocercidae	92	60	92	30	52	45	72	60	72	10	40	1	0.02	0.4	2	0.2	2	20
Piroplasmida	98	30	98	15	65	30	72	30	72	5	40	1	0.02	0.25	-	0.2	2	20
Anaplasma	95	300	94	15	60	30	72	30	72	7	50	1	0.02	0.25	-	0.2	2	20
Rickettsia	98	20	98	10	52	30	72	30	72	5	40	1	0.02	0.25	-	0.2	2	20
Hepatozoon	94	240	94	15	56	30	72	40	72	10	40	1	0.04	0.3	2.5	0.2	2	25

Table 1 – PCR Protocols followed in this study. ID stands for initial denaturation, FE for final extension, T for temperature and D for duration. Temperature was measured in ºCelsius. Duration is presented in seconds except during the final extension stage, where the numbers refer to minutes instead.

		Primers			References
Organism	Gene	Designation	Sequence (5'-3')	Product Size (bp)	
Onchocercidea	ITS-2 rRNA	DIDR-F1	AGTGCGAATTGCAGACGCATTGAG	~550	(Rishniw et al., 2006)
		DIDR-R1	AGCGGGTAATCACGACTGAGTTGA		
Onchocercidea	12S rRNA	12SF	GTTCCAGAATAATCGGCTA	~450	(Casiraghi et al., 2004)
		12SdegR	ATTGACGGATG(AG)TTTGTACC		
Piroplasmida	18s rRNA	RLB-F2	GACACAGGGAGGTAGTGACAA G	~500	(Gubbels et al., 1999) ; (P. T. Matjila, Penzhorn, Bekker, Nijhof, & Jongejan, 2004)
		RLB-R2	CTAAGAATTTCACCTCTGACAGT		
Anaplasma	16S rRNA	AE(Jana)F	GGGGATGATGTCAARTCAGCAC	-	(Tabar et al., 2008)
		AE(Jana)R	CACCAGCTTCGAGTTAAGCCAAT		
Rickettsia	18S rRNA	CS409d	CCTATGGCTATTATGCTTGC	~676	(Krücken et al., 2013) ; (Schreiber et al., 2014) ; (Roux, Rydkina, Eremeeva, & RaoultT, 1997)
		Rmasglta 1065lo	TCAATAAAATATTCATCTTTAAGAGC		
Hepatozoon	18S rRNA	HepF	ATACATGAGCAAAATCTCAAC	~600	(Inokuma, Okuda, Ohno, Shimoda, & Onishi, 2002)
		HepR	CTTATTATTCCATGCTGCAG		

Table 2 - PCR Primers used in the study: the rRNA gene they targeted, their nucleotide sequence, size aproximation and the original publications, responsible for creating them.

For Onchocercidae, screenings of the internal transcribed spacer-2 region of ribosomal RNA (ITS-2 rRNA) a primer pair that spanned this region of the ribosomal DNA was used and it amplified fragments of different length according to the found sequence dimension. DIDR-R1 and DIDR-F1 were used according to Rishniw et al. (2006). A *Dirofilaria immitis* sample was used as a positive control.

When it comes to the 12S rRNA gene, the primers used allowed sequencing of a 450 base pairs (bp) portion of the small subunit ribosomal RNA gene of the mitochondrion and it was designed based on the shared 12S rRNA regions conserved among the nematodes species *Onchocerca volvulus*, *Ascaris suum* and *Caenorhabdites elegans* (Casiraghi et al., 2004). DNA extracted from male adult worms of *Acanthocheilonema viteae* was used as positive control.

For Piroplasmida screening, genus-specific primer pair RLB-F2 and RLB-R2 were used to amplify a partial fragment of approximately 460–540 bp of the 18S ribosomal RNA gene spanning the V4 hyper variable region of *Babesia* and *Theileria* species (Gubbels et al., 1999), with slight modifications. (P. T. Matjila et al., 2004). *Babesia divergens* was used as a positive control.

The PCRs for *Anaplasma* screening were performed with forward primer AE(Jana)F and reverse primer AE(Jana) according to Tabar et al. (2008), with slight modifications. The pair allowed amplification of conserved regions of 16S rRNA gene. As positive control, a known *Anaplasma phagocytophilum* infected sample was used.

The PCRs for *Rickettsia* screening were performed with forward primer CS409d according to Roux et al. (1997) and reverse primer Rmasglta according to Schreiber et al. (2014), using a sample of *R. raoultii* as positive control. Slight modifications were performed according to Krücken et al. (2013).

And lastly, PCRs for *Hepatozoon* screening were performed with forward primer HepF and HepR that were designed to amplify a partial 18S rRNA gene sequence of *Hepatozoon* spp. according to Inokuma et al. (2002), using an *H. canis* infected sample as positive control.

For further characterization of the species, approximately two amplification products per species from samples which showed positive results were purified with DNA Clean & Concentrator TM- 5 Kit (Zymo Research Corporation, Irvine, USA) according to the manufacturer's instructions. Then, purified PCR products were cloned into the StrataClone blunt-end PCR cloning vector ‘pSC-B-amp/kan’ supplied in the StrataCLone Blunt PCR cloning kit (Agilent Technologies, CA, USA) or the TOPO TA Cloning Kit for Sequencing (Thermo Scientific) and recombinant plasmid vectors were transformed into Solopack1 (Agilent Technologies, CA, USA) or One Shot Top10 competent cells according to the manufacturer's instructions. Plasmid DNA was isolated using the Plasmid Mini Prep Kit EasyPrep1Pro (Biozym, Oldendorf, Germany) and clones with inserts were sequenced by LGC Genomics (Berlin).

All obtained sequences will be deposited at the NCBI GenBank™ as soon as the overall project is completed.

5.3 – Sequence and Phylogenetic analyses

For genomic sequence comparison purposes, a common search using Basic Local Alignment Search Tool (BLAST) was performed. BLAST consists of a set of alignment algorithms and it is used to uncover homologies between sequences.

For each PCR-positive sample at least two clones were selected for molecular characterization by purification, cloning and sequencing, which then allowed sequence comparison to those present in the NCBI GenBank™ using nucleotide-nucleotide Basic Local Alignment Search Tool (BLASTn) (https://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al., 1990). If identical or nearly identical sequences were found in GenBank™, the pathogen was considered to belong to this species and if no virtually identical sequence could be identified the sequences were considered to belong to a new pathogen species. In the latter, further phylogenetic analyses were conducted to identify the closest known relatives of alleged pathogen.

For the construction of the phylogenetic trees, the ClustalX program (version 1.81 for Windows) was used to align both the ITS-2 and the 12S rRNA sequences of *Acanthocheilonema*. The alignment was manually edited in BioEdit (version 5.0.9) and truncated to the size of the smallest sequence and then the two resulting phylogenetic trees were visualized in Mega6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The sequences that showed the highest similarities to the query ones in NCBI GenBank™ were the ones used to construct the trees.

5.4 – Statistical analyses

For frequencies of pathogens, 95% confidence intervals (CIs) were calculated as Wilson-Score values using the propCI function of the R package prevalence 0.4.0 in R version 3.3.1 and applying R studio 1.1.383 as graphical user interface.

6 – Results

6.1 – Frequency of pathogens

Bat-eared foxes did not have any positive results for *Onchocercidae*, Piroplasmida and *Rickettsia*, but they showed a high percentage of infection with *Anaplasma* and one co-infection with *Hepatozoon* as well.

In brown hyenas, the most frequent pathogen genus was *Anaplasma* sp., followed by Piroplasmida, *Hepatozoon* sp., Onchocercidae and *Rickettsia* sp. Spotted hyenas displayed very similar results, with the exception that these animals had more positive samples for Onchocercidae when in comparison with *Hepatozoon*. Also, all spotted hyena samples tested were positive for *Anaplasma* sp.

As for cheetahs, the results revealed a high percentage of Piroplasmida positive samples. *Hepatozoon,* Onchocercidae and *Anaplasma* were also highly prevalent, but *Rickettsia* was found in less samples.

Overall, the most prevalent pathogens found were Piroplasmida, closely followed by *Anaplasma*. *Hepatozoon* and Onchocercidae were also highly present and, lastly, *Rickettsia* numbers were relatively low. The complete examined samples searched agents and prevalence rates are presented below, in Table 3.

		Onchocercidae			Piroplasmida			*Anaplasma*			*Rickettsia*			*Hepatozoon*		
	N	n	%	CI (%)	n	%	CI (%)	n	%	CI (%)	N	%	CI (%)	n	%	CI (%)
Bat eared foxes	9	0	0	-	0	0	-	8	88.9	56.5-98	0	0	-	2	22.2	6.3-54.7
Brown hyenas	17	8	47.1	26.2-69	13	76.5	52.7-90.4	14	82.4	59-93.8	1	5.9	1-27	11	64.7	41.3-82.7
Spotted hyenas	19	9	47.4	27.3-68.3	11	57.9	36.3-76.9	19	100	87.5-1	3	15.8	5.5-37.6	8	42.1	23.1-63.7
Cheetahs	85	39	45.9	35.7-56.4	64	75.3	65.2-83.2	37	43.5	33.5-54.1	9	10.6	5.7-18.9	41	48.2	37.9-58.7
Total	130	56	43.1	34.9-51.7	88	67.7	59.2-75.1	78	60	51.4-68	13	10	5.9-16.4	62	47.7	39.3-56.2

Table 3 - Frequency of pathogens found in the study and percentages according to the animal species

6.2 – Gel Electrophoresis

Examination of amplification success using gel electrophoresis was conducted for all PCRs that were performed. Thus, the following figures (Figure 7-10) are just a fraction of these PCRs and are merely representative of the obtained results.

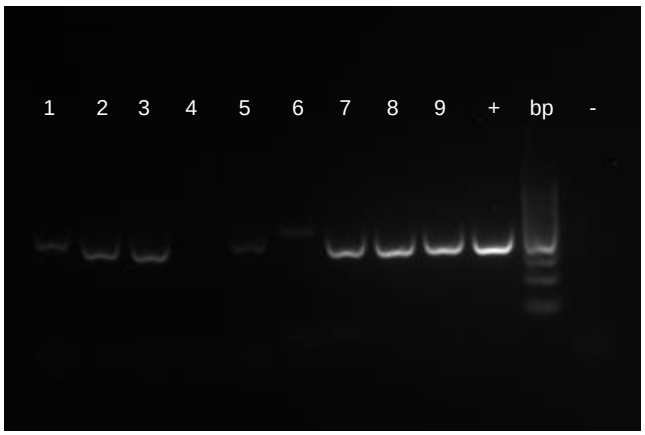


Figure 12 - Onchocercidae PCR from spotted hyenas' samples. A visible band at around the same height in the gel as the positive control means a positive sample. The first nine slots are of spotted hyena samples and the remainder three are, from left to right, the positive control (+), a 100 base pair (bp) ladder marker and the negative control (-). Agarose used was a 1.5% solution.

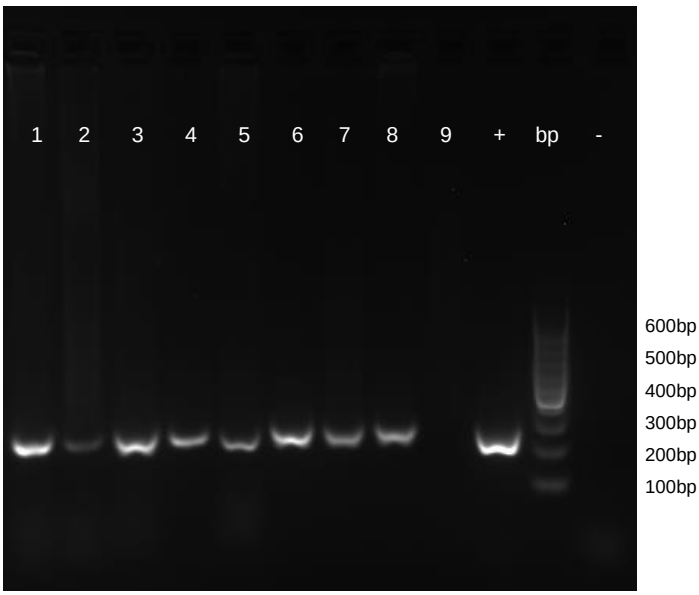


Figure 11 - *Anaplasma* PCR for bat-eared foxes' samples. Positive results are represented through a visible band at around the same height as the positive control. The first nine slots are of spotted hyena samples and the remainder three are, from left to right, the positive control (+), a 100 base pair (bp) ladder marker and the negative control (-). Agarose used was a 1.5%

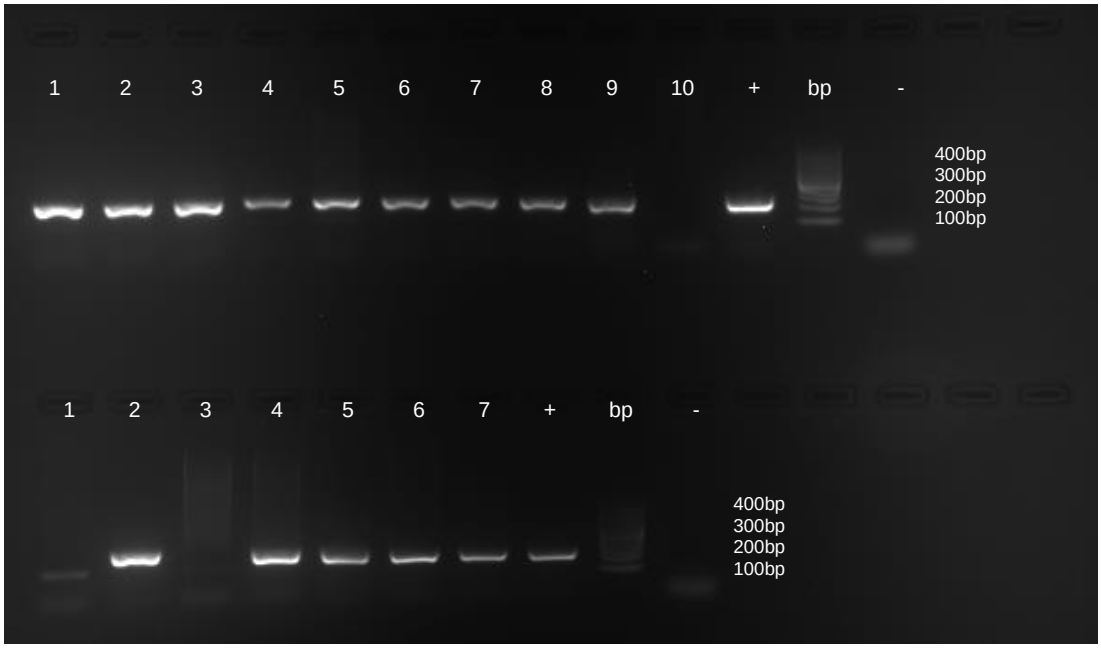


Figure 13 - *Anaplasma* PCR for brown hyenas' samples. Positive results are represented through a visible band at around the same height as the positive control. In each row the last three slots represent, from left to right, the positive control (+), a 100 base pair (bp) ladder marker and the negative control (-). Agarose used was a 1.5% solution.

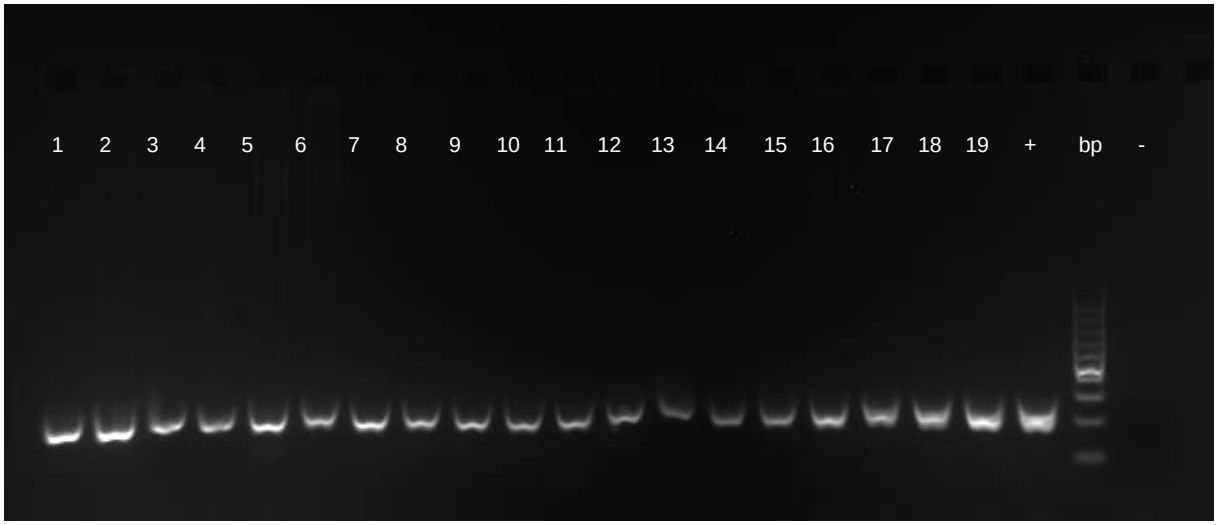


Figure 14 - *Anaplasma* PCR for spotted hyenas' samples. Positive samples are represented through a visible band at around the same height as the positive control. The last three slots represent, from left to right, the positive control (+), a 100 base pair (bp) ladder marker and the negative control (-) and the remainder ones are spotted hyena samples. Agarose used was a 1.5% solution.

6.3 – Phylogenetic Analysis

In depth phylogenetic analysis was only performed with the hyena species, both brown and spotted, but with none of the other animals.

Exceptionally, all *Hepatozoon* positive samples were sequenced and, unfortunately none of the Piroplasmida ones. The main reason is that at first, both hyena species showed zero positive results for Piroplasmida, but after repetition of the tests, the results changed, but time was very scarce and it did not allow further studies.

The Onchocercidae sequences showed high homology with *Acanthocheilonema reconditum* and *Acanthocheilonema dracunculoides* in both hyena species. The positive samples were then tested again based on a new protocol that targeted the small subunit ribosomal RNA gene of the mitochondrion (12S rRNA) with very similar sequencing results and phylogenetic trees were created with both ITS-2 and 12S rRNA genes, shown below in Figure 11 and 12, respectively.

Only one sequence was used to create the tree, but all sequenced positive brown hyena samples for filarids were identical to one another, and the same goes for the spotted hyena sequences. BLASTn searches showed no identical sequences in the public database.

As it is shown in Figure 13, the *Acanthocheilonema* sequences belonging to Cheetahs had very similar results to the Hyenas, but they were closer in proximity to the *Acanthocheilonema* found in Spotted Hyenas than to the one found in Brown Hyenas, as revealed in Figure 14.

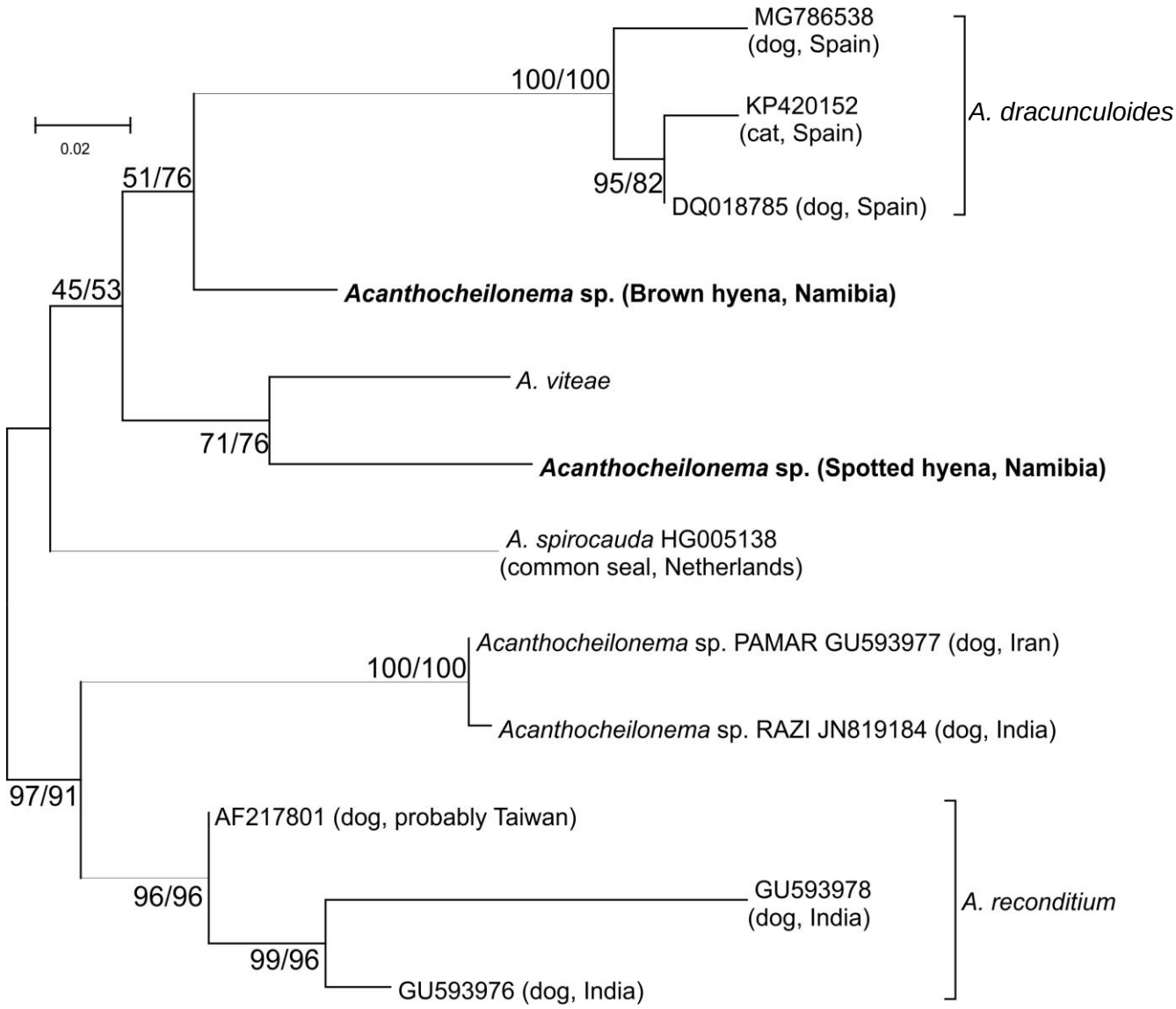


Figure 15 – Maximum-likelihood phylogenetic analysis of the *Acanthocheilonema* isolates based on ITS-2 rRNA gene sequences. The sequences obtained in the present study are printed bold. GenBank accession numbers of related sequences are shown next to species names, host where it was isolated from and location. Bootstrap percentages from 1000 replicates per analysis are shown for clades that were supported in the analysis. Numbers before and after the slash represents node support values obtained by the Shimodaira–Hasegawa likelihood ratio test and bootstrapping, respectively.

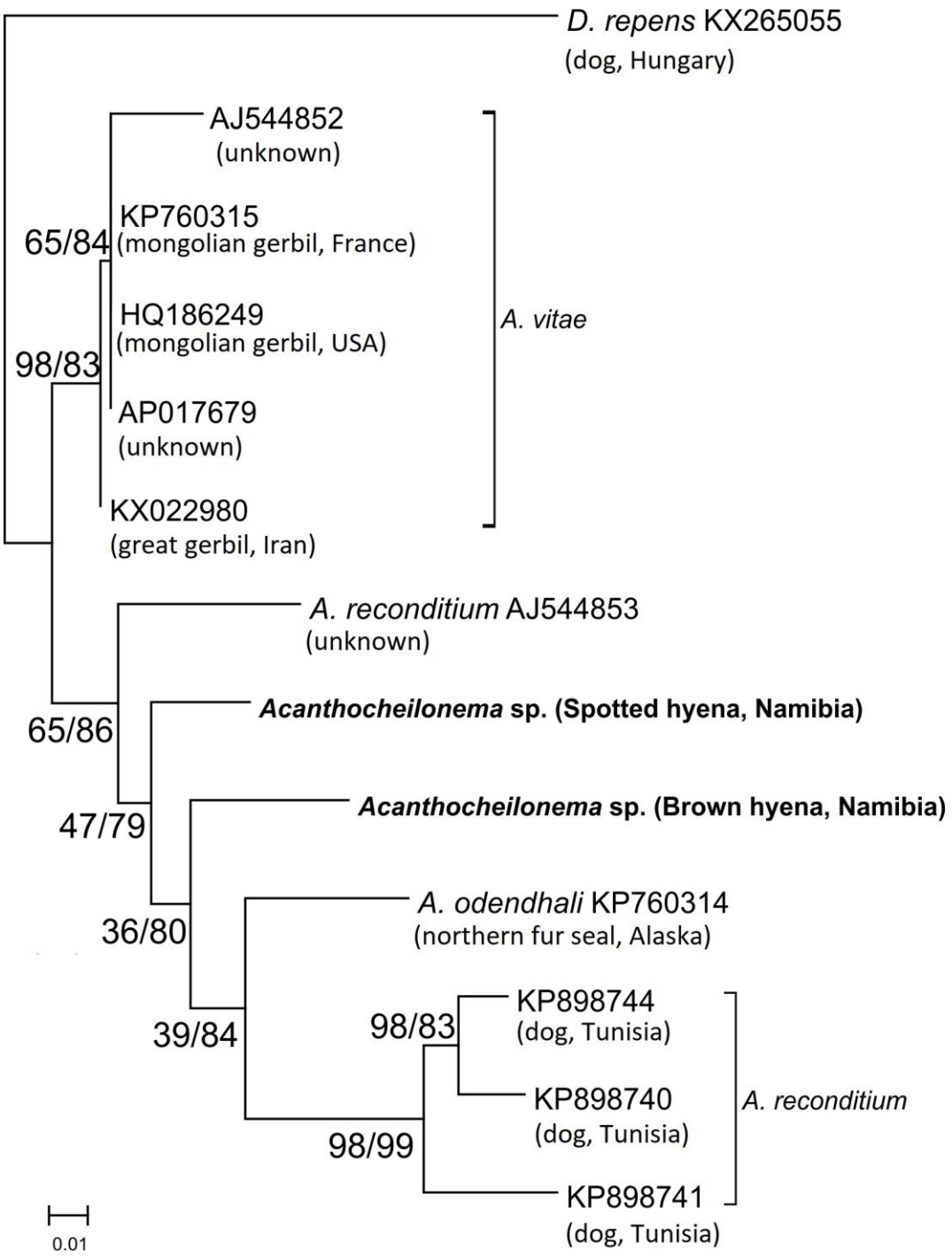


Figure 16 – Maximum-likelihood phylogenetic analysis of the *Acanthocheilonema* isolates based on 12S rRNA gene sequences. The sequences obtained in the present study are printed bold. GenBank accession numbers of related sequences are shown next to species names, host where it was isolated from and location. Bootstrap percentages from 1000 replicates per analysis are shown for clades that were supported in the analysis. Numbers before and after the slash represents node support values obtained by the Shimodaira–Hasegawa likelihood ratio test and bootstrapping, respectively.

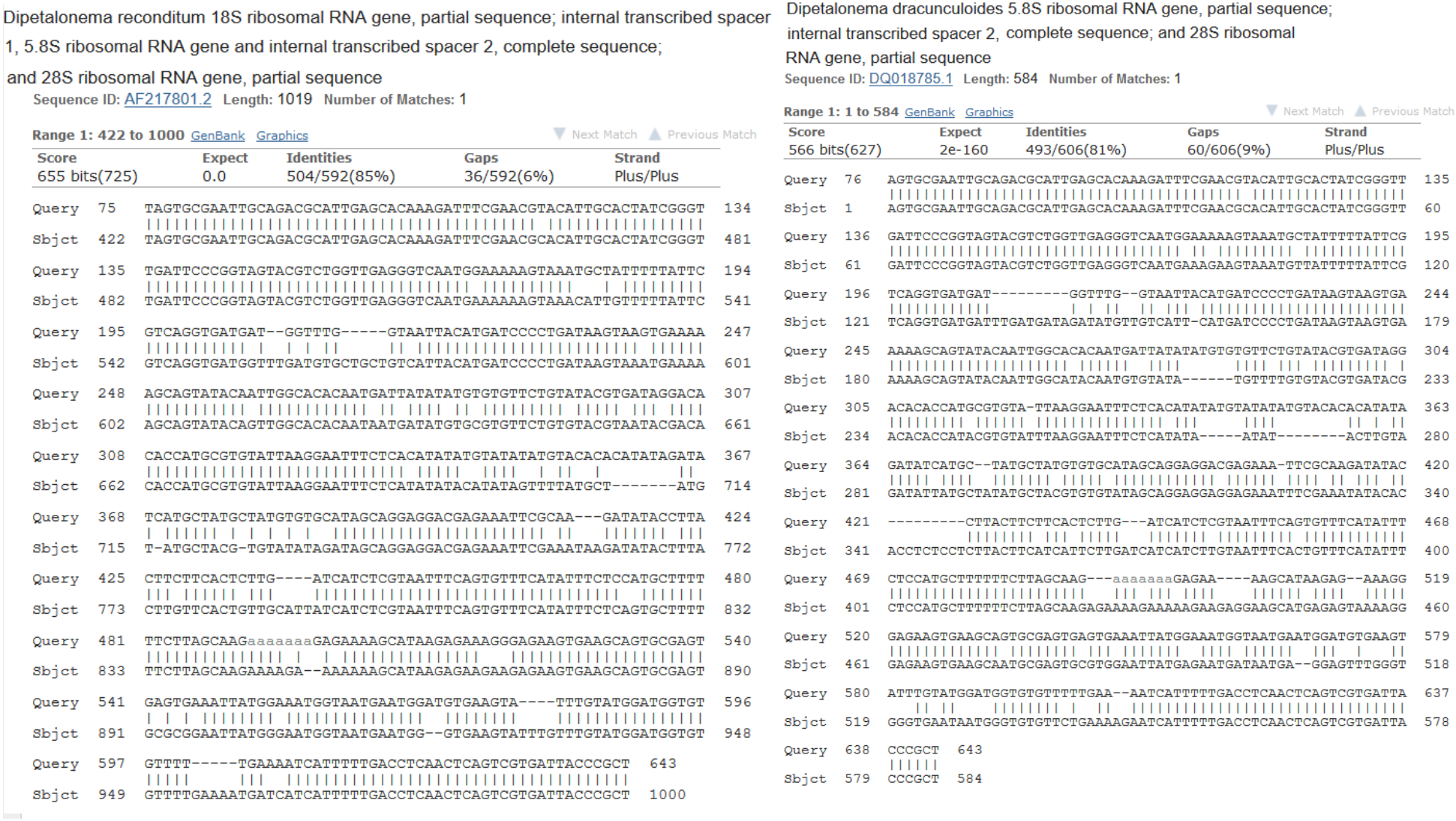


Figure 17 – BLASTn comparison for cheetahs' *Acanthocheilonema* sequences showcasing high similarity with partial *Acanthocheilonema* (former *Dipetalonema*) *reconditum.*and *Acantocheilonema dracunculoides* ITS-2 ribosomal RNA gene sequences.

Figure 18 –BLASTn comparison between one cheetahs' *Acanthocheilonema* sequence and two belonging to spotted and brown hyenas.

The *Anaplasma* sequences were mostly similar with *Anaplasma phagocytophilum* and *Anaplasma platys* in all of the studied species. Multiple 16S primers were used to attempt a differentiation between these last species, but conclusions were not reached. Figures 15 to 17 show the BLAST results for the obtained sequences.

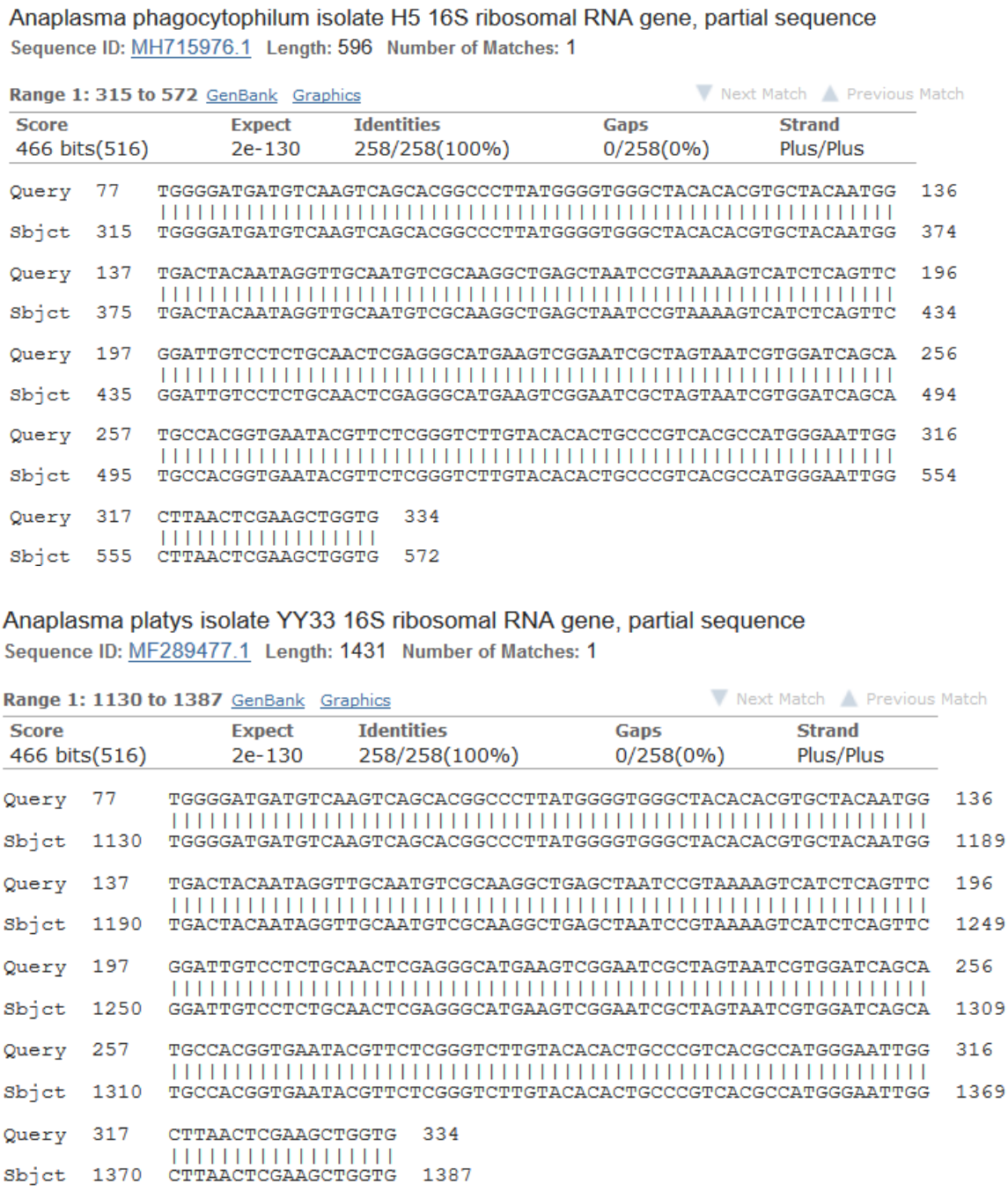


Figure 19 – BLASTn comparison for bat-eared foxes' *Anaplasma* sequences showcasing high similarity with partial 16S ribosomal RNA gene *sequences* of *Anaplasma phagocytophium* (at the top) and *Anaplasma platys* (on the bottom).

Anaplasma phagocytophilum isolate H5 16S ribosomal RNA gene, partial sequence

Sequence ID: MH715976.1 Length: 596 Number of Matches: 1

Range 1: 315 to 572 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
466 bits(516)	1e-130	258/258(100%)	0/258(0%)	Plus/Plus

```
Query  72   TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  131
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  374

Query  132  TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  191
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  434

Query  192  GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  251
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  494

Query  252  TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  311
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  554

Query  312  CTTAACTCGAAGCTGGTG  329
            ||||||||||||||||||
Sbjct  555  CTTAACTCGAAGCTGGTG  572
```

Anaplasma platys isolate YY33 16S ribosomal RNA gene, partial sequence

Sequence ID: MF289477.1 Length: 1431 Number of Matches: 1

Range 1: 1130 to 1387 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
466 bits(516)	1e-130	258/258(100%)	0/258(0%)	Plus/Plus

```
Query  72    TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  131
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1130  TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  1189

Query  132   TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  191
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1190  TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  1249

Query  192   GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  251
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1250  GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  1309

Query  252   TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  311
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1310  TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  1369

Query  312   CTTAACTCGAAGCTGGTG  329
             ||||||||||||||||||
Sbjct  1370  CTTAACTCGAAGCTGGTG  1387
```

Figure 20 – BLASTn comparison for brown hyenas' *Anaplasma* sequences showcasing high similarity with partial *Anaplasma phagocytophium* (at the top) *and Anaplasma platys* 16S ribosomal RNA gene sequences (on the bottom).

Anaplasma phagocytophilum isolate H5 16S ribosomal RNA gene, partial sequence

Sequence ID: MH715976.1 Length: 596 Number of Matches: 1

Range 1: 315 to 572 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
466 bits(516)	1e-130	258/258(100%)	0/258(0%)	Plus/Plus

```
Query  77   TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  136
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  315  TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  374

Query  137  TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  196
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  375  TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  434

Query  197  GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  256
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  435  GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  494

Query  257  TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  316
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  495  TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  554

Query  317  CTTAACTCGAAGCTGGTG  334
            ||||||||||||||||||
Sbjct  555  CTTAACTCGAAGCTGGTG  572
```

Anaplasma platys isolate YY33 16S ribosomal RNA gene, partial sequence

Sequence ID: MF289477.1 Length: 1431 Number of Matches: 1

Range 1: 1130 to 1387 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
466 bits(516)	1e-130	258/258(100%)	0/258(0%)	Plus/Plus

```
Query  77    TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  136
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1130  TGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGG  1189

Query  137   TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  196
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1190  TGACTACAATAGGTTGCAATGTCGCAAGGCTGAGCTAATCCGTAAAAGTCATCTCAGTTC  1249

Query  197   GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  256
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1250  GGATTGTCCTCTGCAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCA  1309

Query  257   TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  316
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1310  TGCCACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGG  1369

Query  317   CTTAACTCGAAGCTGGTG  334
             ||||||||||||||||||
Sbjct  1370  CTTAACTCGAAGCTGGTG  1387
```

Figure 21 – BLASTn comparison for spotted hyenas' *Anaplasma* sequences showcasing high similarity with partial 16S ribosomal RNA gene sequences of *Anaplasma phagocytophium* (at the top) *and Anaplasma platys* (on the bottom).

Figures 18 and 19 reveal the attained sequence comparison between the different studied animal species.

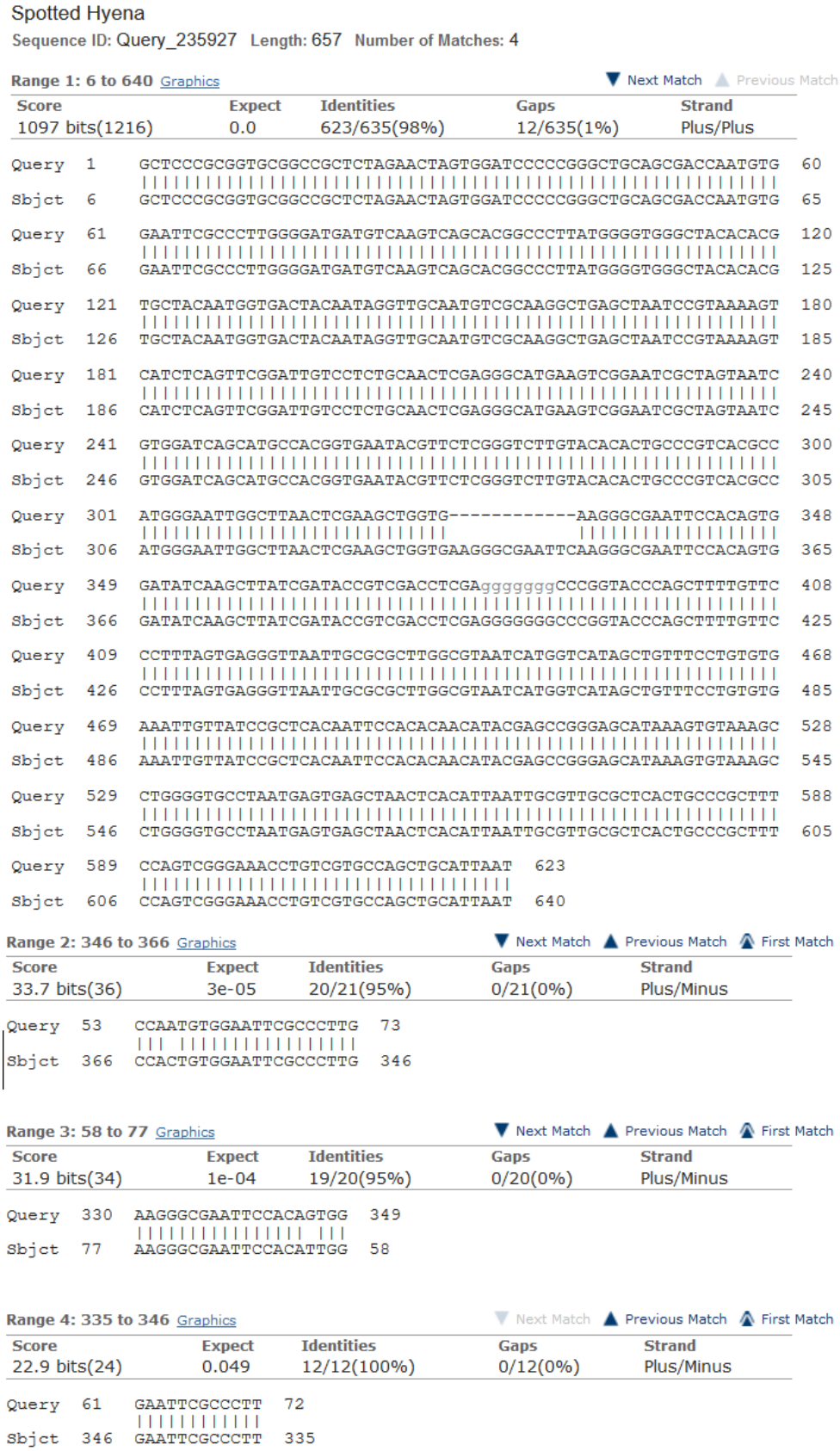


Figure 22 – BLASTn comparison between one brown hyena's *Anaplasma* sequence and one belonging to a spotted hyena.

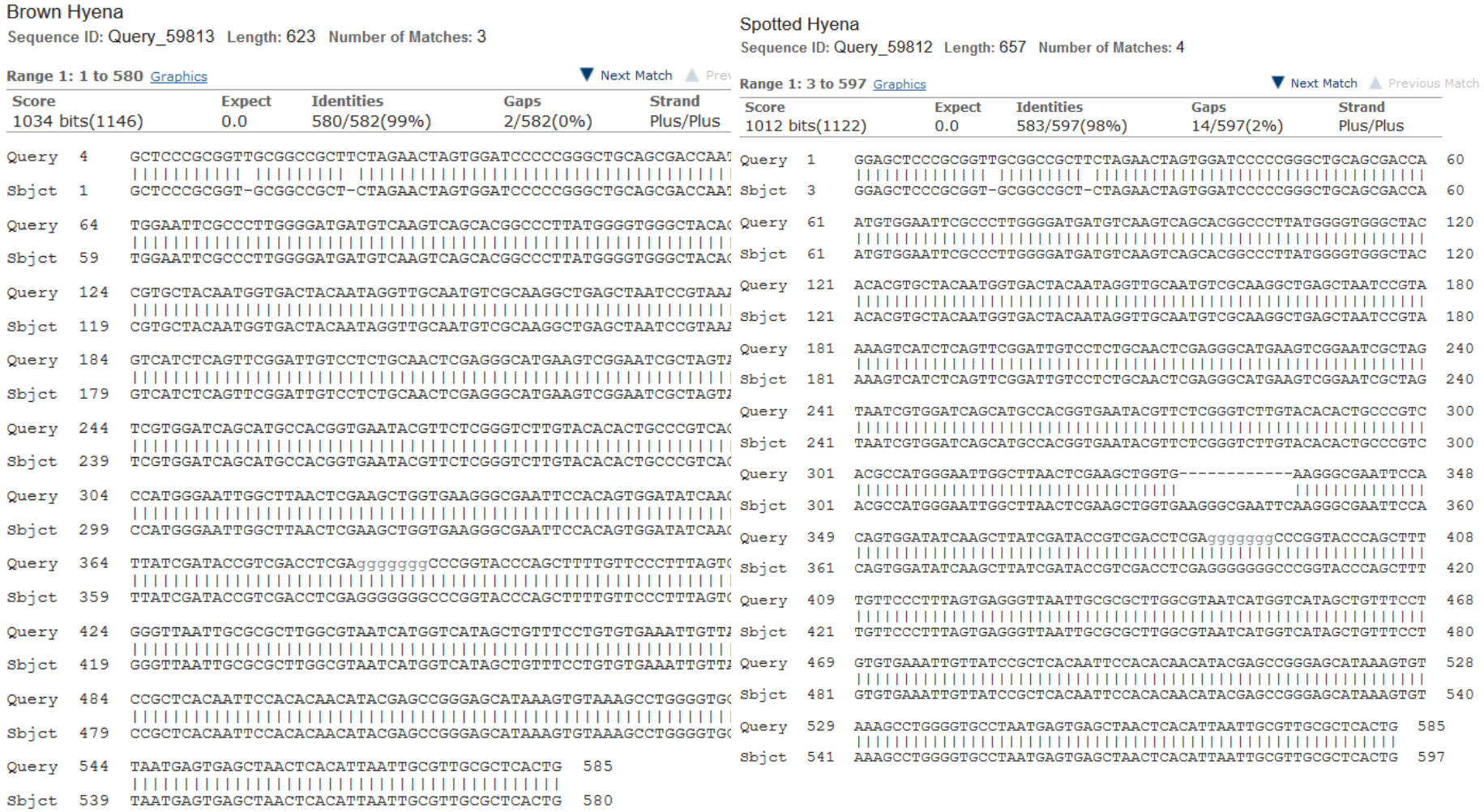
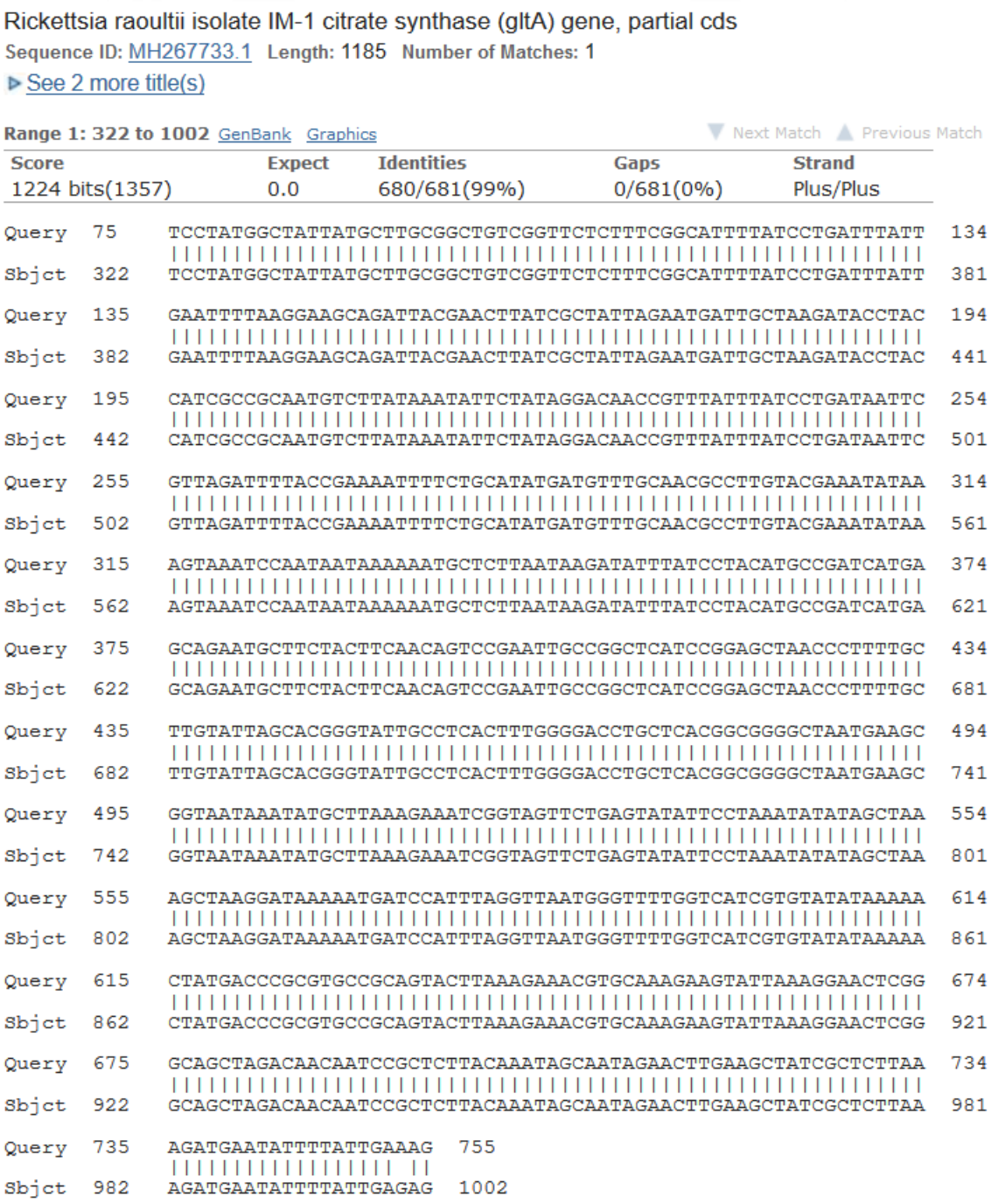


Figure 23 – BLASTn comparison between one bat-eared fox's*Anaplasma* sequence and two belonging to both hyena species, brown hyena on the left and spotted on the right.

As for the *Rickettsia* in cheetahs, the results showed high homology with *Rickettsia raoulti* (Figure 20).



Rickettsia raoultii isolate IM-1 citrate synthase (gltA) gene, partial cds

Sequence ID: MH267733.1 Length: 1185 Number of Matches: 1

See 2 more title(s)

Range 1: 322 to 1002 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1224 bits(1357)	0.0	680/681(99%)	0/681(0%)	Plus/Plus

```
Query  75    TCCTATGGCTATTATGCTTGCGGCTGTCGGTTCTCTTTCGGCATTTTATCCTGATTTATT  134
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  322   TCCTATGGCTATTATGCTTGCGGCTGTCGGTTCTCTTTCGGCATTTTATCCTGATTTATT  381

Query  135   GAATTTTAAGGAAGCAGATTACGAACTTATCGCTATTAGAATGATTGCTAAGATACCTAC  194
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  382   GAATTTTAAGGAAGCAGATTACGAACTTATCGCTATTAGAATGATTGCTAAGATACCTAC  441

Query  195   CATCGCCGCAATGTCTTATAAATATTCTATAGGACAACCGTTTATTTATCCTGATAATTC  254
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  442   CATCGCCGCAATGTCTTATAAATATTCTATAGGACAACCGTTTATTTATCCTGATAATTC  501

Query  255   GTTAGATTTTACCGAAAATTTTCTGCATATGATGTTTGCAACGCCTTGTACGAAATATAA  314
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  502   GTTAGATTTTACCGAAAATTTTCTGCATATGATGTTTGCAACGCCTTGTACGAAATATAA  561

Query  315   AGTAAATCCAATAATAAAAAATGCTCTTAATAAGATATTTATCCTACATGCCGATCATGA  374
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  562   AGTAAATCCAATAATAAAAAATGCTCTTAATAAGATATTTATCCTACATGCCGATCATGA  621

Query  375   GCAGAATGCTTCTACTTCAACAGTCCGAATTGCCGGCTCATCCGGAGCTAACCCTTTTGC  434
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  622   GCAGAATGCTTCTACTTCAACAGTCCGAATTGCCGGCTCATCCGGAGCTAACCCTTTTGC  681

Query  435   TTGTATTAGCACGGGTATTGCCTCACTTTGGGGACCTGCTCACGGCGGGGCTAATGAAGC  494
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  682   TTGTATTAGCACGGGTATTGCCTCACTTTGGGGACCTGCTCACGGCGGGGCTAATGAAGC  741

Query  495   GGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGTATATTCCTAAATATATAGCTAA  554
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  742   GGTAATAAATATGCTTAAAGAAATCGGTAGTTCTGAGTATATTCCTAAATATATAGCTAA  801

Query  555   AGCTAAGGATAAAAATGATCCATTTAGGTTAATGGGTTTTGGTCATCGTGTATATAAAAA  614
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  802   AGCTAAGGATAAAAATGATCCATTTAGGTTAATGGGTTTTGGTCATCGTGTATATAAAAA  861

Query  615   CTATGACCCGCGTGCCGCAGTACTTAAAGAAACGTGCAAAGAAGTATTAAAGGAACTCGG  674
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  862   CTATGACCCGCGTGCCGCAGTACTTAAAGAAACGTGCAAAGAAGTATTAAAGGAACTCGG  921

Query  675   GCAGCTAGACAACAATCCGCTCTTACAAATAGCAATAGAACTTGAAGCTATCGCTCTTAA  734
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  922   GCAGCTAGACAACAATCCGCTCTTACAAATAGCAATAGAACTTGAAGCTATCGCTCTTAA  981

Query  735   AGATGAATATTTTATTGAAAG  755
             |||||||||||||||||  ||
Sbjct  982   AGATGAATATTTTATTGAGAG  1002
```

Figure 24 – BLASTn comparison between cheetahs' *Rickettsia* sequences showcasing high similarity with partial *Rickettsia raoultii* 18S ribosomal RNA gene sequences.

The *Hepatozoon* positive samples were all sequenced and the brown hyaenas were only infected with *Hepatozoon felis* (Figure 21), whilst the spotted hyenas presented a mixed infection, 45% with *Hepatozoon felis* (Figure 22) and the other 55% with *Hepatozoon canis* (Figure 18). The *Hepatozoon felis* infecting both hyena species seems to be the same one, as it is shown in Figure 23.

Hepatozoon felis clone 8533 18S ribosomal RNA gene, partial sequence

Sequence ID: KC138533.1 Length: 1373 Number of Matches: 1

Range 1: 115 to 782 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1169 bits(1296)	0.0	660/668(99%)	0/668(0%)	Plus/Plus

```
Query  18   CTTATACATGAGCAAAATCTCAACTTTTTTAGGAGAGATGCATTTATTAGATAAAAAATC  77
            ||  ||||||||||||||||  ||||||||||||||||||||||||||||||||||||||
Sbjct  115  CTAATACATGAGCAAAATCTTAACTTTTTTAGGAGAGATGCATTTATTAGATAAAAAATC  174

Query  78   AATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACTTAGCAAATCGCATAGT  137
            ||||||||||||||||||||||||||||||||||||||||| |||||  ||||||||||||||||
Sbjct  175  AATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAATAACTCAGCAAATCGCATAGT  234

Query  138  GAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTG  197
            |||||||  ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  235  GAAAACCGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTG  294

Query  198  GCTTACCGTGGCAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTG  257
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  295  GCTTACCGTGGCAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTG  354

Query  258  AGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATTCTAACAGC  317
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  355  AGAAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATTCTAACAGC  414

Query  318  ATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAATGCTTTGTAATTGGA  377
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  415  ATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAATGCTTTGTAATTGGA  474

Query  378  ATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAG  437
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  475  ATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAG  534

Query  438  CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAAAGCTCGTAG  497
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  535  CCGCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAAAGCTCGTAG  594

Query  498  TTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGTGGTATCTTGGTGTGTTT  557
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  595  TTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGTGGTATCTTGGTGTGTTT  654

Query  558  TTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTCAGGATT  617
            |||||||||||||||||||||  ||||||||||||||||||||||||||  ||  ||||||
Sbjct  655  TTAGCAATAATGTCCTTTGAAATGTTTTTTACTTCATTGTAATAAATTATATTTAGGATT  714

Query  618  TTTACTTTGAGAAAATTAGAGTGTTTCTAGCAGGCTAATGCTTTGAATACTGCAGCATGG  677
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  715  TTTACTTTGAGAAAATTAGAGTGTTTCTAGCAGGCTAATGCTTTGAATACTGCAGCATGG  774

Query  678  AATAATAA  685
            ||||||||
Sbjct  775  AATAATAA  782
```

Figure 25 – BLASTn comparison for brown hyenas' *Hepatozoon* sequences showcasing almost an entire identity with partial 18S ribosomal RNA gene sequences *Hepatozoon felis*.

Hepatozoon canis isolate J2 18S ribosomal RNA gene, partial sequence

Sequence ID: KJ572976.1 Length: 668 Number of Matches: 1

Range 1: 1 to 668 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1187 bits(1316)	0.0	664/668(99%)	0/668(0%)	Plus/Plus

```
Query  21   TATACATGAGCAAAATCTCAACTTTATTAGAAGAGACGCATTTATTAGATAAAAAGCCAG  80
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TATACATGAGCAAAATCTCAACTTTATTAGAAGAGACGCATTTATTAGATAAAAAGCCAG  60

Query  81   TTCATGCTTTTACAGTATGAAAATTGGTGATTTATAATAACTTAGCAAATCGCAAAGTGA  140
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  61   TTCATGCTTTTACAGTATGAAAATTGGTGATTTATAATAACTTAGCAAATCGCAAAGTGA  120

Query  141  AAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGC  200
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  AAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGC  180

Query  201  TTACCGTGGCAGTGACGGTTAACGGGGGATTAGGGTTCAATTCCGGAGAGGGAGCCTGAG  260
            |||||||||||||||||||||||||||||||||||| ||||||||||||||||||||||
Sbjct  181  TTACCGTGGCAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAG  240

Query  261  AAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATTCTAACAGTTT  320
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  241  AAACGGCTACCACATCTAAGGAAGGCAGCAGGCGCGCAAATTACCCAATTCTAACAGTTT  300

Query  321  GAGAGAGGTAGTAACGAGAAATAACAATACAAGGCAGTCAAAATGCTTTGTAATTGGAAT  380
            ||||||||||||||| |||||||||||||||||||| ||||||||||||||||||||||
Sbjct  301  GAGAGAGGTAGTAACAAGAAATAACAATACAAGGCAGTTAAAATGCTTTGTAATTGGAAT  360

Query  381  GATAGAAATTTAAACCCTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCC  440
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  361  GATAGAAATTTAAACCCTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCC  420

Query  441  GCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAAAGCTCGTAGTT  500
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  421  GCGGTAATTCCAGCTCCAATAGCGTATATTAAAATTGTTGCAGTTAAAAAGCTCGTAGTT  480

Query  501  GAAGTTCTGCTGAAAGTAACCGGTCTGCTTTTAATAAAAGTGGTATCTTGGTATGTATTT  560
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  481  GAAGTTCTGCTGAAAGTAACCGGTCTGCTTTTAATAAAAGTGGTATCTTGGTATGTATTT  540

Query  561  AGCAATGATGTTCTTTGAAGTGTTTTTTACTTTATTGTAATAAAGCATATTCAGGACTTT  620
            |||||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  541  AGCAATGATGTCCTTTGAAGTGTTTTTTACTTTATTGTAATAAAGCATATTCAGGACTTT  600

Query  621  TACTTTGAGAAAATTAGAGTGTTTCTAGCAGGCTAACGCTTTGAATACTGCAGCATGGAA  680
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  601  TACTTTGAGAAAATTAGAGTGTTTCTAGCAGGCTAACGCTTTGAATACTGCAGCATGGAA  660

Query  681  TAATAAGA  688
            ||||||||
Sbjct  661  TAATAAGA  668
```

Hepatozoon felis clone 8533 18S ribosomal RNA gene, partial sequence

Sequence ID: KC138533.1 Length: 1373 Number of Matches: 1

Range 1: 130 to 729 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1043 bits(1156)	0.0	592/600(99%)	1/600(0%)	Plus/Minus

```
Query  1    TTTTCTCAA-GTAAAAATCCTGAACATAATTTATTACAATGAAGTAAAAAATATTTCAAA  59
            ||||||||| |||||||||||  || ||||||||||||||||||||||||||| ||||||||
Sbjct  729  TTTTCTCAAAGTAAAAATCCTAAATATAATTTATTACAATGAAGTAAAAAACATTTCAAA  670

Query  60   GGACATTATTGCTAAAAACACACCAAGATACCACCTTTATTAAAAGCAGACCGGTTATTT  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  669  GGACATTATTGCTAAAAACACACCAAGATACCACCTTTATTAAAAGCAGACCGGTTATTT  610

Query  120  TTAGCAGAAATTCAACTACGAGCTTTTTAACTGCAACAATTTTAATATACGCTATTGGAG  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  609  TTAGCAGAAATTCAACTACGAGCTTTTTAACTGCAACAATTTTAATATACGCTATTGGAG  550

Query  180  CTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGATACTTTAAAAAGTGT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  549  CTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGATACTTTAAAAAGTGT  490

Query  240  TTAAATTTCTATCATTCCAATTACAAAGCATTTTAACTGCCTTGTATTGTTATTTCTTGT  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  489  TTAAATTTCTATCATTCCAATTACAAAGCATTTTAACTGCCTTGTATTGTTATTTCTTGT  430

Query  300  CACTACCTCTCTTATGCTGTTAGAATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTAGAT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  429  CACTACCTCTCTTATGCTGTTAGAATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTAGAT  370

Query  360  GTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCCCCGTTAACCGTC  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  369  GTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCCCCGTTAACCGTC  310

Query  420  ACTGCCACGGTAAGCCAATACCATACCGTCGAAAGCTGATAGGTCAGAAACTTGAATGAT  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  309  ACTGCCACGGTAAGCCAATACCATACCGTCGAAAGCTGATAGGTCAGAAACTTGAATGAT  250

Query  480  TTATCGCCTGTTTTCACTATGCGATTTGCTAAGTTACTGTAATTCACCAAATTCCATACT  539
            |||||||| |||||||||||||||||||| ||||| ||||||||||||||||||||||||
Sbjct  249  TTATCGCCGGTTTTCACTATGCGATTTGCTGAGTTATTGTAATTCACCAAATTCCATACT  190

Query  540  TTAAAAGCATGTATTGATTTTTTATCTAATAAATGCATCTCTCCTAAAAAAGTTGAGATT  599
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||||
Sbjct  189  TTAAAAGCATGTATTGATTTTTTATCTAATAAATGCATCTCTCCTAAAAAAGTTAAGATT  130
```

Figure 26 – BLASTn comparison for spotted hyenas' *Hepatozoon* sequences showcasing almost an entire identity with both partial *Hepatozoon canis* (on the left) and *Hepatozoon felis* 18S ribosomal RNA gene sequences(on the right). These belong to different animals.

Brown Hyena

Sequence ID: Query_12055 Length: 1071 Number of Matches: 1

Range 1: 33 to 632 Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1101 bits(596)	0.0	599/600(99%)	1/600(0%)	Plus/Minus

```
Query  1    TTTTCTC-AAGTAAAAATCCTGAACATAATTTATTACAATGAAGTAAAAAATATTTCAAA  59
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  632  TTTTCTCAAAGTAAAAATCCTGAACATAATTTATTACAATGAAGTAAAAAATATTTCAAA  573

Query  60   GGACATTATTGCTAAAAACACACCAAGATACCACCTTTATTAAAAGCAGACCGGTTATTT  119
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  572  GGACATTATTGCTAAAAACACACCAAGATACCACCTTTATTAAAAGCAGACCGGTTATTT  513

Query  120  TTAGCAGAAATTCAACTACGAGCTTTTTAACTGCAACAATTTTAATATACGCTATTGGAG  179
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  512  TTAGCAGAAATTCAACTACGAGCTTTTTAACTGCAACAATTTTAATATACGCTATTGGAG  453

Query  180  CTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGATACTTTAAAAAGTGT  239
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  452  CTGGAATTACCGCGGCTGCTGGCACCAGACTTGCCCTCCAATTGATACTTTAAAAAGTGT  393

Query  240  TTAAATTTCTATCATTCCAATTACAAAGCATTTTAACTGCCTTGTATTGTTATTTCTTGT  299
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  392  TTAAATTTCTATCATTCCAATTACAAAGCATTTTAACTGCCTTGTATTGTTATTTCTTGT  333

Query  300  CACTACCTCTCTTATGCTGTTAGAATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTAGAT  359
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  332  CACTACCTCTCTTATGCTGTTAGAATTGGGTAATTTGCGCGCCTGCTGCCTTCCTTAGAT  273

Query  360  GTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCCCCGTTAACCGTC  419
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  272  GTGGTAGCCGTTTCTCAGGCTCCCTCTCCGGAATCGAACCCTAATCCCCCGTTAACCGTC  213

Query  420  ACTGCCACGGTAAGCCAATACCATACCGTCGAAAGCTGATAGGTCAGAAACTTGAATGAT  479
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  212  ACTGCCACGGTAAGCCAATACCATACCGTCGAAAGCTGATAGGTCAGAAACTTGAATGAT  153

Query  480  TTATCGCCTGTTTTCACTATGCGATTTGCTAAGTTACTGTAATTCACCAAATTCCATACT  539
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  152  TTATCGCCTGTTTTCACTATGCGATTTGCTAAGTTACTGTAATTCACCAAATTCCATACT  93

Query  540  TTAAAAGCATGTATTGATTTTTTATCTAATAAATGCATCTCTCCTAAAAAAGTTGAGATT  599
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  92   TTAAAAGCATGTATTGATTTTTTATCTAATAAATGCATCTCTCCTAAAAAAGTTGAGATT  33
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Figure 27 – BLASTn comparison between one spotted hyena's -*Hepatozoon felis* sequence and one brown hyena's *Hepatozoon felis* sequence.

7 – Discussion

Wildlife have many positive roles in human life. They play a critical role in holding together numerous terrestrial, freshwater, and marine ecosystems on which the health of our biosphere depends, they are important sources of food and animal products for a high number of people, and they are also of considerable cultural, spiritual, and economic significance. Sadly, they are increasingly subject to a diversity of stressors, like climate change, diseases, habitat disruption and harvesting for food or for control of pest populations, justifying the high priority that should be given to deepening our knowledge of wildlife parasitology, in order to define the actual and potential roles of key parasites in wildlife health, especially at the population level and in endangered species (MacPhee & Greenwood, 2013; Polley & Thompson, 2015).

Whilst the integrative thinking on human and animal health has come from ancient times, it was only in the 20th century that the American epidemiologist C.W. Schwabe comprehensively revisited the concept of "One Medicine" (commonly referred to as "One Health") (Zinsstag, Schelling, Waltner-Toews, & Tanner, 2011). This concept simply defends a seamless interaction between veterinary and human medicine as a way of working together for the benefit of domestic, wild animal and human health and the global environment (Day, 2011; Crozier & Schulte-Hostedde, 2014; Thompson & Polley, 2014; Robertson et al., 2014).

Wild animals are frequently considered in One Health contexts as reservoirs of emerging diseases or food security, and not as fellow inhabitants of a changing environment with shared risks (Taylor et al., 2001). And even though these animals are, indeed, a major source of emerging human pathogens and the main reservoirs of pathogens of medical and veterinary concern (Daszak et al., 2001; Kruse, Kirkemo, & Handeland, 2004; Thompson et al., 2010; Peter Daszak, 2013; Polley & Thompson, 2015), wildlife parasitology can actually offer other relevant insights into One Health (Thompson et al., 2010; Day, 2011; Thompson, 2013; Jenkins, Simon, Bachand, & Stephen, 2015; Polley & Thompson, 2015; Thompson & Polley, 2015; Kofer et al., 2017). The understanding of the ecological dynamics of a parasite shared among species is crucial for assessing and managing risks to one of the species, especially when host generalism comes into play. Furthermore, the parasitic diseases of wildlife have implications for wildlife conservation, evaluation of wildlife-domestic interactions and for determining their public health significance (Thompson et al., 2010; MacPhee & Greenwood, 2013; Peter Daszak, 2013; Polley & Thompson, 2015; Aguirre, 2016; Espinaze et al., 2018). In fact, prioritizing wildlife parasites in a One Health context involves consideration of whether parasites in wildlife represent a risk to human health and whether they demonstrate potential to adversely affect wildlife populations of conservation concern, those key to ecosystem stability and function, or human communities who rely on wildlife (Jenkins et al., 2015).

Parasites are ubiquitous in wildlife populations (Polley & Thompson, 2015), and their presence does not necessarily mean that the animals are unhealthy, quite the opposite actually, as parasites can serve as indicators of high biodiversity and intact trophic relationships in healthy ecosystems

(Hudson, Dobson, & Lafferty, 2006). However, there is mounting evidence that infectious agents can significantly impact local population dynamics, by causing temporary or permanent declines, reduce host fitness in the wild, interact with other population processes and shape community structure (Abbott, 2006; Hudson et al., 2006; Wyatt et al., 2008; Robinson et al., 2010; Cameron et al., 2011; MacPhee & Greenwood, 2013; Williams et al., 2014). Also, though a parasite may not generally be considered as population-limiting, it is important to remember it may have greater significance in the case of threatened species, or in a wildlife population experiencing the cumulative effects of other pathogens and stressors (Nijhof et al., 2005; Schnittger et al., 2012; Daszak, 2013).

Recently, the significant impact parasites can have on wildlife population dynamics has emerged as a critical issue in the conservation of threatened species (Hudson, Dobson, & Newborn, 1998; Tompkins & Begon, 1999; Albon et al., 2002; Newey & Thirgood, 2004; Moller J. T., 2007; Pedersen, Jones, Nunn, & Altizer, 2007; Aguirre & Tabor, 2008; Burthe et al., 2008; Hawlena, Bashary, Abramsky, Khokhlova, & Krasnov, 2008). Still, the majority of zoonotic parasites for which wildlife is the main reservoir are characterised by having little clinical impact on their hosts (Thompson, 2013; Williams et al., 2014; Burroughs et al., 2017).

Detection and measurement of the health effects of parasites on wildlife, particularly at the population level, is extremely difficult in the absence of obvious “clinical” disease or death (MacPhee & Greenwood, 2013; Watson, 2013; Polley & Thompson, 2015). And it is also quite challenging to establish definitive evidence for population level effects of parasites in free-ranging wildlife (Daszak, 2013), especially considering the poor data environment and the difficulty of accessing the effect a specific variable has on a dynamic population (McCallum, 2016). Taking spotted hyenas for example, the role their parasites play in ecological or population processes is totally unknown, particularly in light of the paucity of published information confirming clinical infections in this species, with diseases such as canine distemper and tuberculosis in the absence of described clinical signs (Alexander et al., 2010; Williams et al., 2014; Bohm & Höner, 2015). This could perhaps point to an immune system that is highly developed, since spotted hyenas are scavengers of carcasses and predators, and are also regularly exposed to a myriad of different pathogens within any ecosystem. Still, there is very little evidence of visible signs of clinical infection of diseases, which might be an indication of their resilience and ability to adapt (Flies, Mansfield, Grant, Weldele, & Holekamp, 2015; Burroughs et al., 2017).

Another major impediment to the large-scale surveillance of wildlife populations has been the difficulties of assessing infection status in living animals (Thompson et al., 2010) and also the great difficulty of collecting samples of free-raging wild animals, especially alive ones. This has been alleviated with the development of molecular tools that can provide sufficient information through non-invasive sampling of living animals (Thompson et al., 2010), but these studies involving wildlife, especially in their natural settings still depend on complex and costly logistical support, that may be hard to find (Polley & Thompson, 2015). The small number of samples available, particularly for

wildlife living in conservation areas, is one of the major impediments and a key reason why this type of studies is not performed more often and thoroughly.

In addition, high prevalence alone is not enough to conclude about effects on wildlife on a population level nor to demonstrate the importance of a species as a reservoir. In order to determine eventual conservation threats for endangered carnivores and to better understand their role in the epidemiology of diseases it is imperative that research be conducted to determine the exact classification of the causative agent and to identify the vectors and/or reservoirs for a large number of agents (Alvarado-Rybak et al., 2016). Their role as carriers of parasites and their ability to serve as sources of infection to vectors still needs elucidation (Burroughs et al., 2017), and these data would deepen the knowledge on the dynamics of parasitic pathogens and would help determine potential distribution areas of the disease.

Basically, understanding parasites and parasitic diseases today requires a detailed knowledge of biochemical, molecular, and immunological aspects as well as population genetics, epidemiology, evolutionary ecology, and disease ecology (Kofer et al., 2017). But despite the enormous contribution of molecular biology in this area, especially to the discovery of new species or strains of pathogens (Duh et al., 2010; Pacheco et al., 2011; Alvarado-Rybak et al., 2016), genetic data must always be interpreted with caution (Dantas-Torres et al., 2012).

In this study, bat-eared foxes were almost not infected with the vector-borne pathogens searched, but the failure to detect parasites in these animals could have been linked with a too low prevalence to detect within our samples, which in this particular animal species was quite small. Furthermore, sampled animals may have had little contact with vectors or areas inhabited with infected animals.

Until the present year, veterinary health studies in Namibia have been mostly centred around livestock and game farms, which are fairly important to the country's economy (Schneider, 2012; OIE, 2018), and very little attention has been paid to the health of small companion animals (Noden & Van der Colf 2013 cited by Noden & Soni, 2015) and wildlife. To date, there is no published record of the distribution of dogs and cats and their specific vector-borne diseases throughout the country and this lack of information means it remains unclear whether or not these species are acting as reservoirs for infectious diseases to other animals, as well as human populations (Noden & Soni, 2015). Likewise, the knowledge on wildlife parasitology is quite lacking in the African continent and Namibia is not an exception to this.

7.1 – Onchocercidae

As for the obtained results, the epidemiology of *Acanthocheilonema dracunculoides* (Cobbold, 1970) usually involves domestic and some sylvatic canines, like aardwolves, red fox and spotted hyenas (Schwan & Schroter, 2006). So the presence of a similar organism in the blood samples examined in this study was not surprising, especially considering that Schwan & Schroter (2006) reported two clinical cases of *A. dracunculoides* in dogs from Windhoek, Namibia, which shows the parasite is

already present in the country. Also, the principal intermediate hosts are the house fly (*Hippobosca longipennis*) and the brown dog tick (*Rhipicephalus sanguineus*), which is the main tick species reported on dogs throughout central and southern Namibia (Matthee et al., 2010).

As for *Acanthocheilonema reconditum* (Grassi, 1889), it is a common filarial parasite of dogs in many geographical areas (Siwila, Mwase, Nejsum, & Simonsen, 2015) and it has been isolated from some wild canids, as well as spotted and brown hyenas (Sonin 1985 cited by Schwan, 2009). So far there have been no reports of its existence in Namibia, though it is relatively common in Zambia (Siwila et al., 2015), and it has been previously reported from Kenya (Albrechtová et al., 2011), Uganda (Bwangamoi and Isyagi, 1973 cited by Siwila et al., 2015)), Mozambique (Schwan & Durand, 2002) and South Africa (Schwan, 2009). It is transmitted by fleas (*Ctenocephalides canis* and *Ctenocephalides felis*) and lice (*Heterodoxus spiniger* and *Trichodectes canis)* (Noden & Soni, 2015).

It remains very difficult to establish how much rRNA gene sequence variation must exist for a source organism to be considered a different species or to be considered merely a variant and/or a genotype of a species (Allsopp & Allsopp, 2006).

In this particular case, as the morphology of the parasite was not studied, nor the possible vectors or its role in clinical disease, it is not feasible to draw a definitive line between previously described new species. Alternatively, the detected specimen could represent only a variant of *Acanthocheilonema dracunculoides* or *Acanthocheilonema reconditum.* However, since two different genes, with separate rates of variability were studied, the molecular characterization allowed a better distinction of isolates and an improved understanding of genomic homologies and differences, as well as phylogenetic proximity. This led us to consider a high probability of this *Acanthocheilonema* species being a new, not described one, but further studies will be necessary to prove this fact. In addition, many of these previously reported *Acanthocheilonema* were only identified morphologically, and it is not known whether or not they share the same genetics as the ones found in both hyena species in Namibia.

When it comes to the *Acanthocheilonema* present in the cheetahs, the fact that it shows a higher homology with the one present in spotted hyenas can be of interest, though it can be due to the fact this species is more prevalent than brown hyenas.

Both *A. reconditum* and *A. dracunculoides* are widely regarded as apathogenic (Noden & Soni, 2015; E. V. Schwan, 2009), but there is some evidence reported from Spain, Kenya, Uganda and Namibia that suggests the latter may not be as innocuous as generally assumed (E. V. Schwan, 2009). This fact further supports the need to perform additional analysis of this apparent novel parasite.

7.2 – Piroplasmida

Out of the order Piroplasmida, *Babesia* spp. are one of the most important protozoal vector-borne agents of disease of small companion animals throughout the world (Baneth et al., 2016). A wide

variety of *Babesia* species has been vastly reported in varied species of African carnivores (Collett, 2000; B. Penzhorn, Kjemtrup, & Lopez-Rebollar, 2001; A. M. Bosman et al., 2007, 2013; Munson et al., 2008; Paul Tshepo Matjila, Leisewitz, Jongejan, Bertschinger, & Penzhorn, 2008;), including domestic canines and felines throughout southern Africa (Chitanga, Gaff, & Mukaratirwa, 2014).

Previous reports from hosts of the family Hyaenidae include *Babesia alberti* described from the spotted hyena (Van den Berge 1937 cited by Williams et al., 2014) and *Babesia* sp. related to *B. lengau* (Burroughs et al., 2017), the first piroplasm ever to be reported in brown hyenas. *Babesia lengau* was firstly described by Bosman, Oosthuizen, Peirce, Venter, & Penzhorn (2010), and was initially thought to be exclusive to cheetahs. But more recently has been found in two domestic cats in South Africa (Bosman et al., 2013) and also a *Babesia* detected from clinically ill domestic sheep in Greece was reported to be 99 % similar to *B. lengau* (Giadinis et al., 2012).

Asymptomatic infections with *Babesia* sp. are common (Lopez-Rebollar et al., 1999; Penzhorn et al., 2001; Penzhorn, 2006; Bosman et al., 2007, 2010; Githaka et al., 2012), but this parasite can cause disease in cases of highly parasitized, immunosuppressed or stressed hosts (Banie L. Penzhorn, 2006; Munson et al., 2008;).

Overall, its presence in the studied animals was as anticipated, and it would be of most interest to sequence the obtained results in order to compare them to the ones recently found in the same country in some of the same species (Burroughs et al., 2017).

7.3 – *Anaplasma*

Anaplasma has been reported in dogs in South Africa (Inokuma et al., 2005; Matjila, Leisewitz, Oosthuizen, Jongejan, & Penzhorn, 2008), but these pathogens have yet to be reported in Namibia, even though they have been found in domestic canines and felines throughout the remaining southern part of Africa (Chitanga et al., 2014).

Anaplasma phagocytophilum, a considered emerging pathogen of humans, horses and dogs worldwide, was recently reported from South Africa (Kolo et al., 2016), but previously to that a closely related but distinct species had been reported from domestic dogs also in South Africa (Inokuma et al., 2005). As for *Anaplasma platys,* it was found in dogs in Congo (Sanogo et al., 2003), the Ivory Coast and Kenya (Matei et al., 2016) and in domestic and wild ruminants in South Africa (Berggoetz et al., 2014). It is, then, very possible for a variant of any of these parasites to be found in Namibia, since the distance between these sites is not that great, even though there have been no reports of any species of *Anaplasma* in the country.

Moreover, since both these species have zoonotic potential (Matei et al., 2016; Penzhorn et al., 2018), posing a known human-health risk, the presence of closely related organisms may be cause for concern and should inquire further studies.

The obtained sequences shared equal homologies with both *Anaplasma* species and though multiple 16S primers were used to attempt a differentiation between these, conclusions were, unfortunately, not reached.

7.4 – *Rickettsia*

As for *Rickettsia*, in southern Africa there have been reports of *Rickettsia conorii and Rickettsia africae* (Pretorius, Jensenius & Birtles 2004 cited by Noden & Soni, 2015), two Rickttsiae belonging to the tick-transmitted spotted fever group. However, the presence of these species has not been shown in Namibia in dogs and cats, but there has been serological evidence of humans affected (Noden & Van der Colf 2013 cited by Noden, Tshavuka, Van Der Colf, Chipare, & Wilkinson, 2014). *Rickettsia typhi* (typhus group) and *Rickettsia felis* (spotted fever group) are Rickettsiae of public health importance transmitted by fleas (Azad et al. 1997 cited by Noden & Soni, 2015), generally not associated with companion animals. However, recently, dogs and cats were implicated as reservoirs for infection in Europe (Nogueras et al., 2013), thus posing a risk for zoonotic transmission to human populations and both species have been reported in Zimbabwe and South Africa (Matthewman et al. 1997a cited by Noden & Soni, 2015).

However, the parasite found in cheetahs showed high homology with *Rickettsia raoultii*, an emerging spotted fever group Rickettsiae transmitted by *Dermacentor* ticks (Mediannikov et al., 2008). Firstly reported in Europe (Mediannikov et al., 2008), it has recently spread to Asia, with cases of human infections in China (Jia et al., 2014), and it has also been reported in Morocco, the only known African appearance, in *Dermacentor marginatus* ticks (Sarih et al., 2008).

More studies would be necessary to verify this presence, including comparing these sequences with the other positive samples for *Rickettsia* after sequencing. Yet, this is something that must be taken into consideration, even with low percentage of infected animals, due to the clinical relevance it may withhold, as it is known to be a human pathogen.

7.5 – *Hepatozoon*

Hepatozoon has been reported in domestic (Chitanga et al., 2014) and also wild felines and canines (Paul Tshepo Matjila et al., 2008; Williams et al., 2014) throughout southern Africa. It has been shown to be quite prevalent among free-ranging African carnivores, including spotted hyenas (McCully et al., 1975; Averbeck et al., 1990; Peirce, Laurenson, & Gascoyne, 1995; Van Heerden & Mills, 1995; East et al., 2008; Williams et al., 2014), so the results of this study were expected.

Still, even though asymptomatic infections are more common (Averbeck et al., 1990; McCully et al., 1975; Van Heerden & Mills, 1995), a non-*H. canis* species was reported to cause clinical disease in spotted hyenas in Tanzania (East et al., 2008) and also significant lesions attributed to hepatozoonosis were described in Kruger National Park in South Africa (McCully et al., 1975), which

means the presence of these parasites should not be discarded as irrelevant as it may have further implications on the animal populations.

The different species are not considered to be specific to the suborders of the Carnivora, and both *H. canis* and *H. felis* have been reported in both canids and felids (Gad Baneth et al., 2013).This is consistent with the findings of this study, where brown hyenas were only infected with *Hepatozoon felis*, whilst the spotted ones presented a mixed infection.

8 – Conclusion

This study revealed insights into vector-borne pathogens infecting free-ranging bat-eared foxes (*Otocyon megalotis*), brown hyenas (*Parahyaena brunnea*), spotted hyenas (*Crocuta crocuta*) and cheetahs (*Acinonyx jubatus*) from Namibia.

Overall, the carnivore species were found to harbour parasites belonging, by prevalence order, to the Piroplasmida order and to the families Anaplasmataceae, Hepatozoidae, Onchocercidae and Rickettsiaceae.

This study also provided a preliminary phylogenetic analysis on some of the found parasites. Obtained filaroid sequences showed high homologies with both *Acanthocheilonema reconditum* and *Acanthocheilonema dracunculoides* and the resulting phylogenetic tree allowed for the study of the relationship amongst these related taxa. For Anaplasmataceae, the sequencing results indicated high similarity with both *Anaplasma phagocytophilum* and *Anaplasma platys*, Rickettsiaceae found displayed high homologies wi*th Rickettsia raoultii* and the Hepatozoidae infection showed to be a mixed one with bo*th Hepatozoon ca*nis a*nd Hepatozoon fe*lis.

In conclusion, this dissertation was only a small portion of a much larger set of studies, and it contributed with more information regarding vector-borne pathogens of wild African carnivores, more specifically of Namibia. However, the remaining samples are still presently being studied, so hopefully with time we will know which specific parasites are currently infecting these animals in this country.

Future studies should focus on better understanding the epidemiology of vectors, determine transmission routes, infection dynamics, parasite diversity, and host specificity of these vector-borne parasites and also the risk of transmission between domestic animals, wildlife and humans.

9 – Bibliography

Abbott, I. (2006). Mammalian faunal collapse in Western Australia, 1875-1925: The hypothesised role of epizootic disease and a conceptual model of its origin, introduction, transmission, and spread. *Australian Zoologist*, *33*(4), 530–561. https://doi.org/10.7882/AZ.2006.024

Abdul-Ghani, R., Al-Mekhlafi, A. M., & Karanis, P. (2012). Loop-mediated isothermal amplification (LAMP) for malarial parasites of humans: Would it come to clinical reality as a point-of-care test? *Acta Tropica*, *122*(3), 233–240. https://doi.org/10.1016/j.actatropica.2012.02.004

Aguirre, A. A. (2016). Parasitic diseases of wildlife and domestic animals: new trends of disease emergence, (May).

Aguirre, A. A., & Tabor, G. M. (2008). Global factors driving emerging infectious diseases: Impact on wildlife populations. *Annals of the New York Academy of Sciences*, *1149*, 1–3. https://doi.org/10.1196/annals.1428.052

Albon, S. D., Stien, A., Irvine, R. J., Langvatn, R., Ropstad, E., & Halvorsen, O. (2002). The role of parasites in the dynamics of a reindeer population. *Proceedings of the Royal Society B: Biological Sciences*, *269*(1500), 1625–1632. https://doi.org/10.1098/rspb.2002.2064

Albrechtová, K., Sedlák, K., PetrŽelková, K. J., Hlaváč, J., Mihalca, A. D., Lesingirian, A., … Modrý, D. (2011). Occurrence of filaria in domestic dogs of Samburu pastoralists in Northern Kenya and its associations with canine distemper. *Veterinary Parasitology*, *182*(2–4), 230–238. https://doi.org/10.1016/j.vetpar.2011.05.042

Alexander, K. A., McNutt, J. W., Briggs, M. B., Standers, P. E., Funston, P., Hemson, G., … van Vuuren, M. (2010). Multi-host pathogens and carnivore management in southern Africa. *Comparative Immunology, Microbiology and Infectious Diseases*, *33*(3), 249–265. https://doi.org/10.1016/j.cimid.2008.10.005

Allsopp, M. T. E. P., & Allsopp, B. A. (2006). Molecular sequence evidence for the reclassification of some Babesia species. *Annals of the New York Academy of Sciences*, *1081*, 509–517. https://doi.org/10.1196/annals.1373.076

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology*, *215*(3), 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2

Alvarado-Rybak, M., Solano-Gallego, L., & Millán, J. (2016). A review of piroplasmid infections in wild carnivores worldwide: importance for domestic animal health and wildlife conservation. *Parasites and Vectors*, *9*(1), 1–19. https://doi.org/10.1186/s13071-016-1808-7

Álvarez, I., & Wendel, J. F. (2003). Ribosomal ITS sequences and plant phylogenetic inference. *Molecular Phylogenetics and Evolution*, *29*(3), 417–434. https://doi.org/10.1016/S1055-7903(03)00208-2

Anderson, R. M., & May, R. M. (1978). Regulation and Stability of Host-Parasite Population Interactions: I. Regulatory Processes. *Source: Journal of Animal Ecology Journal of Animal Ecology Journal of Animal Ecology*, *47*(47), 219–247. https://doi.org/10.2307/3933

Averbeck, G. a, Bjork, K. E., Packer, C., & Herbst, L. (1990). Prevalence of hematozoans in lions (Panthera leo) and cheetah (Acinonyx jubatus) in Serengeti National Park and Ngorongoro Crater, Tanzania. *Journal of Wildlife Diseases*, *26*(3), 392–394. https://doi.org/10.7589/0090-3558-26.3.392

Baldauf, S. L. (2003). Phylogeny for the faint of heart: A tutorial. *Trends in Genetics*, *19*(6), 345–351. https://doi.org/10.1016/S0168-9525(03)00112-4

Baneth, G., Thamsborg, S. M., Otranto, D., Guillot, J., Blaga, R., Deplazes, P., & Solano-Gallego, L. (2016). Major Parasitic Zoonoses Associated with Dogs and Cats in Europe. *Journal of Comparative Pathology*, *155*(1), S54–S74. https://doi.org/10.1016/j.jcpa.2015.10.179

Baneth, Gad, Samish, M., Alekseev, E., Aroch, I., & Shkap, V. (2001). Transmission of Hepatozoon canis to dogs by naturally-fed or percutaneously-injected Rhipicephalus sanguineus ticks. *J. Parasitol*, *87*(3), 606–611. https://doi.org/10.1645/0022-3395(2001)087[0606:TOHCTD]2.0.CO;2

Baneth, Gad, Sheiner, A., Eyal, O., Hahn, S., Beaufils, J. P., Anug, Y., & Talmi-Frank, D. (2013). Redescription of Hepatozoon felis (Apicomplexa: Hepatozoidae) based on phylogenetic analysis, tissue and blood form morphology, and possible transplacental transmission. *Parasites and Vectors*, *6*(1), 1–10. https://doi.org/10.1186/1756-3305-6-102

Bartsch, S. M., Hotez, P. J., Asti, L., Zapf, K. M., Bottazzi, M. E., Diemert, D. J., & Lee, B. Y. (2016). The Global Economic and Health Burden of Human Hookworm Infection. *PLoS Neglected Tropical Diseases*, *10*(9). https://doi.org/10.1371/journal.pntd.0004922

Belbachir, F., Pettorelli, N., Wacher, T., Belbachir-Bazi, A., & Durant, S. M. (2015). Monitoring rarity: The critically endangered Saharan cheetah as a flagship species for a threatened ecosystem. *PLoS ONE*, *10*(1), 1–15. https://doi.org/10.1371/journal.pone.0115136

Berggoetz, M., Schmid, M., Ston, D., Wyss, V., Chevillon, C., Pretorius, A. M., & Gern, L. (2014). Protozoan and bacterial pathogens in tick salivary glands in wild and domestic animal environments in South Africa. *Ticks and Tick-Borne Diseases*, *5*(2), 176–185. https://doi.org/10.1016/j.ttbdis.2013.10.003

Bohm, T., & Höner, O. . (2015). Crocuta crocuta. The IUCN Red List of Threatened Species 2015: e.T5674A45194782. Retrieved from http://dx.doi.org/10.2305/IUCN.UK.2015-2.RLTS.T5674A45194782.en

Bosman, A. M., Oosthuizen, M. C., Peirce, M. A., Venter, E. H., & Penzhorn, B. L. (2010). Babesia lengau sp. nov., a novel Babesia species in Cheetah (Acinonyx jubatus, Schreber, 1775) populations in South Africa. *Journal of Clinical Microbiology*, *48*(8), 2703–2708. https://doi.org/10.1128/JCM.02266-09

Bosman, A. M., Oosthuizen, M. C., Venter, E. H., Steyl, J., Gous, T. A., & Penzhorn, B. L. (2013). Babesia lengau associated with cerebral and haemolytic babesiosis in two domestic cats. *Parasites and Vectors*, *6*(1), 1–6. https://doi.org/10.1186/1756-3305-6-128

Bosman, A. M., Venter, E. H., & Penzhorn, B. L. (2007). Occurrence of Babesia felis and Babesia leo in various wild felid species and domestic cats in Southern Africa, based on reverse line blot analysis. *Veterinary Parasitology*, *144*(1–2), 33–38. https://doi.org/10.1016/j.vetpar.2006.09.025

Bosman, Anna Mari, Oosthuizen, M. C., Peirce, M. A., Venter, E. H., & Penzhorn, B. L. (2010). Babesia lengau sp. nov., a novel Babesia species in Cheetah (Acinonyx jubatus, Schreber, 1775) populations in South Africa. *Journal of Clinical Microbiology*, *48*(8), 2703–2708. https://doi.org/10.1128/JCM.02266-09

Boydston, E. E., Morelli, T. L., & Holekamp, K. E. (2001). Sex differences in territorial behaviour exhibited by the spotted hyena (Hyaenidae, Crocuta crocuta). *Ethology*, *107*, 369–385. https://doi.org/10.1046/j.1439-0310.2002.00781.x

Breuer, T. (2005). Diet choice of large carnivores in northern Cameroon Diet choice of large carnivores in northern Cameroon, (APRIL 2005), 97–106. https://doi.org/10.1111/j.1365-2028.2005.00551.x

Brocklesby, D. W., & Vidler, B. O. (1965). Some parasites of East African wild animals. *African Journal of Ecology*. https://doi.org/10.1111/j.1365-2028.1965.tb00745.x

Broekhuis, F., Cozzi, G., Valeix, M., Mcnutt, J. W., & Macdonald, D. W. (2013). Risk avoidance in sympatric large carnivores: Reactive or predictive? *Journal of Animal Ecology*, *82*(5), 1098–1105. https://doi.org/10.1111/1365-2656.12077

Broomhall, L. S., Mills, M. G. L., & du Toit, J. T. (2003). Home range and habitat use by cheetahs (Acinonyx jubatus) in the Kruger National Park. *Journal of Zoology*, *261*(2), S0952836903004059. https://doi.org/10.1017/S0952836903004059

Burgener, N., & Gusset, M. (2003). The feeding habits of brown hyaenas (Hyaena brunnea) on a game ranch in Limpopo Province, South Africa. *African Zoology*, *38*(1), 181–184. https://doi.org/10.1080/15627020.2003.11657207

Burroughs, R. E. J., Penzhorn, B. L., Wiesel, I., Barker, N., Vorster, I., & Oosthuizen, M. C. (2017). Piroplasms in brown hyaenas (Parahyaena brunnea) and spotted hyaenas (Crocuta crocuta) in Namibia and South Africa are closely related to Babesia lengau. *Parasitology Research*, *116*(2), 685–692. https://doi.org/10.1007/s00436-016-5334-5

Burthe, S., Telfer, S., Begon, M., Bennett, M., Smith, A., & Lambin, X. (2008). Cowpox virus infection in natural field vole *Microtus agrestis* populations: significant negative impacts on survival. *Journal of Animal Ecology*, *77*(1), 110–119. https://doi.org/10.1111/j.1365-2656.2007.01302.x.Cowpox

Cameron, S. A., Lozier, J. D., Strange, J. P., Koch, J. B., Cordes, N., Solter, L. F., & Griswold, T. L. (2011). Patterns of widespread decline in North American bumble bees. *Proceedings of the National Academy of Sciences*, *108*(2), 662–667. https://doi.org/10.1073/pnas.1014743108

Casiraghi, M., Bain, O., Guerrero, R., Martin, C., Pocacqua, V., Gardner, S. L., … Bandi, C. (2004). Mapping the presence of Wolbachia pipientis on the phylogeny of filarial nematodes: Evidence for symbiont loss during evolution. *International Journal for Parasitology*, *34*(2), 191–203. https://doi.org/10.1016/j.ijpara.2003.10.004

Chare, E. R., & Holmes, E. C. (2006). A phylogenetic survey of recombination frequency in plant RNA viruses, 933–946. https://doi.org/10.1007/s00705-005-0675-x

Charruau, P., Fernandes, C., Orozco-Terwengel, P., Peters, J., Hunter, L., Ziaie, H., … Burger, P. A. (2011). Phylogeography, genetic structure and population divergence time of cheetahs in Africa and Asia: Evidence for long-term geographic isolates. *Molecular Ecology*, *20*(4), 706–724. https://doi.org/10.1111/j.1365-294X.2010.04986.x

Chauvin, A., Moreau, E., Bonnet, S., Plantard, O., & Malandrin, L. (2009). Babesia and its hosts: Adaptation to long-lasting interactions as a way to achieve efficient transmission. *Veterinary Research*, *40*(2). https://doi.org/10.1051/vetres/2009020

Chitanga, S., Gaff, H., & Mukaratirwa, S. (2014). Tick-borne pathogens of potential zoonotic importance in the southern African Region. *Journal of the South African Veterinary Association*, *85*(1), 1–3. https://doi.org/10.4102/jsava.v85i1.1084

Cleaveland, S., Laurenson, M. K., & Taylor, L. H. (2001). Diseases of humans and their domestic mammals: Pathogen characteristics, host range and the risk of emergence. *Philosophical*

Transactions of the Royal Society B: Biological Sciences, *356*(1411), 991–999. https://doi.org/10.1098/rstb.2001.0889

Coleman, A. W. (2003). ITS2 is a double-edged tool for eukaryote evolutionary comparisons. *Trends in Genetics*, *19*(7), 370–375. https://doi.org/10.1016/S0168-9525(03)00118-5

Coleman, A. W. (2009). Is there a molecular key to the level of “biological species” in eukaryotes? A DNA guide. *Molecular Phylogenetics and Evolution*, *50*(1), 197–203. https://doi.org/10.1016/j.ympev.2008.10.008

Collett, M. G. (2000). Survey of canine babesiosis in South Africa. *J.S.Afr.Vet.Assoc.*, *71*(1019-9128 (Print)), 180–186. https://doi.org/10.4102/jsava.v71i3.710

Colwell, D. D., Dantas-Torres, F., & Otranto, D. (2011). Vector-borne parasitic zoonoses: Emerging scenarios and new perspectives. *Veterinary Parasitology*, *182*(1), 14–21. https://doi.org/10.1016/j.vetpar.2011.07.012

Conceição-Silva, F. M., Abranches, P., Silva-Pereira, M. C. D., & Janz, J. G. (1988). Hepatozoonosis in Foxes From Portugal. *Journal of Wildlife Diseases*, *24*(2), 344–347. https://doi.org/10.7589/0090-3558-24.2.344

Cooper, A. B., Pettorelli, N., & Durant, S. M. (2007). Large carnivore menus: factors affecting hunting decisions by cheetahs in the Serengeti. *Animal Behaviour*, *73*(4), 651–659. https://doi.org/10.1016/j.anbehav.2006.06.013

Cooper, S. M., Holekamp, K. E., & Smale, L. (1999). A seasonal feast: long-term analysis of feeding behaviour in the spotted hyaena (*Crocuta crocuta*). *African Journal of Ecology*, *37*(2), 149–160. https://doi.org/10.1046/j.1365-2028.1999.00161.x

Crozier, G. K. D., & Schulte-Hostedde, A. I. (2014). The ethical dimensions of wildlife disease management in an evolutionary context. *Evolutionary Applications*, *7*(7), 788–798. https://doi.org/10.1111/eva.12171

Cumming, G. S., & Van Vuuren, D. P. (2006). Will climate change affect ectoparasite species ranges? *Global Ecology and Biogeography*, *15*(5), 486–497. https://doi.org/10.1111/j.1466-822X.2006.00241.x

Czupryna, A. M., Brown, J. S., Bigambo, M. A., Whelan, C. J., Mehta, S. D., Santymire, R. M., … Faust, L. J. (2016). Ecology and demography of free-Roaming domestic dogs in rural villages near serengeti national park in Tanzania. *PLoS ONE*, *11*(11), 1–24. https://doi.org/10.1371/journal.pone.0167092

Dalerum, F., Roux, A., Vries, L. De, & Kamler, J. (2016). Otocyon megalotis – Bat-eared Fox Assessment Rationale, (October 2017).

Dantas-Torres, F. (2007). Rocky Mountain spotted fever. *The Lancet Infectious Diseases*, *7*(11), 724–732. https://doi.org/10.1016/S1473-3099(07)70261-X

Dantas-Torres, F., Chomel, B. B., & Otranto, D. (2012). Ticks and tick-borne diseases: A One Health perspective. *Trends in Parasitology*, *28*(10), 437–446. https://doi.org/10.1016/j.pt.2012.07.003

Dantas-Torres, F., & Otranto, D. (2013). Dirofilariosis in the Americas: A more virulent Dirofilaria immitis? *Parasites and Vectors*, *6*(1), 1–9. https://doi.org/10.1186/1756-3305-6-288

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2001). Anthropogenic environmental change and

the emergence of infectious diseases in wildlife. *Acta Tropica*, *78*(2), 103–116. https://doi.org/10.1016/S0001-706X(00)00179-0

Daszak, Peter. (2013). Emerging Infectious Diseases of Wildlife — Threats to Biodiversity and Human Health, *443*(2000). https://doi.org/10.1126/science.287.5452.443

Day, M. J. (2011). One health: the importance of companion animal vector-borne diseases. *Parasites & Vectors*, *4*(1), 49. https://doi.org/10.1186/1756-3305-4-49

Delsuc, F., Brinkmann, H., & Philippe, H. (2005). Phylogenomics and the Reconstruction of the Tree of Life, *6*(May). https://doi.org/10.1038/nrg1603

Duh, D., Punda-Polic, V., Avsic-Zupanc, T., Bouyer, D., Walker, D. H., Popov, V. L., … Strus, J. (2010). Rickettsia hoogstraalii sp. nov., isolated from hardand soft-bodied ticks. *International Journal of Systematic and Evolutionary Microbiology*, *60*(4), 977–984. https://doi.org/10.1099/ijs.0.011049-0

Durant, S. M., Wacher, T., Bashir, S., Woodroffe, R., De Ornellas, P., Ransom, C., … Pettorelli, N. (2014). Fiddling in biodiversity hotspots while deserts burn? Collapse of the Sahara's megafauna. *Diversity and Distributions*, *20*(1), 114–122. https://doi.org/10.1111/ddi.12157

Durant, S., Mitchell, N., Ipavec, A., & Groom, R. (2015). Acinonyx jubatus. The IUCN Red List of Threatened Species 2015: e.T219A50649567. *Mammal Species*. https://doi.org/10.1644/1545-1410(2005)771[0001:AJ]2.0.CO;2

Durant, Sarah M. (1998). Comparative refuges and coexsistance: an example from Serengeti carnivores. *Journal of Animal Ecology*, *67*(3), 370–386. https://doi.org/10.1046/j.1365-2656.1998.00202.x

Durant, Sarah M. (2000). Predator avoidance, breeding experience and reproductive success in endangered cheetahs, Acinonyx jubatus. *Animal Behaviour*, *60*(1), 121–130. https://doi.org/10.1006/anbe.2000.1433

Durant, Sarah M., Bashir, S., Maddox, T., & Laurenson, M. K. (2007). Relating long-term studies to conservation practice: The case of the serengeti cheetah project. *Conservation Biology*, *21*(3), 602–611. https://doi.org/10.1111/j.1523-1739.2007.00702.x

Durant, Sarah M., Craft, M. E., Foley, C., Hampson, K., Lobora, A. L., Msuha, M., … Pettorelli, N. (2010). Does size matter? An investigation of habitat use across a carnivore assemblage in the Serengeti, Tanzania. *Journal of Animal Ecology*, *79*(5), 1012–1022. https://doi.org/10.1111/j.1365-2656.2010.01717.x

East, M. L., Wibbelt, G., Lieckfeldt, D., Ludwig, A., Goller, K., Wilhelm, K., … Hofer, H. (2008). A Hepatozoon Species Genetically Distinct From H. Canis Infecting Spotted Hyenas in the Serengeti Ecosystem, Tanzania. *Journal of Wildlife Diseases*, *44*(1), 45–52. https://doi.org/10.7589/0090-3558-44.1.45

Eloff, F. C. (1964). On the predatory habits of lions and hyaenas. *Koedoe*. https://doi.org/10.4102/koedoe.v7i1.805

Espinaze, M. P. A., Hellard, E., Horak, I. G., & Cumming, G. S. (2018). Domestic mammals facilitate tick-borne pathogen transmission networks in South African wildlife. *Biological Conservation*, *221*(December 2017), 228–236. https://doi.org/10.1016/j.biocon.2018.03.017

Estrada-Peña, A., Ayllón, N., & de la Fuente, J. (2012). Impact of climate trends on tick-borne pathogen transmission. *Frontiers in Physiology*, *3 MAR*(March), 1–12.

https://doi.org/10.3389/fphys.2012.00064

Farhadinia, B. M. S. (2004). The Last Stronghold : Cheetah in Iran. *Spring*, *40*(June 2003), 11–14. Retrieved from http://wildlife.ir/Files/library/IranianCheetah_Farhadinia2004.pdf

Farhadinia, M. S., Hunter, L. T. B., Jourabchian, A., Hosseini-Zavarei, F., Akbari, H., Ziaie, H., … Jowkar, H. (2017). The critically endangered Asiatic cheetah Acinonyx jubatus venaticus in Iran: a review of recent distribution, and conservation status. *Biodiversity and Conservation*, *26*(5), 1027–1046. https://doi.org/10.1007/s10531-017-1298-8

Flies, A. S., Mansfield, L. S., Grant, C. K., Weldele, M. L., & Holekamp, K. E. (2015). Markedly elevated antibody responses in wild versus captive spotted hyenas show that environmental and ecological factors are important modulators of immunity. *PLoS ONE*, *10*(10), 1–18. https://doi.org/10.1371/journal.pone.0137679

Furstenburg, D. (2012). Focus on the Brown Hyaena Parahyaena brunnea, (April).

Gasaway, W. C., Mossestad, K. T., & Stander, P. E. (1989). Demography of spotted hyaenas in an arid savanna, Etosha National Park, south west Africa/Namibia. *Madoqua*, *16*(2), 121–127.

Giadinis, N. D., Chochlakis, D., Kritsepi-Konstantinou, M., Makridaki, E., Tselentis, Y., Kostopoulou, D., … Psaroulaki, A. (2012). Haemolytic disease in sheep attributed to a Babesia lengau-like organism. *Veterinary Record*, *170*(6), 2011–2013. https://doi.org/10.1136/vr.100394

Githaka, N., Konnai, S., Kariuki, E., Kanduma, E., Murata, S., & Ohashi, K. (2012). Molecular detection and characterization of potentially new Babesia and Theileria species/variants in wild felids from Kenya. *Acta Tropica*, *124*(1), 71–78. https://doi.org/10.1016/j.actatropica.2012.06.013

Gottelli, D., Wang, J., Bashir, S., & Durant, S. M. (2007). Genetic analysis reveals promiscuity among female cheetahs. *Proceedings of the Royal Society B: Biological Sciences*, *274*(1621), 1993–2001. https://doi.org/10.1098/rspb.2007.0502

Gray, R. D., & Atkinson, Q. D. (2003). Language-tree divergence times support the Anatolian theory of Indo-European origin, *426*(November), 435–439. https://doi.org/10.1029/2001GC000192

Gros, P. M. (2002). The status and conservation of the cheetah Acinonyx jubatus in Tanzania. *Biological Conservation*, *106*, 177–185.

Gubbels, J. M., De Vos, A. P., Van Der Weide, M., Viseras, J., Schouls, L. M., De Vries, E., & Jongejan, F. (1999). Simultaneous detection of bovine Theileria and Babesia species by reverse line blot hybridization. *Journal of Clinical Microbiology*, *37*(6), 1782–1789.

Hajibabaei, M., Singer, G. A. C., Hebert, P. D. N., & Hickey, D. A. (2007). DNA barcoding: how it complements taxonomy, molecular phylogenetics and population genetics. *Trends in Genetics*, *23*(4), 167–172. https://doi.org/10.1016/j.tig.2007.02.001

Hawlena, H., Bashary, D., Abramsky, Z., Khokhlova, I. S., & Krasnov, B. R. (2008). Programmed versus stimulus-driven antiparasitic grooming in a desert rodent. *Behavioral Ecology*, *19*(5), 929–935. https://doi.org/10.1093/beheco/arn046

Hayward, M. W., Hofmeyr, M., O'Brien, J., & Kerley, G. I. H. (2006). Prey preferences of the cheetah (Acinonyx jubatus) (Felidae: Carnivora): morphological limitations or the need to capture rapidly consumable prey before kleptoparasites arrive? *Journal of Zoology*, *270*(4),

615–627. https://doi.org/10.1111/j.1469-7998.2006.00184.x

Hayward, Matt W., & Hayward, G. J. (2007). Activity patterns of reintroduced lion *Panthera leo* and spotted hyaena *Crocuta crocuta* in the Addo Elephant National Park, South Africa. *African Journal of Ecology*, *45*(2), 135–141. https://doi.org/10.1111/j.1365-2028.2006.00686.x

Hoch, H., & Strickland, K. (2014). Canine and feline dirofilariasis, *109*, 70–78.

Hoffmann, M. (2014). Otocyon megalotis. The IUCN Red List of Threatened Species 2014: e.T15642A46123809. Retrieved from http://dx.doi.org/10.2305/IUCN.UK.2014-1.RLTS.T15642A46123809.en

Holder, M., & Lewis, P. O. (2003). Phylogeny Estimation: Traditional and Bayesian Approaches, *4*(April). https://doi.org/10.1038/nrg1044

Holekamp, K. E., Smale, L., Berg, R., & Cooper, S. M. (1997). Hunting rates and hunting success in the spotted hyena (Crocuta crocuta). *Journal of Zoology*, *242*(1), 1–15. https://doi.org/10.1111/j.1469-7998.1997.tb02925.x

Holekamp, Kay E., & Dloniak, S. M. (2010). Intraspecific variation in the behavioral ecology of a tropical carnivore, the spotted hyena. *Advances in the Study of Behavior*, *42*(C), 189–229. https://doi.org/10.1016/S0065-3454(10)42006-9

Holmes, J. C. (1996). Parasites as threats to biodiversity in shrinking ecosystems. *Biodiversity and Conservation*, *5*(8), 975–983. https://doi.org/10.1007/BF00054415

Höner, O. P., Wachter, B., East, M. L., & Hofer, H. (2002). The Response of Spotted Hyaenas to Long-Term Changes in Prey Populations : Functional Response and Interspecific Kleptoparasitism Author (s): Oliver P . Honer , Bettina Wachter , Marion L . East , Heribert Hofer Published by : British Ecological Society. *Journal of Animal Ecology*, *71*(2), 236–246. Retrieved from http://www.jstor.org/stable/2693441

Höner, O. P., Wachter, B., Hofer, H., Wilhelm, K., Thierer, D., Trillmich, F., … East, M. L. (2010). The fitness of dispersing spotted hyaena sons is influenced by maternal social status. *Nature Communications*, *1*(5), 1–7. https://doi.org/10.1038/ncomms1059

Hudson, P., Dobson, A., & Newborn, D. (1998). Prevention of population cycles by parasites removal. *Science*, *282*(December), 2256–2258.

Hudson, P. J., Dobson, A. P., & Lafferty, K. D. (2006). Is a healthy ecosystem one that is rich in parasites? *Trends in Ecology and Evolution*, *21*(7), 381–385. https://doi.org/10.1016/j.tree.2006.04.007

Hulsman, A., Dalerum, F., Swanepoel, L., Ganswindt, A., Sutherland, C., & Paris, M. (2010). Patterns of scat deposition by brown hyaenas Hyaena brunnea in a mountain savannah region of South Africa. *Wildlife Biology*, *16*(4), 445–451. https://doi.org/10.2981/09-110

Hunter, J. S., Durant, S. M., & Caro, T. M. (2007a). Patterns of scavenger arrival at cheetah kills in Serengeti National Park Tanzania. *African Journal of Ecology*, *45*(3), 275–281. https://doi.org/10.1111/j.1365-2028.2006.00702.x

Hunter, J. S., Durant, S. M., & Caro, T. M. (2007b). To flee or not to flee: Predator avoidance by cheetahs at kills. *Behavioral Ecology and Sociobiology*, *61*(7), 1033–1042. https://doi.org/10.1007/s00265-006-0336-4

Hunter, L., Jowkar, H., Ziaie, H., Schaller, G., Balme, G., Walzer, C., … Christie, S. (2007).

Conserving the Asiatic Cheetah in Iran: Launching the First Radio-Telemetry Study. *Cat News*, *46*(Spring 2007), 8–11. https://doi.org/10.1017/CBO9781107415324.004

Inokuma, H., Okuda, M., Ohno, K., Shimoda, K., & Onishi, T. (2002). Analysis of the 18S rRNA gene sequence of a Hepatozoon detected in two Japanese dogs. *Veterinary Parasitology*, *106*(3), 265–271. https://doi.org/10.1016/S0304-4017(02)00065-1

Inokuma, H., Oyamada, M., Kelly, P. J., Jacobson, L. A., Fournier, P. E., Itamoto, K., … Brouqui, P. (2005). Molecular detection of a new Anaplasma species closely related to Anaplasma phagocytophilum in canine blood from South Africa. *Journal of Clinical Microbiology*, *43*(6), 2934–2937. https://doi.org/10.1128/JCM.43.6.2934-2937.2005

Jenkins, E. J., Simon, A., Bachand, N., & Stephen, C. (2015). Wildlife parasites in a One Health world. *Trends in Parasitology*, *31*(5), 174–180. https://doi.org/10.1016/j.pt.2015.01.002

Jia, N., Zheng, Y. C., Ma, L., Huo, Q. B., Ni, X. B., Jiang, B. G., … Cao, W. C. (2014). Human infections with Rickettsia raoultii, China. *Emerging Infectious Diseases*, *20*(5), 866–868. https://doi.org/10.3201/eid2005.130995

Jones, K. E., Pattel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *44*(2), 319–335. https://doi.org/10.1038/nature06536.

Jongejan, F., & Uilenberg, G. (2004). The global importance of ticks. *Parisitology*, *129*, 3–14. https://doi.org/10.1017/S0031182004005967

Junker, K., Horak, I. G., & Penzhorn, B. (2015). History and development of research on wildlife parasites in southern Africa, with emphasis on terrestrial mammals, especially ungulates. *International Journal for Parasitology: Parasites and Wildlife*, *4*(1), 50–70. https://doi.org/10.1016/j.ijppaw.2014.12.003

Kamler, J. F., Gray, M. M., Oh, A., & Macdonald, D. W. (2013). Genetic structure, spatial organization, and dispersal in two populations of bat-eared foxes. *Ecology and Evolution*, *3*(9), 2892–2902. https://doi.org/10.1002/ece3.683

Keller, A., Förster, F., Müller, T., Dandekar, T., Schultz, J., & Wolf, M. (2010). Including RNA secondary structures improves accuracy and robustness in reconstruction of phylogenetic trees. *Biology Direct*, *5*, 1–12. https://doi.org/10.1186/1745-6150-5-4

Kelly, P., Marabini, L., Dutlow, K., Zhang, J., Loftis, A., & Wang, C. (2014). Molecular detection of tick-borne pathogens in captive wild felids, Zimbabwe. *Parasites and Vectors*, *7*(1), 1–6. https://doi.org/10.1186/s13071-014-0514-6

Kent, V. T., & Hill, R. A. (2013). The importance of farmland for the conservation of the brown hyaena Parahyaena brunnea. *Oryx*, *47*(3), 431–440. https://doi.org/10.1017/S0030605312001007

Kocan, a a, Cummings, C. a, Panciera, R. J., Mathew, J. S., Ewing, S. a, & Barker, R. W. (2000). Naturally occurring and experimentally transmitted Hepatozoon americanum in coyotes from Oklahoma. *Journal of Wildlife Diseases*, *36*(1), 149–153. https://doi.org/10.7589/0090-3558-36.1.149

Kofer, J., Hofer, H., & Hartmann, S. (2017). Next-Generation Parasitologists: Structured Training Programs Meet Educational Challenges. *Trends in Parasitology*, *33*(6), 423–425. https://doi.org/10.1016/j.pt.2017.03.008

Kolo, A. O., Sibeko-Matjila, K. P., Maina, A. N., Richards, A. L., Knobel, D. L., & Matjila, P. T. (2016). Molecular detection of zoonotic rickettsiae and Anaplasma spp. in domestic dogs and their ectoparasites in Bushbuckridge, South Africa. *Vector Borne and Zoonotic Diseases*, *16*(4), 1–19.

Kolowski, J. M., & Holekamp, K. E. (2009). Ecological and anthropogenic influences on space use by spotted hyaenas. *Journal of Zoology*, *277*(1), 23–36. https://doi.org/10.1111/j.1469-7998.2008.00505.x

Kolowski, Joseph M., Katan, D., Theis, K. R., & Holekamp, K. E. (2007). Daily Patterns of Activity in the Spotted Hyena. *Journal of Mammalogy*, *88*(4), 1017–1028. https://doi.org/10.1644/06-MAMM-A-143R.1

Krücken, J., Schreiber, C., Maaz, D., Kohn, M., Demeler, J., Beck, S., … Von Samson-Himmelstjerna, G. (2013). A novel high-resolution melt PCR assay discriminates Anaplasma phagocytophilum and "Candidatus Neoehrlichia mikurensis." *Journal of Clinical Microbiology*, *51*(6), 1958–1961. https://doi.org/10.1128/JCM.00284-13

Kruse, H., Kirkemo, A. M., & Handeland, K. (2004). Wildlife as source of zoonotic infections. *Emerging Infectious Diseases*, *10*(12), 2067–2072. https://doi.org/10.3201/eid1012.040707

Kruuk, H. (1972). The spotted hyena; a study of predation and social behavior. *Wildlife Behavior and Ecology*. https://doi.org/10.2307/1379154

Kuhn, B. F., Skinner, J. D., & Wiesel, I. (2008). Diet of brown hyaenas (Parahyaena brunnea) on the Namibian coast. *Transactions of the Royal Society of South Africa*, *63*(2), 150–159. https://doi.org/10.1080/00359190809519219

Kuntzsch, V., & Nel, J. A. J. (1992). Diet of bat-eared foxes Otrocyon megalotis in the Karoo. *Koedoe*.

Lightner, L. K., & Reardon, M. J. (1983). Dipetalonema dracunculoides in dogs and spotted hyena (Crocuta crocuta) in the Turkana district of Kenya. *Proceedings of the Helminthological Society of Washington*, *50*(2), 333–335.

Lindsey, P. A., Havemann, C. P., Lines, R., Palazy, L., Price, A. E., Retief, T. A., … van der Waal, C. (2013). Determinants of Persistence and Tolerance of Carnivores on Namibian Ranches: Implications for Conservation on Southern African Private Lands. *PLoS ONE*, *8*(1). https://doi.org/10.1371/journal.pone.0052458

Lopez-Rebollar, L. M., Penzhorn, B. L., de Waal, D. T., & Lewis, B. D. (1999). A possible new piroplasm in lions from the Republic of South Africa. *Journal of Wildlife Diseases*, *35*(1), 82–85. https://doi.org/10.7589/0090-3558-35.1.82

Maas, B. (1993). Bat-eared fox behavioural ecology and the incidence of rabies in the Serengeti National Park. *The Onderstepoort Journal of Veterinary Research*, *60*(4), 389–393. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/7777325

MacPhee, R. D. E., & Greenwood, A. D. (2013). Infectious Disease, Endangerment, and Extinction. *International Journal of Evolutionary Biology*, *2013*, 1–9. https://doi.org/10.1155/2013/571939

Mallon, D. (2007). Cheetahs in Central Asia: A historical summary. *Cat News*, (1959), 4–7.

Manati, A. R., & Nogge, G. (2008). Cheetahs in Afghanistan. *Cat News*, *49*(Schreber 1776), 18. Retrieved from Manati_&_Nogge_2008_Cheetahs_in_Afghanistan.pdf

Marker, L. (2005). Overview of the global wild cheetah population. *Animal Keeper's Forum*, *32*(7/8), 284–288. Retrieved from Marker_2005_The_wild_cheetah_population.pdf

Marker, L. L., Dickman, A. J., Mills, M. G. L., Jeo, R. M., & Macdonald, D. W. (2008). Spatial ecology of cheetahs on north-central Namibian farmlands. *Journal of Zoology*, *274*(3), 226–238. https://doi.org/10.1111/j.1469-7998.2007.00375.x

Marker, Laurie L, Muntifering, J. R., Dickman, A. J., Mills, M. G. L., & Macdonald, D. W. (2003). Quantifying prey preferences of free-ranging Namibian cheetahs. *South African Journal of Wildlife Research*, *33*(April), 43–53. Retrieved from http://reference.sabinet.co.za/webx/access/journal_archive/03794369/3393.pdf

Matei, I. A., D'Amico, G., Yao, P. K., Ionica, A. M., Kanyari, P. W. N., Daskalaki, A. A., … Mihalca, A. D. (2016). Molecular detection of Anaplasma platys infection in free-roaming dogs and ticks from Kenya and Ivory Coast. *Parasites and Vectors*, *9*(1), 1–8. https://doi.org/10.1186/s13071-016-1443-3

Matijatko, V., Torti, M., & Schetters, T. P. (2012). Canine babesiosis in Europe: How many diseases? *Trends in Parasitology*, *28*(3), 99–105. https://doi.org/10.1016/j.pt.2011.11.003

Matjila, P. T., Leisewitz, A. L., Oosthuizen, M. C., Jongejan, F., & Penzhorn, B. L. (2008). Detection of Theileria sp . infections in dogs in South Africa . *Veterinary Parasitology*, *157*(1–2), 34–40.

Matjila, P. T., Penzhorn, B. L., Bekker, C. P. J., Nijhof, A. M., & Jongejan, F. (2004). Confirmation of occurrence of Babesia canis vogeli in domestic dogs in South Africa. *Veterinary Parasitology*, *122*(2), 119–125. https://doi.org/10.1016/j.vetpar.2004.03.019

Matjila, Paul Tshepo, Leisewitz, A. L., Jongejan, F., Bertschinger, H. J., & Penzhorn, B. L. (2008). Molecular detection of Babesia rossi and Hepatozoon sp. in African wild dogs (Lycaon pictus) in South Africa. *Veterinary Parasitology*, *157*(1–2), 123–127. https://doi.org/10.1016/j.vetpar.2008.07.016

Matthee, S., Lovely, C., Gaugler, A., Beeker, R., Venter, H. R., & Horak, I. G. (2010). Ixodid ticks on domestic dogs in the Northern Cape Province of South Africa and in Namibia. *Journal of the South African Veterinary Association*, *81*(2), 126–128. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/21247023

Maude, G., & Mills, M. G. L. (2005). The Comparative Ecology of the Brown Hyaena (Hyaena in Lagkadigadi National Park and a Neighbouring Community Cattle Area in Botswana. *South African Journal of Wildlife Research*, *35*(June), 201–214.

May R. M. (1995). The cheetah controversy. *Nature*, 309–310.

McCallum, H. (2016). Models for managing wildlife disease. *Parasitology*, *143*(7), 805–820. https://doi.org/10.1017/S0031182015000980

McCallum, H., & Dobson, A. (1995). Detecting disease and parasite threats to endangered species and ecosystems. *Trends in Ecology & Evolution*, *10*(5), 190–194. https://doi.org/10.1016/S0169-5347(00)89050-3

McCully, R. M., Basson, P. A., Bigalke, R. D., De Vos, V., & Young, E. (1975). Observations on naturally acquired hepatozoonosis of wild carnivores and dogs in the Republic of South Africa. *The Onderstepoort Journal of Veterinary Research*, *42*(4), 117–133. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/1221330

Mediannikov, O., Matsumoto, K., Samoylenko, I., Drancourt, M., Roux, V., Rydkina, E., … Fournier, P. E. (2008). Rickettsia raoultii sp. nov., a spotted fever group rickettsia associated with Dermacentor ticks in Europe and Russia. *International Journal of Systematic and Evolutionary Microbiology*, *58*(7), 1635–1639. https://doi.org/10.1099/ijs.0.64952-0

Medlar, A., Aivelo, T., & Löytynoja, A. (2014). Séance: Reference-based phylogenetic analysis for 18S rRNA studies. *BMC Evolutionary Biology*, *14*(1), 1–9. https://doi.org/10.1186/s12862-014-0235-7

Menotti-Raymond, M., & O'Brien, S. J. (1993). Dating the genetic bottleneck of the African cheetah. *Proceedings of the National Academy of Sciences*, *90*(8), 3172–3176. https://doi.org/10.1073/pnas.90.8.3172

Mills, G., & Hofer, H. (1998). Hyaenas. Status survey and conservation action plan, 164.

Mills, M. G. L. (1984). Prey selection and feeding habits of the large carnivores in the southern Kalahari. *Koedoe*. https://doi.org/10.4102/koedoe.v27i2.586

Mills, M. G. L., Broomhall, L. S., & Du Toit, J. T. (2004). Cheetah Acinonyx jubatus feeding ecology in the Kruger National Park and a comparison across African savanna habitats: is the cheetah only a successful hunter on open grassland plains? *Wildlife Biology*, *10*, 177–186. https://doi.org/10.1016/j.tibtech.2011.06.009

Moller J. T., A. P. & N. (2007). Malaria and Risk of Predation: A Comparative Study of Birds. *Ecology*, *88*(4), 871–881. https://doi.org/10.1890/06-0747

Munson, L., Terio, K. A., Kock, R., Mlengeya, T., Roelke, M. E., Dubovi, E., … Packer, C. (2008). Climate extremes promote fatal co-infections during canine distemper epidemics in African lions. *PLoS ONE*, *3*(6), 5–10. https://doi.org/10.1371/journal.pone.0002545

Nei, M., & Rooney, A. P. (2005). Concerted and Birth-and-Death Evolution of Multigene Families. *Annual Review of Genetics*, *39*(1), 121–152. https://doi.org/10.1146/annurev.genet.39.073003.112240

Nel, J. A. J. (1993). The Bat-Eared Fox - a Prime Candidate for Rabies Vector. *Onderstepoort Journal of Veterinary Research*, *60*(4), 395–397.

Nel, J. A. J., & Mackie, A. J. (1990). Food and foraging behaviour of bat-eared foxes in the south-eastern Orange Free State. *South African Journal of Wildlife Research*, *20*(4), 162–166. https://doi.org/10.1080/19397030902947041

Nel, J. A. J., Mills, M. G. L., & Vanaarde, R. J. (1984). Fluctuating group size in bat-eared foxes (Otocyon megalotis) in the Southwestern Kalahari. *Journal of Zoology*, *203*(JUN), 294–298.

Newey, S., & Thirgood, S. (2004). Parasite-mediated reduction in fecundity of mountain hares. *Proceedings of the Royal Society B: Biological Sciences*, *271*(SUPPL. 6), 413–415. https://doi.org/10.1098/rsbl.2004.0202

Nicholson, W. L., Allen, K. E., McQuiston, J. H., Breitschwerdt, E. B., & Little, S. E. (2010). The increasing recognition of rickettsial pathogens in dogs and people. *Trends in Parasitology*, *26*(4), 205–212. https://doi.org/10.1016/j.pt.2010.01.007

Nielsen, R., Bustamante, C., Clark, A. G., Glanowski, S., Sackton, T. B., Hubisz, M. J., … Cargill, M. (2005). A Scan for Positively Selected Genes in the Genomes of Humans and Chimpanzees, *3*(6). https://doi.org/10.1371/journal.pbio.0030170

Nijhof, A. M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W. H., Lawrence, A., … Jongejan, F. (2005). Molecular Characterization of Theileria Species Associated with Mortality in Four Species of African Antelopes Molecular Characterization of Theileria Species Associated with Mortality in Four Species of African Antelopes. *Journal of Clinical Microbiology*, *43*(12), 5907–5911. https://doi.org/10.1128/JCM.43.12.5907

Noden, B. H., & Soni, M. (2015). Vector-borne diseases of small companion animals in Namibia: Literature review, knowledge gaps and opportunity for a One Health approach. *Journal of the South African Veterinary Association*, *86*(1), 1–7. https://doi.org/10.4102/jsava.v86i1.1307

Noden, B. H., Tshavuka, F. I., Van Der Colf, B. E., Chipare, I., & Wilkinson, R. (2014). Exposure and risk factors to Coxiella burnetii, spotted fever group and typhus group rickettsiae, and Bartonella henselae among volunteer blood donors in Namibia. *PLoS ONE*, *9*(9). https://doi.org/10.1371/journal.pone.0108674

Nogueras, M. M., Pons, I., Ortuño, A., Miret, J., Pla, J., Castellà, J., & Segura, F. (2013). Molecular Detection of Rickettsia typhi in Cats and Fleas. *PLoS ONE*, *8*(8), 1–8. https://doi.org/10.1371/journal.pone.0071386

O'Brien, S. J., Wildt, D. E., Bush, M., Caro, T. M., FitzGibbon, C., Aggundey, I., & Leakey, R. E. (1987). East African cheetahs: evidence for two population bottlenecks? *Proceedings of the National Academy of Sciences*, *84*(2), 508–511. https://doi.org/10.1073/pnas.84.2.508

O'Brien, Stephen J., & Johnson, W. E. (2005). Big Cat Genomics. *Annual Review of Genomics and Human Genetics*, *6*(1), 407–429. https://doi.org/10.1146/annurev.genom.6.080604.162151

Otranto, D. (2018). Arthropod-borne pathogens of dogs and cats: From pathways and times of transmission to disease control. *Veterinary Parasitology*, *251*(November 2017), 68–77. https://doi.org/10.1016/j.vetpar.2017.12.021

Otranto, D., Dantas-Torres, F., & Breitschwerdt, E. B. (2009). Managing canine vector-borne diseases of zoonotic concern - read part two. *Trends in Parasitology*, *25*(5), 228–235. https://doi.org/10.1016/j.pt.2009.02.005

Owens, M. J., & Owens, D. D. (1978). Feeding ecology and its influence on social organization in Brown hyenas (Hyaena brunnea, Thunberg) of the Central Kalahari Desert. *African Journal of Ecology*, *16*(2), 113–135. https://doi.org/10.1111/j.1365-2028.1978.tb00433.x

Pacheco, R. C., Moraes-Filho, J., Marcili, A., Richtzenhain, L. J., Szabó, M. P. J., Catroxo, M. H. B., Labruna, M. B. (2011). Rickettsia monteiroi sp. nov., infecting the tick Amblyomma incisum in Brazil. *Applied and Environmental Microbiology*, *77*(15), 5207–5211. https://doi.org/10.1128/AEM.05166-11

Pedersen, A. B., Jones, K. E., Nunn, C. L., & Altizer, S. (2007). Infectious diseases and extinction risk in wild mammals. *Conservation Biology*, *21*(5), 1269–1279. https://doi.org/10.1111/j.1523-1739.2007.00776.x

Peirce, M. A., Laurenson, M. K., & Gascoyne, S. C. (1995). Hepatozoonosis in cheetahs and wild dogs in the Serengeti ecosystem. *African Journal of Ecology*. Retrieved from http://europepmc.org/abstract/med/7629782%5Cnhttp://www.ncbi.nlm.nih.gov/pubmed/7629782

Penzhorn, B., Kjemtrup, A. M., & Lopez-Rebollar, L. M. (2001). Babesia leo N. Sp. from Lions in the Kruger National Park, South Africa, and Its Relation to Other Small Piroplasms. *The Journal of Parasitology*, *87*(5), 1191–1194. https://doi.org/10.1645/0022-3395(2001)087

Penzhorn, Banie L. (2006). Babesiosis of wild carnivores and ungulates. *Veterinary Parasitology*, *138*(1–2), 11–21. https://doi.org/10.1016/j.vetpar.2006.01.036

Penzhorn, Barend L. (2011). Why is Southern African canine babesiosis so virulent? An evolutionary perspective. *Parasites and Vectors*, *4*(1), 2–7. https://doi.org/10.1186/1756-3305-4-51

Penzhorn, Barend L., Netherlands, E. C., Cook, C. A., Smit, N. J., Vorster, I., Harrison-White, R. F., & Oosthuizen, M. C. (2018). Occurrence of Hepatozoon canis (Adeleorina: Hepatozoidae) and Anaplasma spp. (Rickettsiales: Anaplasmataceae) in black-backed jackals (Canis mesomelas) in South Africa. *Parasites and Vectors*, *11*(1), 1–7. https://doi.org/10.1186/s13071-018-2714-y

Pfeil, B. E. P., Schlueter, J. A. S., Shoemaker, R. C. S., & Doyle, J. J. D. (2005). Placing Paleopolyploidy in Relation to Taxon Divergence : A Phylogenetic Analysis in Legumes Using 39 Gene Families, *54*(3), 441–454. https://doi.org/10.1080/10635150590945359

Philippe, H., & Douady, C. J. (2003). Horizontal gene transfer and phylogenetics. *Current Opinion in Microbiology*, *6*(5), 498–505. https://doi.org/10.1016/j.mib.2003.09.008

Piesman, J., & Eisen, L. (2002). Prevention of tick-borne diseases. *Medical Clinics of North America*, *86*(2), 219–238. https://doi.org/10.1016/S0025-7125(03)00084-1

Piron, M., Fisa, R., Casamitjana, N., López-Chejade, P., Puig, L., Vergés, M., … Sauleda, S. (2007). Development of a real-time PCR assay for Trypanosoma cruzi detection in blood samples. *Acta Tropica*, *103*(3), 195–200. https://doi.org/10.1016/j.actatropica.2007.05.019

Polley, L. (2005). Navigating parasite webs and parasite flow: Emerging and re-emerging parasitic zoonoses of wildlife origin. *International Journal for Parasitology*, *35*(11–12), 1279–1294. https://doi.org/10.1016/j.ijpara.2005.07.003

Polley, L., & Thompson, A. (2015). Parasites and wildlife in a changing world. *Trends in Parasitology*, *31*(4), 123–124. https://doi.org/10.1016/j.pt.2015.03.001

Pompei, S., Loreto, V., & Tria, F. (2012). Phylogenetic Properties of RNA Viruses. *PLoS ONE*, *7*(9), 1–10. https://doi.org/10.1371/journal.pone.0044849

Purchase, G. K., & du Toit, J. T. (2000). The use of space and prey by cheetahs in Matusadonha National Park, Zimbabwe. *South African Journal of Wildlife Research*.

Ramírez, S. R., Gravendeel, B., Singer, R. B., Marshall, C. R., & Pierce, N. E. (2007). Dating the origin of the Orchidaceae from a fossil orchid with its pollinator, *448*(August), 1042–1045. https://doi.org/10.1038/nature06039

Ray, J. C., Hunter, L., & Zigouris, J. (2005). Setting Conservation and Research Priorities for Larger African Carnivores, (24).

Rhyan, J. C., & Spraker, T. R. (2010). Emergence of diseases from wildlife reservoirs. *Veterinary Pathology*, *47*(1), 34–39. https://doi.org/10.1177/0300985809354466

Rishniw, M., Barr, S. C., Simpson, K. W., Frongillo, M. F., Franz, M., & Dominguez Alpizar, J. L. (2006). Discrimination between six species of canine microfilariae by a single polymerase chain reaction. *Veterinary Parasitology*, *135*(3–4), 303–314. https://doi.org/10.1016/j.vetpar.2005.10.013

Robertson, L. J., Utaaker, K. S., Goyal, K., & Sehgal, R. (2014). Keeping parasitology under the

one health umbrella. *Trends in Parasitology*, *30*(8), 369–373. https://doi.org/10.1016/j.pt.2014.06.002

Robinson, R. A., Lawson, B., Toms, M. P., Peck, K. M., Kirkwood, J. K., Chantrey, J., … Cunningham, A. A. (2010). Emerging infectious disease leads to rapid population declines of common british birds. *PLoS ONE*, *5*(8). https://doi.org/10.1371/journal.pone.0012215

Rohland, N., Pollack, J. L., Nagel, D., Beauval, C., Airvaux, J., Pääbo, S., & Hofreiter, M. (2005). The population history of extant and extinct hyenas. *Molecular Biology and Evolution*, *22*(12), 2435–2443. https://doi.org/10.1093/molbev/msi244

Rostro-García, S., Kamler, J. F., & Hunter, L. T. B. (2015). To kill, stay or flee: The effects of lions and landscape factors on habitat and kill site selection of cheetahs in South Africa. *PLoS ONE*, *10*(2), 1–20. https://doi.org/10.1371/journal.pone.0117743

Roux, V., Rydkina, E., Eremeeva, M., & RaoultT, D. (1997). Citrate Synthase Gene Comparison, a New Tool for Phylogenetic Analysis, and Its Application for the Rickettsiae. *International Journal of Systematic Bacteriology*, *47*(2), 252–261. https://doi.org/10.1099/00207713-47-2-252

Sanogo, Y. O., Davoust, B., Inokuma, H., Camicas, J.-L., Parola, P., & Brouqui, P. (2003). First evidence of Anaplasma platys in Rhipicephalus sanguineus (Acari: Ixodida) collected from dogs in Africa. *The Onderstepoort Journal of Veterinary Research*, *70*(3), 205–212. https://doi.org/http://dx.doi.org/10.1006/cryo.1998.2118

Sarih, M., Socolovschi, C., Boudebouch, N., Hassar, M., Raoult, D., & Parola, P. (2008). Spotted Fever Group Rickettsiae in Ticks , Morocco, *14*(7), 1067–1073. https://doi.org/10.3201/eid1407.070096

Schneider, H. P. (2012). The history of veterinary medicine in Namibia. *Journal of the South African Veterinary Association*, *83*(1), 1–11. https://doi.org/10.4102/jsava.v83i1.4

Schnittger, L., Rodriguez, A. E., Florin-Christensen, M., & Morrison, D. A. (2012). Babesia: A world emerging. *Infection, Genetics and Evolution*, *12*(8), 1788–1809. https://doi.org/10.1016/j.meegid.2012.07.004

Schreiber, C., Krücken, J., Beck, S., Maaz, D., Pachnicke, S., Krieger, K., … Von Samson-Himmelstjerna, G. (2014). Pathogens in ticks collected from dogs in Berlin/Brandenburg, Germany. *Parasites and Vectors*, *7*(1), 1–10. https://doi.org/10.1186/s13071-014-0535-1

Schwan, E. V. (2009). Filariosis of domestic carnivores in Gauteng, KwaZulu-Natal and Mpumalanga provinces, South Africa, and Maputo province, Mozambique, (July).

Schwan, E. V, & Durand, D. T. (2002). Canine filariosis caused by Dirofilaria immitis in Mozambique: a small survey based on the identification of microfilariae. *Journal of the South African Veterinary Association*, *73*(3), 124–126. https://doi.org/10.4102/jsava.v73i3.573

Schwan, E. V, & Schroter, F. G. (2006). First record of Acanthocheilonema dracunculoides from domestic dogs in Namibia. *Journal of the South African Veterinary Association*, *77*(4), 220–221.

Seguel, M., & Gottdenker, N. (2017). The diversity and impact of hookworm infections in wildlife. *International Journal for Parasitology: Parasites and Wildlife*, *6*(3), 177–194. https://doi.org/10.1016/j.ijppaw.2017.03.007

Siegfried, W. (1984). An analysis of faecal pellets of the brown hyaena on the Namib coast. *South*

African Journal of Zoology, *19*(1), 1984. https://doi.org/10.1080/02541858.1984.11447858

Singh, B. (1997). Molecular methods for diagnosis and epidemiological studies of parasitic infections. *International Journal for Parasitology*, *27*(10), 1135–1145. https://doi.org/10.1016/S0020-7519(97)00111-2

Siwila, J., Mwase, E. T., Nejsum, P., & Simonsen, P. E. (2015). Filarial infections in domestic dogs in Lusaka, Zambia. *Veterinary Parasitology*, *210*(3–4), 250–254. https://doi.org/10.1016/j.vetpar.2015.04.009

Skinner, J. D., & van Aarde, R. J. (1981). The distribution and ecology of the brown hyaena Hyaena brunnea and spotted hyaena Crocuta crocuta in the central Namib desert. *Madoqua*, *12*(4), 231–239.

Skinner, J., & Smithers. (1990). Family Felidae - Cats. In: The mammals of the Southern African Subregion, 391–423. Retrieved from http://www.catsg.org/cheetah/05_library/5_3_publications/S/Skinner_&_Smithers_1990_Felidae.pdf

Slater, K., & Muller, K. (2014). The diet of brown hyaenas (Hyaena brunnea) in Shamwari Game Reserve, Eastern Cape, South Africa. *Koedoe*, *56*(1), 1–5. https://doi.org/10.4102/koedoe.v56i1.1143

Smith, J. E., Kolowski, J. M., Graham, K. E., Dawes, S. E., & Holekamp, K. E. (2008). Social and ecological determinants of fission-fusion dynamics in the spotted hyaena. *Animal Behaviour*, *76*(3), 619–636. https://doi.org/10.1016/j.anbehav.2008.05.001

Solano-Gallego, L., Sainz, Á., Roura, X., Estrada-Peña, A., & Miró, G. (2016). A review of canine babesiosis: The European perspective. *Parasites and Vectors*, *9*(1), 1–18. https://doi.org/10.1186/s13071-016-1596-0

Song, J., Shi, L., Li, D., Sun, Y., Niu, Y., Chen, Z., … Chen, S. (2012). Extensive Pyrosequencing Reveals Frequent Intra-Genomic Variations of Internal Transcribed Spacer Regions of Nuclear Ribosomal DNA. *PLoS ONE*, *7*(8). https://doi.org/10.1371/journal.pone.0043971

Stein, A. B., Fuller, T. K., & Marker, L. L. (2013). Brown Hyaena Feeding Ecology on Namibian Farmlands. *South African Journal of Wildlife Research*, *43*(1), 27–32. https://doi.org/10.3957/056.043.0115

Stuart, C. T., & Shaughnwssy, P. D. (1984). Content of Hyaena brunnea and Canis mesomelas scats from southern coastal Namibia. *Mammalia*, *48*(4), 611–612.

Tabar, M. D., Altet, L., Francino, O., Sánchez, A., Ferrer, L., & Roura, X. (2008). Vector-borne infections in cats: Molecular study in Barcelona area (Spain). *Veterinary Parasitology*, *151*(2–4), 332–336. https://doi.org/10.1016/j.vetpar.2007.10.019

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution*, *30*(12), 2725–2729. https://doi.org/10.1093/molbev/mst197

Taubenberger, J. K. (2006). Was the 1918 pandemic caused by a bird flu?, *440*(April), 2005–2007. https://doi.org/10.1038/nature04823

Taylor, L. H., Latham, S. M., & Woolhouse, M. E. J. (2001). Risk factors for human disease emergence. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *356*(1411), 983–989. https://doi.org/10.1098/rstb.2001.0888

Thompson, R. C.A., Lymbery, A. J., & Smith, A. (2010). Parasites, emerging disease and wildlife conservation. *International Journal for Parasitology*, *40*(10), 1163–1170. https://doi.org/10.1016/j.ijpara.2010.04.009

Thompson, R. C.Andrew. (2013). Parasite zoonoses and wildlife: One health, spillover and human activity. *International Journal for Parasitology*, *43*(12–13), 1079–1088. https://doi.org/10.1016/j.ijpara.2013.06.007

Thompson, R. C.Andrew, Kutz, S. J., & Smith, A. (2009). Parasite zoonoses and wildlife: Emerging issues. *International Journal of Environmental Research and Public Health*, *6*(2), 678–693. https://doi.org/10.3390/ijerph6020678

Thompson, R. C.Andrew, & Polley, L. (2014). Parasitology and One Health. *International Journal for Parasitology: Parasites and Wildlife*, *3*(3), A1–A2. https://doi.org/10.1016/j.ijppaw.2014.09.002

Thompson, R. C.Andrew, & Polley, L. (2015). Parasites of wildlife - Special issue. *International Journal for Parasitology: Parasites and Wildlife*, *4*(2), 197. https://doi.org/10.1016/j.ijppaw.2015.03.001

Thomson, G. R., & Meredith, C. D. (1993). Rabies in bat-eared foxes in South Africa, *403*, 399–403.

Thorn, M., Green, M., Bateman, P. W., Waite, S., & Scott, D. M. (2011). Brown hyaenas on roads: Estimating carnivore occupancy and abundance using spatially auto-correlated sign survey replicates. *Biological Conservation*, *144*(6), 1799–1807. https://doi.org/10.1016/j.biocon.2011.03.009

Thorn, M., Green, M., Keith, M., Marnewick, K., Bateman, P. W., Cameron, E. Z., & Scott, D. M. (2011). Large-scale distribution patterns of carnivores in Northern South Africa: Implications for conservation and monitoring. *Oryx*, *45*(4), 579–586. https://doi.org/10.1017/S0030605311000123

Tilson, R. L., & Henschel, J. R. (1986). Spatial arrangement of spotted hyaena groups in a desert environment, Namibia. *African Journal of Ecology*, *24*(3), 173–180. https://doi.org/10.1111/j.1365-2028.1986.tb00358.x

Tompkins, D. M., & Begon, M. (1999). Parasites can regulate wildlife populations. *Parasitology Today*, *15*(8), 311–313. https://doi.org/10.1016/S0169-4758(99)01484-2

Trinkel, M., & Kastberger, G. (2005). Competitive interactions between spotted hyenas and lions in the Etosha National Park, Namibia. *African Journal of Ecology*, *43*(3), 220–224. https://doi.org/10.1111/j.1365-2028.2005.00574.x

Van der Weyde, L. K., Mbisana, C., & Klein, R. (2018). Multi-species occupancy modelling of a carnivore guild in wildlife management areas in the Kalahari. *Biological Conservation*, *220*(February), 21–28. https://doi.org/10.1016/j.biocon.2018.01.033

Van Heerden, J., & Mills, M. G. L. (1995). An investigation into the health status and diseases of wild dogs (Lycaon pictus) in the Kruger National Park. *Journal of the South African Veterinary Association*, *66*(1), 18–27. https://doi.org/10.1111/j.1572-0241.1993.tb02958.x

Watson, M. J. (2013). What drives population-level effects of parasites? Meta-analysis meets life-history. *International Journal for Parasitology: Parasites and Wildlife*, *2*(1), 190–196. https://doi.org/10.1016/j.ijppaw.2013.05.001

Weisburg, W. G., Barns, S. M., Pelletier, D. A., & Lane, D. J. (1991). 16S ribosomal DNA amplification for phylogenetic study. *Weisburg, WG*, *173*(2), 697–703. https://doi.org/n.a.

Westbury, M. V, Hartmann, S., Barlow, A., Wiesel, I., Leo, V., Welch, R., … Hofreiter, M. (2018). Extended and Continuous Decline in Effective Population Size Results in Low Genomic Diversity in the World's Rarest Hyena Species, the Brown Hyena. *Molecular Biology and Evolution*, *35*(5), 1225–1237. https://doi.org/10.1093/molbev/msy037

Whelan, S., Liò, P., & Goldman, N. (2001). Molecular phylogenetics: state-of-the- art methods for looking into the past. *Trends in Genetics*, *17*, 262–272. https://doi.org/10.1016/S0168-9525(01)02272-7

Wiesel, I. (2010). Killing of Cape fur seal (Arctocephalus pusillus pusillus) pups by brown hyenas (Parahyaena brunnea) at mainland breeding colonies along the coastal Namib Desert. *Acta Ethologica*, *13*(2), 93–100. https://doi.org/10.1007/s10211-010-0078-1

Wiesel, I. (2015). Parahyaena brunnea. The IUCN Red List of Threatened Species 2015: e.T10276A82344448. Retrieved from http://dx.doi.org/10.2305/IUCN.UK.2015-4.RLTS.T10276A82344448.en

Williams, B. M., Berentsen, A., Shock, B. C., Teixiera, M., Dunbar, M. R., Becker, M. S., & Yabsley, M. J. (2014). Prevalence and diversity of Babesia, Hepatozoon, Ehrlichia, and Bartonella in wild and domestic carnivores from Zambia, Africa. *Parasitology Research*, *113*(3), 911–918. https://doi.org/10.1007/s00436-013-3722-7

Wright, H. W. Y. (2006). Paternal den attendance is the best predictor of offspring survival in the socially monogamous bat-eared fox. *Animal Behaviour*, *71*(3), 503–510. https://doi.org/10.1016/j.anbehav.2005.03.043

Wyatt, K. B., Campos, P. F., Gilbert, M. T. P., Kolokotronis, S. O., Hynes, W. H., DeSalle, R., … Greenwood, A. D. (2008). Historical mammal extinction on Christmas Island (Indian Ocean) correlates with introduced infectious disease. *PLoS ONE*, *3*(11). https://doi.org/10.1371/journal.pone.0003602

Yabsley, M. J., & Shock, B. C. (2013). Natural history of Zoonotic Babesia: Role of wildlife reservoirs. *International Journal for Parasitology: Parasites and Wildlife*, *2*(1), 18–31. https://doi.org/10.1016/j.ijppaw.2012.11.003

Zanet, S., Trisciuoglio, A., Bottero, E., De Mera, I. G. F., Gortazar, C., Carpignano, M. G., & Ferroglio, E. (2014). Piroplasmosis in wildlife: Babesia and Theileria affecting free-ranging ungulates and carnivores in the Italian Alps. *Parasites and Vectors*, *7*(1), 1–7. https://doi.org/10.1186/1756-3305-7-70

Zinsstag, J., Schelling, E., Waltner-Toews, D., & Tanner, M. (2011). From "one medicine" to "one health" and systemic approaches to health and well-being. *Preventive Veterinary Medicine*, *101*(3–4), 148–156. https://doi.org/10.1016/j.prevetmed.2010.07.003

Zuckerkandl, E., & Pauling, L. (1965). Molecules as Documents of Evolutionary History, 357–366.