Organochlorine pesticide exposure levels and haemoparasite infections as health parameters for leopards in South Africa

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Thesis accepted for the degree Doctor of Philosophy in Zoology at the North-West University

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Graduation May 2023

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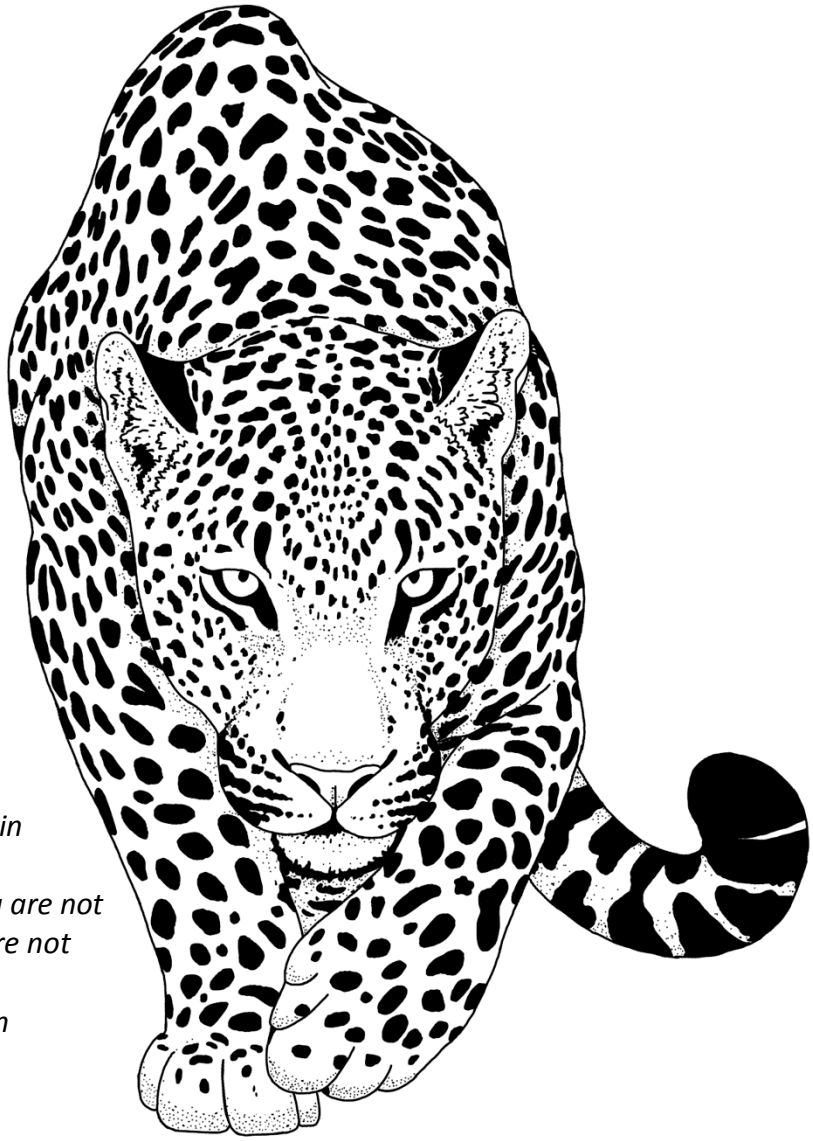
The mighty huntress



Persistent through the ages
living all everywhere about
there but not seen or heard
there you're never found
Pawed prints your only trace
claws unseen, sharp'd to wit
perfect predator, silent hunter
graceful killer, farmer affronter
Rosettes shining in the sun's kiss
dust clouds rising from your pelt
fading into specks rising above
Silent stalker, master at ambush
everyone knows your name
supposed supreme people don your skin
but not, never, your name
Revered where you are and where you are not
because, maybe, you are where you are not
Dedicated mom, provider, teacher
shadow walker among stars and moon
Sometimes you saw into the night
walking alone
in all your might.

M van As - Inspired by J
March 2022

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Preface

At the time of submitting this manuscript in 2022, there's a lack of health-related studies on large carnivores in Africa. Although the African leopard is an iconic animal among researchers and laymen alike, several avenues of its biology are, as of yet, still left unknown to science. This study serves as an initial bridge of these unexplored avenues, as it adds essential knowledge to a slowly growing collection on the inner workings of big cat health, on a global scale.

An integrated philosophy of health provides a framework that integrates existing models and concepts into a tool of health assessment. Blood was specifically selected as a biological data source, as it could be collected from live captive and wild leopards, offered the option of future resampling and could be sustainably used as a method of research on leopard health. This study could be seen as a study of threes: a trilogy integrating three areas of research, namely haematology, haemoparasitology and ecotoxicology, as a means of assessing leopard health. It was also completed with data from a trilogy of sources, including three ex-situ facilities, three in-situ areas representing three core wild leopard populations and involved three leopard phenotypes. As this study shows, an integrated philosophy of leopard health is a valuable novel tool for health assessment, which could potentially be expanded to other large African carnivores in the future.

This manuscript conforms to the thesis format style specified by North West University. Published articles, articles submitted for peer review and sections that still need to be reworked into publication format are hereby presented in this manuscript as a blended compendium. Co-author consent for use of publications as part of this thesis and contributions from each is summarized in Table i.

The following chapters are included in this dissertation:

- Chapter 1 (unpuplished): General introduction.
- Chapter 2 (unpublished): General materials and methods.
- Chapter 3 (unpublished): Haematological characterization of leopards.
- Chapter 4 (published): Intraleukocytic haemoparasites of leopards.
    - Sections of this chapter has been published in the following peer-reviewed publication:
      VAN AS, M., NETHERLANDS, E. C. & SMIT, N. J. 2020. Molecular characterisation and morphological description of two new species of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina: Hepatozoidae) infecting leukocytes of African leopards *Panthera pardus pardus* (L.). *Parasites & Vectors* 13:1–16. BioMed Central.

- Chapter 5 (submitted for peer review): Possible life cycle stages of a *Hepatozoon* species in an *Ixodes* tick.
    - Some sections of this chapter has been submitted for peer review:
      VAN AS, M., VAN AS, J., COOK, C.A. & SMIT, N.J. 2022. Morphologies of life cycle stages of a *Hepatozoon* species (Apicomplexa: Adeleorina: Hepatozoidae) in an *Ixodes* tick (Arthropoda: Ixodida: Ixodidae) from an infected African leopard. *International Journal for Parasitology: Parasites and Wildlife*. Under review.

- Chapter 6 (published): Organochlorine pesticides in African leopard blood serum.
    - Sections of this chapter has been published in the following peer-reviewed publication:
      VAN AS M., SMIT N. J., WOLMARANS N. J. & WEPENER V. 2022. First record of organochlorine pesticides in blood of wild and captive African leopards, *Panthera pardus pardus* (Linnaeus, 1758). *Frontiers in Environmental Science* 10:938453.

- Chapter 7 (unpublished): An integrated leopard health philosophy.

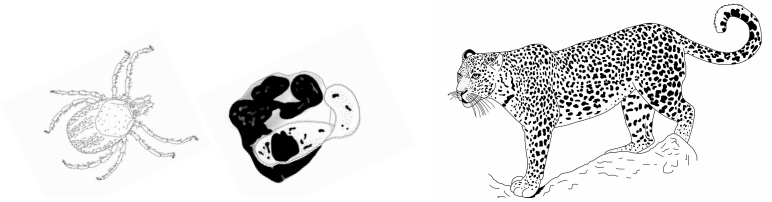

Table *i*. Author contributions and consent for use.

Author	Published/submitted work	Contributions	Consent
Michelle van As	Chapters 4 and 6	First author for papers involved. Conceptualized and designed the studies. Collected all samples and analysed all data. Performed and analysed molecular work together with Ed Netherlands. Wrote original drafts. Review and editing, visualization, project administration.	
Nico J. Smit	Chapters 4 and 6	Analysed molecular work. Co-author for Chapters 4 and 6. Conceptualization, provided resources. Co-author, review and editing, supervision, funding acquisition.	
Victor Wepener	Chapter 6	Conceptualization, formal analysis, resources for methodology, writing—review and editing, supervision.	
Ed C. Netherlands	Chapter 4	Performed molecular work together with Michelle van As. Analysed molecular work. Co-authored publication.	
Nico Wolmarans	Chapter 6	Methodology, validation, writing—review and editing.	

Summary

Compared to aquatic ecosystems, limited information exists on associated health parameters influencing terrestrial vertebrates, specifically large carnivores, and discussions around wildlife health measures are currently still quite rare. The African leopard *Panthera pardus pardus* (Linnaeus, 1758), a popular species for research by ethologists and ecologists, has a noticeable gap in knowledge regarding health status and assessment. Therefore, the main aim of this study was to use a multi-disciplinary approach that combines relatively diverse parameters to establish an integrated approach to the health assessment of ex-situ (captive) and in-situ (wild) South African leopards. Peripheral blood is an informative, non-lethally sampled tissue that can reflect the functioning of a whole organism. Thus, three overarching whole blood-related aspects, namely haematology, haemoparasitology and organochlorine pesticide (OCP) concentrations, were integrated with a newly designed body condition (BC) scoring system, specific to big cats, to evaluate the interaction of these internal and environmental variables from a health parameter perspective.

This study detailed haematological characteristics, confirming great variation in differential blood cell counts and morphologies in leopards, as had been reported for other felids. Differential leukocyte counts of leopards were in the same range as that of other big cats and wild leopards had higher leukocyte counts than captive leopards. Overall, neutrophils were the most numerous circulating leukocyte, followed by lymphocytes and eosinophils. Apicomplexan haemoparasites from the genus *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina: Hepatozoidae) have been widely reported from wild carnivores in Africa, including non-specific reports from leopards, but descriptions of potential developmental stages in naturally infected leopards and associated haematophagous vectors are rare. This study presented detailed morphological and molecular descriptions of two, concurring new haemogregarine species, *Hepatozoon luiperdjie* Van As et al. 2020 and *Hepatozoon ingwe* Van As et al. 2020, along with their associated host cell effects. It also described the first morphological traits of potential hepatozoan life cycle stages within an *Ixodes* tick (Arthropoda: Ixodida: Ixodidae), collected from a naturally infected animal. No clinical symptoms that could be ascribed to hepatozoonosis were observed, confirming the asymptomatic nature of these infections in large carnivores. The first report on baseline OCP concentrations in the blood of African leopards, and relationships between OCP levels, was presented in this study. Captive leopards had a slightly higher mean ΣOCP concentration (901 pg/ml) than wild leopards (768 pg/ml) and OCPs accumulated in the following order DDTs (27%) > HCHs (21%) > Heptachlors (15%) > CHLs (15%) > Drins (14%) > HCB (8%).

Finally, this study put forth a noteworthy interplay in the importance of body condition, haematological characteristics, haemogregarine infections and OCP burdens in captive and wild leopards. The most influential health factor for all leopards was neutrophil-to-lymphocyte ratios (NLRs), followed by other leukocyte counts (lymphocyte, neutrophil, and eosinophil counts in decreasing order of influence) and BC.

Body condition, NLR and OCP factors were the most influential variables for captive leopards, while differential leukocyte counts and haemoparasite infections were the most influential for wild leopards. The results from this study suggested the importance of focusing environmental parasitology studies on terrestrial environments. Ultimately, this thesis has shown that an integrated philosophy of leopard health, merging the variables identified in this study, is valuable as a future tool of health assessment.

Key words

African leopard, haematology, hepatozoonosis, *Hepatozoon*, life cycle, tick vector, organochlorine pesticides, wildlife ecotoxicology, integrated health.

Glossary

A

α–HCH	alpha-Hexachlorocyclohexane
ARC-OVR	Agricultural Research Council-Onderstepoort Veterinary Research Campus

B

BC	Body condition
BCS	Body condition score
β–HCH	beta-Hexachlorocyclohexane
BI	Bayesian Inference
BIC	Bayesian Information Criterion
BLAST	Basic Local Alignment Search Tool
BMI	Body mass index
BW	Body weight

C

CANOCO	Canonical Correspondence Ordination
CAT	Clot Activator Tubes
CDV	Canine distemper virus
CITES	Convention on International Trade in Endangered Species of Wild Fauna and Flora

D

DCC	Differential cell count
DDT	Dichlorodiphenyltrichloroethane
δ–HCH	delta-Hexachlorocyclohexane
DLC	Differential leukocyte count
DNA	Deoxyribonucleic acid

E

EDTA	Ethylenediamine tetraacetic acid

F

FIV	Feline Immunodeficiency Virus

G

γ–HCH	gamma-Hexachlorocyclohexane/ Lindane
GC–ECD	Gas Chromatography with an Electron Capture Detector

H

HCB	Hexachlorobenzene

I

ICZN	International Code of Zoological Nomenclature
IUCN	International Union for Conservation of Nature
IUCN SSC	IUCN Species Surivial Commission

L

LOD	Limit of detection
LOQ	Limit of quantification
LSID	Life Science Identifier

M

MCMC	Markov Chain Monte Carlo
MTBE	Amethyl tert-butyl ether

N

NGO	Non-governmental Organisation
NLR	Neurtophil-to-lymphocyte ratio

O

OC	Organochlorine
OCP	Organochlorine pesticide
OH	One Health
o,p'-DDD	*o,p'*-Dichlorodiphenyldichloroethane
o,p'-DDE	*o,p'*-Dichlorodiphenyldichloroethylene

P

PAUP	Phylogenetic Analysis Using Parsimony
PCA	Principle component analyses
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PCV	Packed cell volume
POP	Persistent Organic Pollutant
p,p'-DDE	*p,p'*-Dichlorodiphenyldichloroethylene

Q

QC	Quality control

R

RBC	Red blood cells/erythrocytes
RDA	Constrained redundancy analysis

S

Sars-CoV2	Severe Acute Respiratory Syndrome Coronavirus 2

T

TOPS	Threatened or Protected Species

U

UNESCO	United Nations Educational, Scientific and Cultural Organization
US	United States
USA	United States of America

W

WBC	White blood cells/leukocytes
WWF	World Wildlife Fund for Nature

CHAPTER 1
General introduction

The least studied of all big cats, no other large carnivore in Africa is as ferocious, elusive and mythical as the leopard *Panthera pardus* (Linnaeus, 1758) (Hes 1991, Bailey 1993, Bothma & Walker 1999, Hancock 2000, Maheshwari 2006). The leopard is the largest spotted cat on this continent (Skinner & Chimimba 2005) and being the least specialized of the African big cats, they are highly adaptable to live in diverse habitats with various outside pressures (Norton et al. 1986, Bailey 1993, Mills & Biggs 1993, Mills & Hes 1997). The grace and strength of the leopard (and all its subspecies) have been admired since the early Roman Empire, when considerable confusion existed regarding this cat's taxonomy (Bailey 1993). Due to its elusiveness, the leopard is the least studied of all the big cats in Africa, although it is a relatively popular subject with South African researchers.

1.1. Taxonomy and phylogeography

Panthera pardus pardus (Linnaeus, 1758)

Taxonomic Notes
Class: Mammalia Linnaeus, 1758
Order: Carnivora Bowdich, 1821
Family: Felidae G. Fischer de Waldheim, 1817
Genus: *Panthera* Oken, 1816
Species: *pardus* (Linnaeus, 1758) Pocock, 1916
Sub-species: *pardus pardus*

Representatives of the subfamily Felinae, or modern cats, have existed since the Miocene era 24 million years ago, but the first known felids date back to the upper Eocene era, 40 million years ago (Estes 1991). Until a few million years ago, sabre-toothed cats dominated the family and modern big cats arising from these as today's predators (Estes 1991).

1.1.1 Genus *Panthera* Oken, 1816

The felid genus *Panthera* Oken 1816, to which the leopard, jaguar *Panthera onca* (Linnaeus, 1758), tiger *Panthera tigris* (Linnaeus, 1758), lion *Panthera leo* (Linnaeus, 1758) and Snow leopard *Panthera uncia* (Schreber, 1775) belong (Fig. 1.1), is considered a fairly young genus (Uphyrkina et al. 2001), suggested to have diverged 2 – 3 million years ago from a common ancestor (Johnson & O'Brien 1997). Fossil evidence as old as 1.5 – 2.0 million years suggests that leopards historically had a much wider distribution than they do today (Maheshwari 2006). The hyoid-bone of all members of the genus *Panthera* is not completely ossified. This characteristic, coupled with relatively long, elastic vocal folds, allows them to roar (Pocock 1916, Peters & Hast 1994, Weissengruber et al. 2002). The only exception in this regard is the Snow leopard, which was placed in the genus *Panthera* due to genetic relatedness to other Pantherines (Johnson et al. 2006). The origin of the modern leopard lineage has previously been estimated at approximately 470 000 – 825 000 years ago in Africa, followed by a migration event across Asia roughly 170 000 – 300 000 years ago (Uphyrkina et al. 2001). However, recent findings by Tseng et al. (2014), who were the first to attempt combining fossil and genetic analysis, revealed that 75% of the divergence events in the Pantherine lineage date back seven million years earlier than previously estimated. Originally described by Carl Linnaeus in 1758 as *Felis pardus* from a specimen collected in Egypt, the leopard was transferred to the genus *Panthera* by R.I. Pocock in 1916 (Bothma & Walker 1999) due to similarity in hyoid-bone structure that enable these cats to roar.

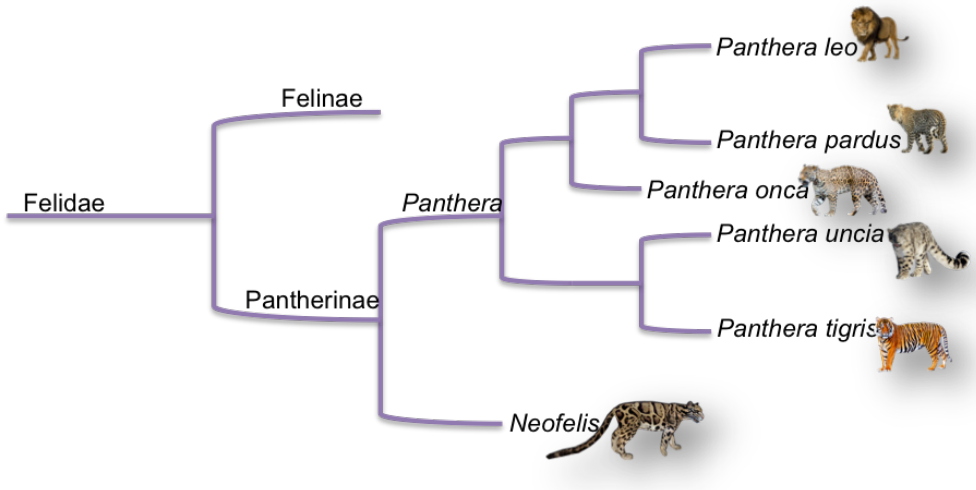

Figure 1.1 Simplified phylogenetic tree of current members of the genus *Panthera*. Digitally adapted from Li et al. 2016.

1.1.2 *Panthera pardus* subspecies

Until the early 1990s, discord remained among scientists regarding the classification of subspecies of leopard (Hes 1991). In the mid 1990s Miththapala et al. (1996) undertook the first

genetic study of leopards to assess the status of leopard subspecies around the world. Preceding this study, 27 leopard subspecies had been described with 13 subspecies occurring in Africa. Miththapala et al. (1996) narrowed these down to nine subspecies occurring globally, with one subspecies limited to the continent of Africa (Table 1.1). Five years later, revisiting data provided by Miththapala et al. (1996), Uphyrkina et al. (2001) confirmed this number of nine subspecies (Table 1.1). Khorozyan et al. (2006) revised the taxonomic status of the Middle Eastern leopards and suggested that some of the subspecies as described before the findings of Miththapala et al. (1996), such as *Panthera pardus ciscaucasica* (syn. *saxicolour*, *transcaucasica*) (Satunin, 1914), *Panthera pardus tulliana* (Valenciennes, 1856) and *Panthera pardus sindica* (syn. *dathei*, *millardi*) Pocock, 1930 should be retained. However, their findings are currently not supported by the majority of the scientific community and therefore the nine subspecies by Miththapala et al. (1996) and Uphyrkina et al. (2001) is still valid. The International Union for Conservation of Nature (IUCN) Redlist also uses the classification of the latter two authors as a tool towards assessing the conservation status of leopards.

1.2 Biogeography of *Panthera pardus*

Despite their important ecological role, the distribution of some of the most wide-ranging African predators has shrunk by more than 76% (Ray et al. 2005). The leopard was the world's most widespread solitary cat until a century ago (Myers 1976, Bailey 1993, Skinner & Chimimba 2005, Hunter et al. 2013), with a global range spanning at least 80 countries, half of which are located in Africa (Estes 1991, Nowell & Jackson 1996, Hunter et al. 2003, Maheshwari 2006, Al-Johany 2007). Their historic range encompassed all of the sub-Saharan and northern Africa, the Middle East, Asia Minor, South and Southeast Asia, extending to the Russian Amur Valley in the Far East. Island ranges included Sri Lanka, Java, Kangean and Zanzibar (see Fig. 1.2) (Myers 1976, Uphyrkina et al. 2001).

The population status of *Panthera pardus* is stable in many protected areas and those existing outside of these areas, do so at far lower densities (Martins et al. 2005). The leopard has a widespread geographic distribution in tropical Asia and Africa, but despite its ability to adapt, its African range has declined by 37% over the past 100 years (Ray et al. 2005). Overall, leopards have experienced a range loss of 97% (Durant et al. 2014). In South Africa's unprotected areas, African leopard distribution has become limited to remote, isolated areas with the notable absence of regular human presence (Norton et al. 1986, Skead et al. 2007).

Today, leopard distribution includes much of Africa and Asia, with some relict populations scattered through the Middle East and south-eastern Europe (Hunter et al. 2003, Maheshwari 2006, Balme et al. 2013), as well as in the majority of protected areas throughout their range (Sunquist & Sunquist 2002) (Fig. 1.2).

Table 1.1 Globally recognized leopard subspecies distribution and conservation status (Miththapala et al. (1996); Uphyrkina et al. (2001), IUCN Red list accessed 2022/11/09).

Current Subspecies	Curent Common name	Previous Subspecies	Previous Common name	Original description	IUCN status
Panthera pardus delacourii	Indochinese leopard	*Panthera pardus delacouri* (Pocock 1930)		Pocock 1930	Near-threatened
Panthera pardus fusca	Indian leopard	*Panthera pardus fusca* (Meyer 1794)	Indian leopard	(Meyer 1794)	Near-threatened
		Panthera pardus millardi (Pocock 1930)	Kashmir leopard		
		Panthera pardus pernigra (Hodgson 1863)	Nepal leopard		
Panthera pardus japonensis	North China leopard	*Panthera pardus japonensis* (Gray 1862)	North Chinese leopard	(Gray 1862)	Near-threatened
Panthera pardus kotiya	Sri Lankan leopard	*Panthera pardus kotiya* (Deraniyagala 1956)	Sri Lankan leopard	Deraniyagala 1956	Endangered
Panthera pardus melas	Javan leopard	*Panthera pardus melas* (Cuvier 1809)	Javan leopard	(Cuvier 1809)	Critically endangered (>250 individuals in the wild)
Panthera pardus nimr	Arabian leopard	*Panthera pardus nimr* (Hemprich & Ehrenberg 1833)	Arabian leopard	(Hemprich & Ehrenberg 1833)	Critically endangered (>200 individuals in the wild)
Panthera pardus orientalis	Amur leopard	*Panthera pardus orientalis* (Schlegel 1857)	Amur leopard	(Schlegel 1857)	Critically endangered (>30 individuals in the wild)
Panthera pardus pardus	African leopard	*Panthera pardus adersi* (Pocock 1932)	Zanzibar leopard	(Linnaeus 1758)	Near-threatened
		Panthera pardus antinorii (de Beaux 1923)	Eritrean leopard		
		Panthera pardus chui (Heller 1913)	Ugandan leopard		
		Panthera pardus iturensis (Allen 1924)	Congo leopard		
		Panthera pardus leopardus (Schreber 1777)	West African forest leopard		

Table 1.1 Globally recognized leopard subspecies distribution and conservation status (Miththapala et al. (1996); Uphyrkina et al. (2001), IUCN Red list accessed 2022/11/09).

Current Subspecies	Curent Common name	Previous Subspecies	Previous Common name	Original description	IUCN status
Panthera pardus pardus	African leopard	*Panthera pardus melanotica* (Gunther 1885)	Cape leopard	(Linnaeus 1758)	Near-threatened
		Panthera pardus nanopardus (Thomas 1904)	Somalian leopard		
		Panthera pardus panthera (Schreber 1777)	Barbary leopard		
		Panthera pardus pardus (Linnaeus, 1758)	North African leopard		
		Panthera pardus reichenowi (Cabrera 1918)	West African leopard		
		Panthera pardus shortridgei (Pocock 1932)	Central African leopard		
		Panthera pardus suahelicus (Neumann 1900)	East African leopard		
Panthera pardus saxicolour	Persian leopard	*Panthera pardus ciscaucasicus* (Satunin 1914)	Caucasus leopard	Pocock 1927	Endangered
	West Asian leopard	*Panthera pardus dathei* (Zukowsky 1964)	Central Persian leopard		
		Panthera pardus sindica (Pocock 1930)	Baluchistan leopard		
	North Persian leopard	*Panthera pardus jarvisi* (Pocock 1932)	Sinai leopard		
	Caucasian leopard	*Panthera pardus saxicolour* (Pocock 1927)	North Persian leopard		
	Central Asian leopard	*Panthera pardus tulliana* (Valenciennes 1856)	Asia Minor leopard		

Fitting their adaptability, leopards inhabit nearly every African habitat, ranging from rainforest to desert and all habitats in-between, being only absent in areas where conflict with humans have driven them away (Bothma & le Riche 1984, Estes 1991, Mills & Hes 1997, Bertram 1999, Spong et al. 2000). All habitat types with annual rainfall above 50 mm seem suitable to support leopards (Monod 1965), but they can also inhabit areas with less rainfall such as the southernmost extension of the Namib Desert, providing that they keep along river courses, such as the Orange River (Stuart & Stuart 1989, Nowell & Jackson 1996). African leopards naturally tend to reach their highest densities in riparian zones (Bailey 1993, Sunquist & Sunquist 2002), but they are also regularly associated with areas of rocky outcrops, forests and mountain ranges (Skinner & Chimimba 2005).

Several leopard population and distribution studies have been completed in Africa, India and the far East (Myers 1976, Sunquist 1983, Martin & de Meulenaer 1988, Johnsingh & Negi 2003, Khorozyan 2003, Baskaya & Bilgili 2004, Wang & Macdonald 2009, Kabir et al. 2013). Johnsingh & Negi (2003) did an easily repeatable transect survey of leopard populations in the Rajaji-Corbett Conservation Unit of northern India. Through the analysis of VORTEX population models for tigers *Panthera tigris tigris* (Linnaeus, 1758) and leopards on Indonesian islands, Wilkinson & O'Regan (2003) discovered that leopard populations were surprisingly more prone to extinction than tiger populations. The main reason for this outcome was attributed to the different breeding biologies of these two cats.

1.2.1 *Panthera pardus pardus* in Africa

The African Leopard *Panthera pardus pardus*, from here on only referred to as leopard, is the most common of the nine leopard subspecies (Miththapala et al. 1996, Uphyrkina et al. 2001). The determination of leopard population size in sub-Saharan Africa has been attempted six times between 1970 and 1992 (Myers 1976, Teer & Swank 1977, Eaton 1978, Hamilton 1981, Martin & de Meulenaer 1988, Shoemaker 1993), of which the research by Myers (1976), Teer & Swank (1977), Eaton (1978) and Karanth et al. (2013) relied mainly on questionnaires and interviews with local communities. Hamilton (1981)'s work focused on leopards in Kenya and was more thorough than that of his predecessors since it was augmented by the author's personal field studies. A few years later, Martin & de Meulenaer (1988) were the first to develop a population model for *P. p. pardus* in South Africa and projected a population of over 23 000 individuals. However, according to Hunter & Balme (2004) these authors' model proved questionable when a similar study undertaken by Peter Norton (1990) came up with about a tenth of the number of leopards in South Africa (~ 2 300).

Different leopard populations throughout Africa exhibit different growth patterns. African leopards have disappeared from most of West Africa's coastal areas (Skinner & Chimimba 2005) and are now virtually extinct in North Africa (Hunter & Balme 2004) (Fig. 1.2). During a camera trap survey conducted by Treves et al. (2010) in the forests of western Uganda, it became

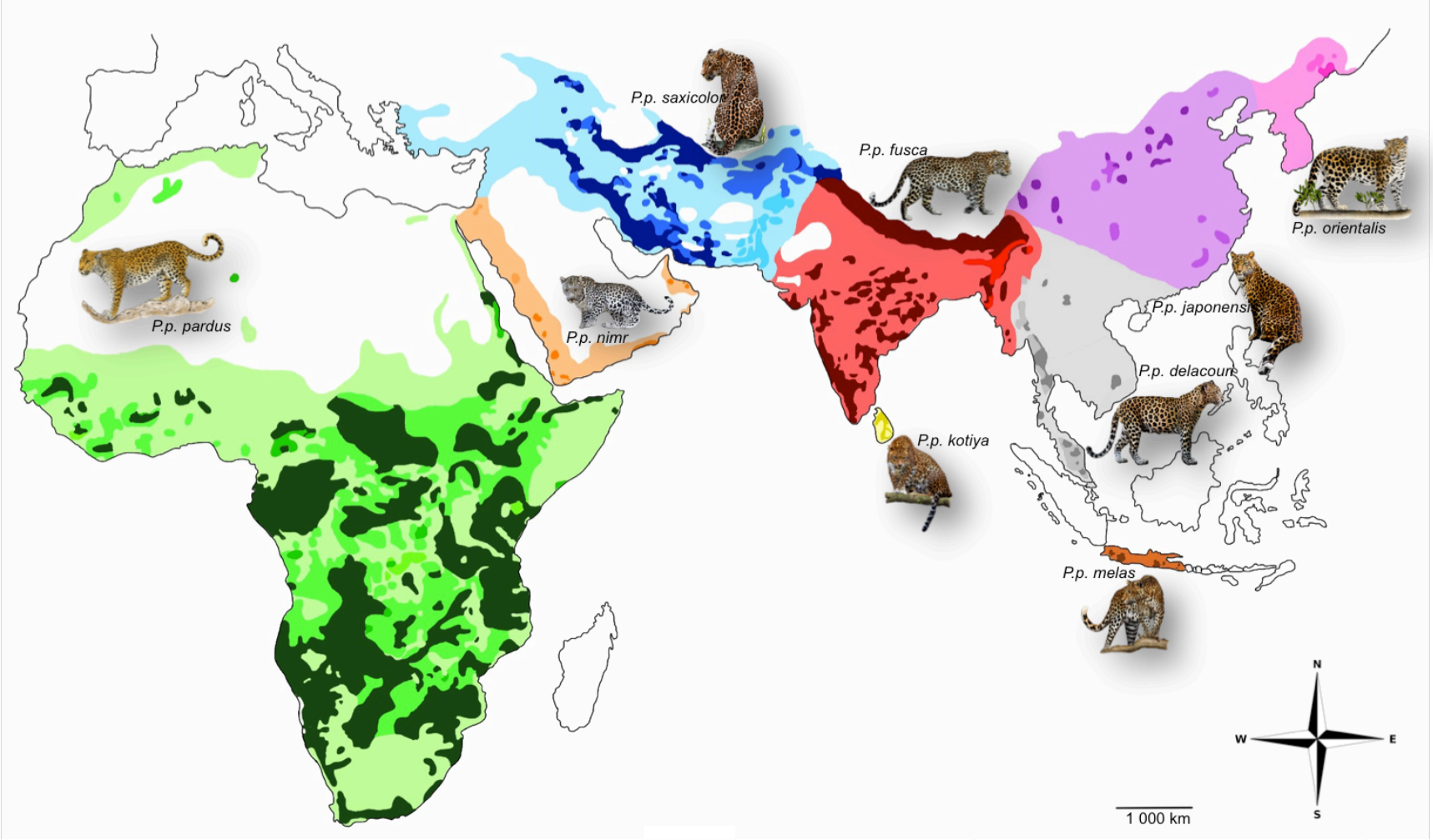

Figure 1.2 Historical and current distribution of current accepted leopard subspecies. Historical range 891 817 km^2; Current known resident range 29 221 km^2 (Durant et al. 2014). The darker the shade the more recent the distribution, with the lightest shades where leopard species have completely disappeared and the darkest shades where populations can still be found. Green – distribution of *Panthera pardus pardus*; Orange – *Panthera pardus nimr*; Blue - *Panthera pardus saxicolour;* Red – *Panthera pardus fusca;* Grey – *Panthera pardus delacouri;* Purple – *Panthera pardus japonensis;* Pink – *Panthera pardus orientalis;* Yellow – *Panthera pardus kotiya;* Brown – *Panthera pardus melas.* Adapted from Miththapala et al. (1996), Gerngross (2019) and Stein et al. (2020).

apparent that the leopard population density is very low and Treves et al. (2010) consequently suggested Ugandan leopards should receive greater conservation attention. On the other hand, Packer et al. (2011) reported a steady rise in the Tanzanian leopard population, which they partly attributed to decreasing demand for Tanzanian leopards as trophies. In the same year, Stein et al. (2011) conveyed claims by north-central Namibian farmers that the leopard population was increasing. They tested this theory by assessing camera trap data combined with telemetry-based estimates on home range sizes. Williams et al. (2013) reported the presence of at least one leopard in the central Namib Desert, along the ephemeral Swakop River in the Namib-Naukluft National Park. Therefore, it seems that leopard density outside of protected areas in arid regions throughout southern Africa is relatively constant (Bothma & le Riche 1984, Stander et al. 1997, Stein et al. 2011).

1.2.2 Leopards in South Africa

In South Africa, leopards roam throughout the Limpopo Province, North West Province, Mpumalanga and Gauteng, with the exception of the Highveld grassland areas in the southern regions of these provinces (see Fig. 1.3) (Mills & Hes 1997, Skinner & Chimimba 2005). According to Skinner & Chimimba (2005), leopards in KwaZulu-Natal occur predominantly in the northeast with a scant distribution in the central and western regions. Intermittent presences have also been reported in the Free State, with signs of activity observed recently in the eastern Free State (M. van As, personal observation). Leopards can also be found in the mountainous areas in the Eastern Cape, along the south coast westwards into the Western Cape, occurring northwards into the northern-most parts of the Northern Cape. Some leopard sightings have also been from the landlocked country of Lesotho (Mills & Hes 1997, Skinner & Chimimba 2005).

South African leopard population sizes differ from region to region (Fig. 1.2), which is due to variation in potential prey densities and those densities' ensuing influence on the size of a leopard's home range (Bothma & Walker 1999). Bailey (1993) projected an average leopard density in the Kruger National Park of 3.5 adults per 100 km^2, with population density increasing in riparian forest zones to a massive 30.3 individuals per km^2. In arid environments, such as South Africa's Kalahari Gemsbok National Park, harbouring smaller potential prey densities, leopard population densities are low with a reported density of 1.25 adults per 100 km^2 (Martin & de Meulenaer 1988).

Daly et al. (2005) identified 10 core African leopard populations in South Africa (Fig. 1.3). It appears that the game reserve network of private ranches and the Kruger National Park, northern KwaZulu-Natal and the Kgalagadi Transfrontier Park sustain the largest leopard populations in South Africa (Fig. 1.3). Fattebert et al. (2013) reported that male leopards could cover more than 350 km during natal dispersal, illustrating the possibility of a functioning metapopulation in Greater Kruger, southern Mozambique, Swaziland and northern KwaZulu-Natal. Chapman & Balme (2010) surveyed a leopard population in the Zululand Rhino Reserve (northern KwaZulu-Natal) by means of camera-traps and found the reserve to be below

carrying capacity. Grey et al. (2013) used the leopard population in the western Soutpansberg Mountains, South Africa, to construct a model predicting the density of leopard populations in

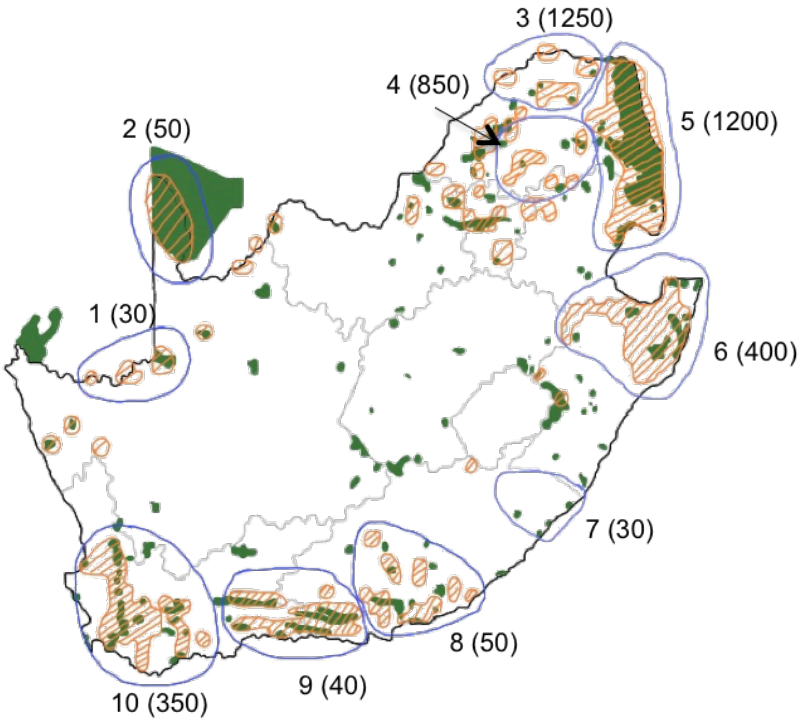

Figure 1.3 Ten core leopard populations and population sizes in South Africa, in relation to formally conserved areas. Orange striped polygons - Leopard observations; Green polygons - Conserved areas of South Africa. Number indicates geographic population. Numbers in brackets indicates estimated population size. 1 – Orange river; 2 – Kalahari; 3 – Northern Limpopo; 4 – Waterberg/Mpumalanga; 5 – Greater Kruger; 6 – Northern KwaZulu-Natal; 7 – Wild Coast; 8 – Eastern Cape Valley; 9 – Eastern Cape Mountain; 10 – Western Cape. Redrawn from Daly et al. (2005) and SA-Venues.com (2013).

mountainous areas. Grey et al. (2013) found that the highest density of sub-Saharan leopards exists in the western Soutpansberg Mountains (see Fig. 1.3 core population number 3), outside state-protected areas.

1.3 Biology and ecology of the South African leopard

1.3.1 Physical appearance and phenotypes

The leopard is the smallest member of the genus *Panthera* and females are smaller and overall more slightly built than males. Full-grown males measure on average from 60 – 70 cm in height and weigh between 35 – 70 kg, with females measuring 57 – 64 cm high and weighing anything from 28 – 58 kg (Estes 1991, Bailey 1993, Stuart & Stuart 2015). These cats have an elongate

body, limbs of moderate length, broad rounded paws and short, round ears. They have a wide head with short, powerful jaws, fitted with long canines (Estes 1991, Bailey 1993, Maheshwari 2006). Prime adult males have a thick, muscular neck-shoulder area with a dewlap hanging under the throat and running along the chest area towards the belly. The leopard is the largest spotted cat in Africa (Skinner & Chimimba 2005) and its pelt is ideally adapted to camouflage the animal in the dappled light of thickets (Bailey 1993). However, there are some colour variations in members of this species, especially between different geographical areas (Mills & Harvey 2001, Uphyrkina et al. 2001). As each human has a unique fingerprint, the spot pattern of each leopard is unique to that individual (Hes 1991, Skinner & Chimimba 2005). There are also three different phenotypic colour morphs in this cat: the so-called regular leopard (regular phenotype), the black leopard (melanistic phenotype) and the red or strawberry leopard (erythristic phenotype) (see Fig. 1.4 for representative examples).

Regular phenotype (regular leopards)

Pelt hues can vary from tawny, deep golden yellow to a light tan colour (Estes 1991, Hes 1991, Bailey 1993, Hancock 2000, Uphyrkina et al. 2001) and are patterned with black rosettes (Fig. 1.4 a). Lower limbs, head, flanks and hind quarters are dotted with solid black spots, and the tail is rosetted on top and whitish underneath with a white tip. Ventral parts of are lighter colour than dorsal areas.

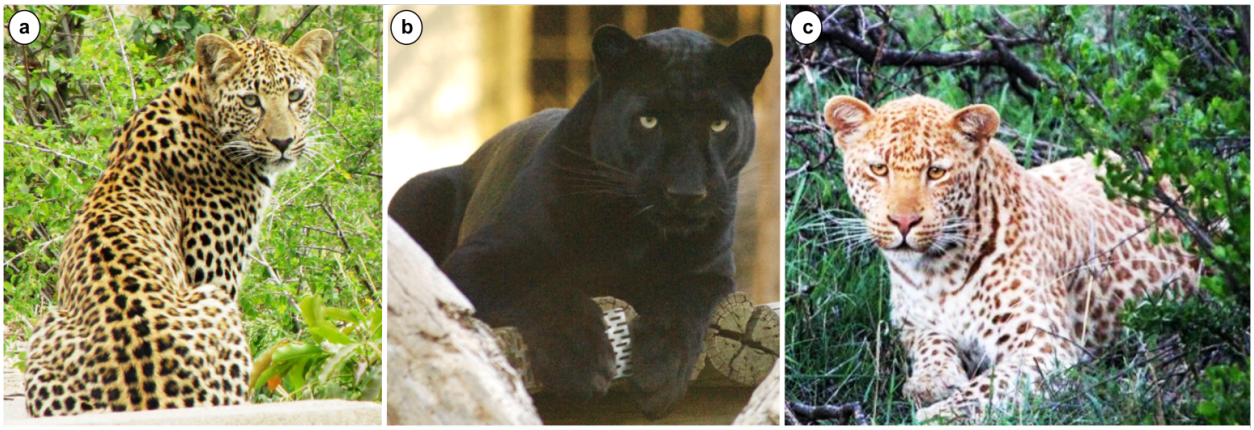

Figure 1.4 The three variations of coat colours in leopards (phenotypes) sampled during this study. **a** Wild leopard female from the Greater Kruger Conservation Area, Mpumalanga, with regular colouration (Michelle van As, 2015). **b** Captive melanistic leopard male from the Bloemfontein Zoo, Free State (Liesl van As, 2013). **c** Wild erytrhistic leopard male from the Lydenburg Area in Mpumalanga (Gerrie Camacho, as shown in Vermaak 2014).

Pocock (1932) observed that the differences in pelt hues of regular African leopards can be associated with the habitat in which these animals live, and he identified four colour variations among regular morphs (see Table 1.2). Pocock's observations suggested that mountain leopards have the darkest pelt colour, followed by the lighter hues of forest leopards, savannah leopards and lastly by the lightest hues of desert leopards. These trends are, however, only representative of leopards from the Congo Basin and equatorial regions of Africa, and are not definitively displayed by leopards in southern Africa.

Table 1.2 Pelt colour variations in African leopards from different habitat types as observed by Pocock (1932).

Habitat type	Description of pelt colour
Warm desert	Pale cream to yellow-brown
Cool desert	Stone coloured, buffish grey
Savannah	Reddish-brown to orange-brown
Lowland rainforest (West Africa)	Deep, dark golden
High altitude forested mountains (equatorial Africa)	Very dark tawny brown

Melanistic phenotype (black leopards)

Melanism is generally defined as the over-distribution of melanin, a dark-coloured pigment, in the skin of an animal. This results in the existence of darker-coloured individuals, compared to the typical phenotype expressed in a given species or population (Kettlewell 1973, Majerus & Mundy 2003). Melanistic leopard phenotypes can appear completely black in colour, especially from a distance (Fig. 1.4 b). However, upon closer inspection, the rosette and spot patterns are still faintly visible against the dark solid background of the pelt. In some individuals, the dark colour may seem reddish-brown, while in others it may seem rich black. The degree of rosette visibility also differs between individuals. Unlike the regular phenotype, these leopards do not have any white colouration on the body and the ventral parts are just as darkly coloured as the dorsal parts. They also only have black noses, whilst regular phenotypes may have a pink nose until it reaches adulthood.

Melanistic leopards, or so-called black leopards, have been reported throughout the range of this cat (Uphyrkina et al. 2001), particularly from humid, densely forested areas (Kawanishi et al. 2010). This trend might suggest melanism in leopards as an adaptive mutation to this habitat type, just as it seems to be in its close relative, the jaguar. Black leopards have been reported from Malaysia, Java (Hunter, 2015), India (Daniel, 1996) and Sri Lanka.

Black African leopards are most commonly reported from the montane forests of the East African Rift Valley, close to the equator, seemingly due to this habitat-associated trend. Black leopards also seem to be exceptionally common in the highland forests of Ethiopia, where these populations don't often get the chance to interbreed with savannah leopards due to the vastness of these forests. Turnbull-Kemp (1967) reported a black leopard from Mount Cameroon in central Africa and Pocock (1932) described the skin and skull of a very dark-coloured male leopard shot in the same region. In the mid-nineties, Bartlett (1994) reported sightings of black leopards in the forests of Mount Kenya and the Aberdare mountain ranges.

He described seeing a black female with the aid of binoculars, as well as the skin of a black leopard shot by a local farmer at Nyeri, Kenya. According to Bartlett (1994), the Game Department Headquarters also had a black leopard skin mounted on their wall, apparently shot at Meru, Kenya. Most recently, a camera trap in Laikipia, Kenya, photographed a black leopard after it was spotted for the first time by locals in 2018 (Wahome 2021, Green 2022). In 2021, a book was published with a collection of black leopard photographs taken with camera traps in that area (Burrard-Lucas 2021).

There have also been some black leopard sightings reported in South Africa since the 1950s. These reports usually originate from the Greater Kruger Conservation Area (see Fig. 1.3 core population number 5), but there have also been reports from Lydenburg (Camacho, G. personal communication) and Sabie (see Fig. 1.3 core population number 4) (Hes 2013), as well as near Pilgrim's Rest (Fig. 1.3 core population number 4) and close to Badplaas in Mpumalanga (Fig. 1.3, just north-north-west outside core population 6). Another unnamed nature reserve in Mpumalanga reported a sighting by 12 people and there was also a report from Hartebeesvlakte (Fig. 1.3 core population number 4) (Brian Jones 1952, as cited by Hes 2013). However, since there is no photographic evidence for these reports, it still needs to be absolutely confirmed that there are wild black leopards in South Africa. Chapters 3 and 6 will address more details on melanism.

Erythristic phenotype (red leopards)

Erythrism is expressed when black pigmentation is replaced by red pigment in an individual, and is highly unusual and subsequently seldom reported from wild leopards (Divyabhanusinh 1993, Sunquist & Sunquist 2014, Hartwell 2015). Erythristic or red leopards are morphologically very similar to the regular phenotypic expression: somewhat paler and more reddish-orange in colour, with the rosettes a light brown to reddish brown instead of the black of the regular phenotype (Fig. 1.4 c). The ventral parts of a red leopard are lighter in colour than the dorsal areas, with a white tip on the tail, just like the regular phenotypes. Recently, Tensen et al. (2022) reported that this phenotype is the resulted expression of a mutated gene identified to be involved in the biosynthesis of melanin.

Red leopards have thus far only been reported in South Africa (Tensen et al. 2022) and twenty red leopards have been recorded in the last four decades, mainly in the northern parts of South Africa (Pirie et al. 2016). According to Pirie et al. (2016), the first documented report of an erythristic leopard was in 2012 from the Madikwe Game Reserve (Fig. 1.3 core population 3), where the borders of Botswana and South Africa's Limpopo and North West Provinces meet. There are only a handful of records of red leopards in the northern parts of South Africa (see Pirie et al. 2016), and only one other report involving five leopards from India (Divyabhanusinh 1993). South African sightings included males, females and a cub of unknown gender, and only one core population as identified by Daly et al. (2005) (see Fig. 1.3 core population 4) are represented in these records. South African sightings occurred in two clusters, one in the area where the borders of Botswana and South Africa's North West and Limpopo Provinces meet

(Fig. 1.3 core population 3), and the other in the Lydenburg area in Mpumalanga (Fig. 1.3 core population 4) (see Table 1.3 for details). The presence of this phenotype in these parts of South Africa may indicate that current leopard populations, especially the Waterberg/Mpumalanga population (Fig. 1.3 core population 4), may be undergoing severe fragmentation (Pirie et al. 2014; 2016), potentially indicating a downwards spiral in genetic variation (Spong et al. 2000).

Table 1.3 Sightings of erythrystic phenotpyes in South Africa (compiled from Pirie et al. 2016 and Daly et al. 2005).

Year of sighting	Gender	Locality	Core leopard population
1990's	?	Close to the border of Botswana and South Africa's North West and Limpopo Provinces	Outside all core populations
2012	M	Close to the border of Botswana and South Africa's North West and Limpopo Provinces	Outside all core populations
2013	F	Road between Lydenburg and Burgersfort	Waterberg/Mpumalanga
2014-2015	F	Lydenburg area	Waterberg/Mpumalanga
2014-2015	M	Lydenburg area	Waterberg/Mpumalanga
2015	M	Close to the border of Botswana and South Africa's North West and Limpopo Provinces	Outside all core populations
2015	F	R36, Lydenburg*	Waterberg/Mpumalanga
2015	Cub	Lydenburg area	Waterberg/Mpumalanga

* sampled during this study

1.3.2 Habitat preferences

Leopards are highly adaptable cats and inhabit nearly every African habitat and are only absent from areas within Africa where they have been driven away by humans (Bothma & le Riche 1984, Estes 1991, Mills & Hes 1997, Bertram 1999, Spong et al. 2000). Leopards occur in habitats ranging from rainforests to deserts, along altitudinal gradients ranging from sea-level coastal areas to high altitude mountains (> 2000 m) and successfully occupy some urban areas (Monod 1965, Estes 1991, Hes 1991, Mills & Hes 1997, Hayward et al. 2006). Monod (1965) hinted that all habitats with annual rainfall > 50 mm seem quite adequate to sustain leopards, but these cats also have viable populations along substantial river courses in desert areas such as the Namib (Stuart & Stuart 1989, Nowell & Jackson 1996). Nevertheless, it has been reported that leopards frequent rocky outcrops, hills and mountain ranges, and reach their highest densities in riparian zones, with sufficient cover and adequately sized prey animals (Estes 1991, Bertram 1999, Sunquist & Sunquist 2002).

1.3.3 General ethological traits

Being territorial, solitary carnivores, leopards only briefly associate with conspecific breeding partners during the mating season and young cubs accompany their mother until they are independent (Estes 1991, Hes 1991, Bailey 1993). A leopard's home range usually excludes conspecifics of the same gender, with both males and females territorial, (Bothma & Walker 1999) and occasional nomads will wander through the ranges of resident individuals (Bailey

1993, Bothma & Walker 1999). According to Bothma & Walker (1999), the size of leopard home ranges vary greatly between different regions and large home ranges are usually found in areas of low rainfall due to a low abundance of potential prey animals. Typical male leopard home range sizes in protected areas have been shown to be substantially larger than that of females (Hamilton 1981, Norton & Lawson 1985, Norton & Henley 1987, Le Roux & Skinner 1989, Bailey 1993).

According to Bailey (1993) and Bothma & Bothma (2006), leopards tend to be more active at night than during the day, and will also rest periodically during the course of the night. However, these activity patterns are influenced by several environmental factors and leopards have been found to be much more diurnal than previously thought. These cats are solitary hunters, stalk-and-pounce specialists, rush at prey at about 60 km/h and usually hunt at night (Mills & Hes 1997, Bertram 1999, Sharma 2004, Schwarz & Fischer 2006, Hayward et al. 2006, Balme et al. 2007). However, a leopard will make full use of any opportunity, even during mid-day (Bothma & Walker 1999). It has been reported that female leopards hunt more efficiently than males for a number of reasons (Bothma & Walker 1999, Bothma & Coertze 2004), for example Bothma & Coertze (2004) reported that motherhood increases the hunting efficiency of females in the Kalahari. This contributes to females usually living longer in the wild than males.

1.3.4 Feeding ecology

Leopards are part of the large carnivore guild (includes lions and hyaenas Genus *Crocuta* Kaup, 1828), have a broad, varied diet and are considered highly opportunistic predators, able to utilize habitats and food resources not favoured by most other members of this guild (Bothma & le Riche 1986, Estes 1991, Hes 1991, Bothma & Walker 1999, Hancock 2000, Hayward et al. 2006). These cats have a broad, generalized diet and will eat whatever food is easiest to obtain, ranging from beetles to animals the size of eland *Taurotragus oryx* (Pallas, 1766) (Norton et al. 1986, Bailey 1993, Chauhan & Goyal 2000, Hayward et al. 2006), rendering leopards the least specialized feeders in the large carnivore guild. A total of 92 prey species taken by leopards in sub-Saharan Africa has been reported by Mills & Harvey (2001), a richness of prey that suggests unselectiveness. Hayward et al. (2006) suggested that the variably body weight of leopards enable this cat to survive on small vertebrates and invertebrates for short periods of time, in the absence of larger prey. This view is supported by records from Mitchell et al. (1965), Hirst (1969), Scheepers & Gilchrist (1991) and Ott (2004), on a variety of species taken as prey.

The easiest obtainable food source is usually also the most common food source at the time in an area, which is commonly reflected in the diet of leopards (Carbone & Gittleman 2002, Breuer 2005, Hayward et al. 2006, 2007). However, leopards are not completely non-selective when selecting prey (Hayward et al. 2006) and predominantly feed on medium sized antelopes (5 – 70 kg) (Kruuk & Turner 1967, Grobler & Wilson 1972, Smith 1977, Bothma & le Riche 1986, Norton & Henley 1987, Le Roux & Skinner 1989, Hes 1991, Bailey 1993, Mills & Hes 1997,

Skinner & Chimimba 2005, Ray et al. 2005, Schwarz & Fischer 2006). Contrary Hirst (1969)'s findings, Bailey (1993), Hayward et al. (2006, 2007) and Balme et al. (2007) agreed that leopards prefer hunting in habitats where it is easier to catch prey to areas where prey was more common. Shultz et al. (2004) suggested that there exists no relationship between prey abundance and leopard predation rates in an area. Yet, Ray et al. (2005) and Marker & Dickman (2005) observed that there in fact does exist a positive correlation between prey availability and leopard density, so this parameter seems to have varying effects on leopard densities in different habitat types and areas. In fact, as Pitman et al. (2013) pointed out, numerous ecological, spatio-temporal and human created factors combine in various degrees of importance to influence the behaviour of leopards.

To successfully maintain body mass, adult leopards generally require 1.6 – 4.9 kg of meat daily (Bothma & le Riche 1986, Bailey 1993, Stander et al. 1997) and therefore they need to successfully kill between 40 – 60 medium sized prey items per year, varying between different geographical areas (Schaller 1972, Le Roux & Skinner 1989, Bailey 1993). Prey is caught with the front paws and killed with a precise, asphyxiating bite to the throat (Hes 1991, Bailey 1993, Mills & Hes 1997). Leopards are known to carefully pluck the hair and feathers from their prey before feeding and they seem to dislike having hair and feathers stuck to their mouth (Skinner & Chimimba 2005). Nevertheless, it is common to find feather and fur remnants in leopard scat (see for example Norton et al. 1986, Rödel 2004, Martins et al. 2011). These cats usually disembowel prey and subsequently start feeding at the soft underbelly between the hind legs. Entrails and scraps will be buried beneath soil, grass and leaves after feeding (Hes 1991, Bailey 1993, Mills & Hes 1997, Hancock 2000). Leopards will also readily take to scavenging if the opportunity presents itself (see (Bailey 1993, Mills & Hes 1997, Van As 2012) and some individuals may become habitual in preferring a single type of prey (Hes 1991, Bailey 1993, Bothma & Walker 1999, Rödel 2004).

1.3.5 Big cat health

Carnivores are well-known to be susceptible to a variety of diseases, with infectious pathogens reported from threatened species such as African Wild dogs *Lycaon pictus* (Temminck, 1820) (Nunn et al. 2003). However, little is known about the frequency in contracting diseases (Nunn et al. 2003). In the early 2000s, Funk et al. (2001), Cleaveland et al. (2002) and Laurenson et al. (2004) illustrated that there exists a lack of epidemiological data concerning carnivore populations, therefore the development of appropriate disease management strategies have not taken place (Winterbach et al. 2013). Nunn et al. (2003)'s comparative study of leukocyte counts and disease risk in carnivores showed significant correlations between elevated leukocyte counts and various ecological and behavioural characteristics, indicating that the basal levels of blood cell counts is closely linked to disease risk.

The two African big cat species, lions and leopards, are highly susceptible to diseases such as canine distemper virus (CDV), feline immunodeficiency virus (FIV), feline parvovirus, feline

coronavirus and feline calicivirus (Barr et al. 1989, Roelke et al. 2009). Big cats (lions, leopards, pumas *Puma concolor* (Linnaeus, 1771)) have also been reported to have thyroid carcinomas (Lombard & Witte 1959, Sagartz et al. 1972, Malmlov et al. 2014). In 2011, the major histocompatibility complex, a crucial component of the immune system and host resistance to pathogens (Kumanovics et al. 2003), has been characterized for free-ranging African leopards in central Namibia by Castro-Prieto et al. (2011). Castro-Prieto et al. (2011)'s study illustrated the relationship between parasite load and the composition of the major histocompatibility complex, which is lacking for the African leopard. Diseases such as hepatozoonosis may also prove detrimental to younger animals, as they are more susceptible due to their still-developing immune systems (Ivanov & Tsachev 2008). Big cats are also susceptible to human viruses, which became apparent amidst the global Covid19 (Sars-CoV2) pandemic. Captive leopards, lions and tigers from ex-situ facilities in India, the United States of America (USA) and South Africa, picked up Sars-CoV2 from humans (Chandrababu & Dey 2021, Behrens 2022, Mendes et al. 2022, Chutel 2022). Some of these cats developed pneumonia, which was mortal in a few cases, illustrating the susceptibility of big cats to viruses and bringing the necessity for integrated health research even more to the front. African felids can be severely affected when there is an exceptionally bad confluence of unfavourable circumstances e.g. when extreme conditions such as droughts and haemoparasite infections coincide within a population. This has been clearly shown by Dybas (2009), when such a 'perfect storm' of severe drought followed by heavy rains and a subsequent severe outbreak of haemoparasites (in this case a *Babesia* sp. Phylum Apicomplexa: Order Piroplasmida: Family Babesiidae) due to a mass bloom of tick-vectors, led to the mortality of one-third of the Serengeti lion population.

Unfortunately, a seamless method to assess animal health does not exist. It is rather the amalgamation of several factors concerning organisms that can provide the most accurate diagnostic image (Todgham & Stillman 2013). To develop effective conservation management strategies, studies on how vertebrates respond to man-made and natural stressors have become vitally important (Todgham & Stillman 2013, Maceda-Veiga et al. 2015, Jorgensen 2016) and something like infectious diseases, that may irrevocably affect wildlife biodiversity, has only recently become concentrated upon by wildlife conservationists (e.g. Thorne & Williams 1988, Macdonald 1993). According to Pavlova et al. (2015), animal health assessment by means of physiological parameters should be an essential emphasis for the development of focused conservation management plans and the challenge to conservation biologists would be to detect disease before it escalates into epidemics (Murray et al. 1999). Although health monitoring of wildlife has increased in popularity in the last decade, baseline data on the parameters of health diagnostics is currently lacking in many wildlife species, including African leopards. This gave rise to the 'One Health Intiative', which promotes cross-disciplinary research to address health issues affecting wildlife, the environment and humans, recognizing that these sectors are 'inextricably linked' in a way that needs to be understood (http://www.onehealthinitiative.com).

1.4. Leopards and humans

1.4.1 Conflict

Large carnivores are especially prone to conflict with humans due to their dietary requirements and the need for large home ranges, which increasingly puts them in contact with humans (Butler 2000, Linnell et al. 2001, Macdonald & Sillero-Zubiri 2002, Graham et al. 2005). The main contributing factors to the global shrinkage of leopard distribution are overhunting (Packer et al. 2011), persecution by humans (Balme et al. 2010b), diminishing prey populations (Henschel et al. 2011) and loss of suitable habitats (Ray et al. 2005). Researchers such as Guy Balme and Luke Hunter, agree that the leopard is the world's most persecuted big cat (Martins et al. 2005). However, the presence of a variety of predators in an area is typically indicative of a balanced system resulting from wise land-use management (Hodkinson et al. 2007) and the presence of these carnivores should therefore rather be viewed as a desired characteristic of environmentally sustainable livestock farms.

Important aspects considered when addressing human-carnivore conflict does not only include studies on the ecological patterns and drivers of livestock predation (Stander 1997, Treves & Naughton-Treves 1999, Chauhan & Goyal 2000, Marker et al. 2003, Treves et al. 2004, Lindsey et al. 2007a), but also address efforts to understand the driving forces and attitudes of the people who are expected by conservationists to share their land with carnivores (Butler 2000, Zimmermann et al. 2005, Holmern et al. 2007, Rahalkar 2008, Selebatso et al. 2008, Kissui 2008, Dar et al. 2009, Hemson et al. 2009). In some extreme cases of conflict, some individual leopards may become habitual man-eaters (Chauhan & Goyal 2000, Santiapillai & Jayewardene 2004, Ray et al. 2005). Sharma (2004) reported that Mumbai, India, was experiencing at that time an influx of leopards from the adjacent Sanjay Gandhi National Park, which are straying out of their natural habitat due to habitat loss and shortage of prey items in the park. Five years later, Nabi et al. (2009) also reported an increase of leopard attacks on humans in Kashmir, India.

Predation on livestock is the main cause of conflict between humans and leopards (Patterson et al. 2004), usually resulting in the removal of the so-called 'problem' leopard or the killing of perceived problem individuals (Mishra 1982, Rahalkar 2008, Kissui 2008). Livestock farming areas surrounding protected areas may potentially become high-conflict areas between leopards and humans. Polisar et al. (2003) and Sangay & Vernes (2008) suggested that certain regions could be regarded as natural predation hotspots where livestock losses are considerably larger than elsewhere. This tendency of predation hotspots, where leopards are prominent livestock predators, has also been reported by Kolowski & Holekamp (2006) from pastoral villages adjacent to the Masai Mara National Reserve, Kenya (Kala & Kothari 2013), from the Binsar Wildlife Sanctuary, India and from the edges of the Serengeti National Park, Tanzania (Holmern et al. 2007). In response to a similar conflict situation, Balme et al. (2010b) assessed the impact of persecution in non-protected areas surrounding the Phinda-Mkhuze

conservation area (KwaZulu-Natal, South Africa) on the protected leopard population. Balme et al. (2010b) found that leopards from protected areas do not necessarily avoid surrounding non-protected areas, rendering the protected population vulnerable to the same pressures as leopards occurring outside of protected areas. The following year, Henschel et al. (2011) reported that direct resource competition between leopards and human bushmeat hunters in the rainforests of central Gabon is driving out the big cat from high-conflict areas.

Leopards have the ability to persist in human-modified habitats where other large felids cannot (Norton et al. 1986, Ray et al. 2005, Athreya & Belsare 2007). Livestock predation by leopards in South Africa is limited, restricted and highly localized to certain well-defined areas (Hodkinson et al. 2007). A study by Swanepoel (2008) in the Waterberg (South Africa) included a survey on farmers' attitudes towards these big cats and showed quite a variation from positive to negative attitudes, which were mainly economically driven either way. Leopards are normally prone to taking small to medium sized livestock such as calves (Grimbeek 1992, Sangay & Vernes 2008), but Kissui (2008) found leopards to prey mostly upon smaller livestock and domestic dogs *Canis familiaris* Linnaeus, 1758 in the Masai steppe, Tanzania. In Maharashtra, India, leopard-human conflict escalated to such a level that Athreya & Belsare (2007) proposed conflict management guidelines for the local communities.

Due to their predation on natural prey as well as livestock, both of high commercial value, leopards are directly persecuted in many regions of South Africa (Martins et al. 2005). Translocation of so-called 'problem' leopards has become a popular means of solving this conflict worldwide (Athreya et al. 2011), and is also a popular approach in South Africa (Mr. G. Camacho, personal observation 2014). However, since the 1980s this practice has proven to be an unsuccessful means of limiting conflict between leopards and humans – instead Rabinowitz (1986) and Athreya et al. (2011) found that translocation can potentially exacerbate human-leopard conflict. Almost 40 years ago, Rabinowitz (1986) suggested that the removal of a so-called 'problem leopard' only serves as a short-term solution to this conflict, since territorial vacancies left open by this practice are likely to quickly fill with other leopards that may potentially also take to livestock depredation in future. Twenty years following the suggestions of Rabinowitz (1986), Athreya (2006) also concluded that relocation is not a lasting solution and only transfers the conflict to the site of release, in the process compromising the welfare of the translocated leopard. In South Africa, Balme et al. (2009) found that territorial males that were removed could be replaced by other nomadic males within a very short period of three months. Another problem posed by translocation is the reintroduction of translocated leopards into suitable areas, as this places a lot of undue stress on established resident populations as well as the translocated animal. In South Africa, translocation of problem animals into the Kruger National Park, a popular practice in previous years, have been prohibited about a decade ago (personal communication Mr. B. Jones 2013 and Mr. G. Camacho 2022).

1.4.2 Positive interactions

The African leopard is ecologically, culturally and economically important. All leopard subspecies are the apex predators throughout large parts of their distribution, with their presence in an environment extremely important with regards to regulation of the population dynamics of prey species. They are also important in regulating the numbers of other predators in the ecological system by means of inter-predator competition (see for example Van As 2012). Di Minin & Moilanen (2013) stated that conservation planning is often dependent on using surrogate species to represent several aspects of biodiversity. African leopards, as part of the charismatic 'Big Five' mammals, can prove to become a highly important conservation-surrogate species, focusing conservation attempts on the biodiversity in the leopard's immediate vicinity. The leopard is a revered creature in the myths and legends of the native people of Africa. The common name for this cat, as well as the traditional and ritualistic values of leopards, vary from region to region within the continent (see Feely 2012). For example, people in Tanzania do not utilize leopards for their rituals (Packer et al. 2011), while tribes in southern Africa over utilize the local leopard populations for traditional purposes.

In terms of economical value, leopards are valuable in two ways: as a hunting trophy or as a tourist attraction. Mossaz et al. (2015) reported that ecotourism and trophy hunting are equally preferred conservation tools in Africa. With the benefits of a hunting-based conservation model widely recognized, there are more than 6 000 exclusive game ranches in South Africa (Flack et al. 2011). By approaching wildlife conservation in this manner, South African wildlife was assigned economic value, which protects it from over-exploitation. By the 1970s the South African game farm industry showed signs of remarkable growth, with professional recreational hunting operations rendering game a more valuable commodity than livestock (Flack et al. 2011).

Male leopards are said the be one of the most desired trophies in Africa (Spong et al. 2000, Lindsey et al. 2007b) and with the highly profitable sport hunting industry growing in Africa and South Africa (Child 2000), conserving and carefully managing leopards can become quite profitable to this sector of the South African economy. South Africa already has an annual income of $65.5 – 137 million from its hunting industry (Damm 2005), and with modern hunters showing increasing interest in practicing this sport only in areas where conservation objectives are met, may greatly assist in the advancement of conservation efforts (Lindsey et al. 2007b). It is relatively easy to hunt leopards in South Africa provided that the correct permits are obtained. To hunt one animal, the monetary value for 2015 was up to $15 000 (African Hunting Safaris 2014). This price tag has increased since and reached around $17 500 daily for a 14 day hunting trip, with an additional $4 800 trophy fee and $2 000 bait fee (Martins 2022). Including accommodation and other additional costs for both trophy hunting and tourism, leopards bring in a substantial amount of international revenue and aid in local job creation every year.

Regulated trophy hunting can create significant regional income and a male leopard specimen is considered the most desired carnivore trophy in Africa. This growing demand by consumers caused CITES to increase leopard-hunting quotas in South Africa in 2008. With this, CITES stipulated that 150 leopards per year may be hunted in this country, opposed to the previous number of 75 (Balme 2009). Yet, accurately sexing leopards during hunts usually prove very challenging and can inadvertently contribute to high female mortality in trophy hunting areas (Spong et al. 2000). Moreover, Spong et al. (2000) suggested that an induced scarcity of males in a population (due to trophy hunting) could mean that an already declining population will additionally forfeit genetic variation. The leopard-hunting quota has changed yearly since 2008 and will be discussed in further detail below.

Leopards are also highly popular photographic subjects for tourists, making these cats important economic contributors to South Africa as one of its Big Five species. Nemangaya (2002) stated that tourists are willing to spend "large amounts of money" to experience a leopard encounter, which in turn funds conservation and plays an important role in creating jobs in South Africa.

1.5. Leopard conservation

1.5.1 Global conservation

Human pressure, land transformation, wildlife economics and veterinary controls are fast becoming key factors in the distribution of large predators (du Toit 1995). Global threats to the leopard population include habitat alteration and loss, unregulated trophy hunting, persecution due to conflict and the fur trade (Uphyrkina & O'Brien 2003, Daly et al. 2005, Martins et al. 2005). According to du Toit (1995), large carnivores are among the first animals to disappear outside protected areas, mis-information and mythical beliefs having a hampering effect on successful leopard conservation (Farhadinia et al. 2011). According to Stander (1998), reliable population estimates are key to all conservation and management projects. In order to formulate successful conservation strategies, the need to involve local communities in studying leopard ecology and developing leopards as an ecotourism product has become important (Stander et al. 1997).

In response to the concerns of the effect of the fur trade on the viability of leopards, the IUCN and World Wildlife Fund for Nature (WWF) commissioned Myers (1976) to assess leopard population status south of the Sahara. During his data-collection phase, Myers (1976) realized the impossibility of obtaining accurate counts for the total number of leopards in an area, and as a result he had to rely on the subjective estimates of his correspondents. Myers (1976) discovered that leopard numbers are likely to follow a continual declining pattern in areas where the use of poison is widely acceptable, but he still predicted that leopards would always manage to subsist, even in the absence of protection. Reports published by the US Fish and

Wildlife service estimated the sub-Saharan leopard population to be in the range of 230 000 to over one million individuals (Carter 1980). Carter (1980) reported that 9 000 leopard skins were imported annually to the USA alone during the late 1960s, but the overruling perception was that regulated sports hunting can provide the surrounding communities with economic incentive to protect leopards.

The global leopard population is increasingly exposed to intensifying pressures due to habitat loss. A considerable area of the world's remaining leopard habitat befalls in countries with significant environmental, economic and social problems (Bailey 1993). Wildlife conservation programs in Africa, being home to many poor people, face additional pressures from expanding zones of overgrazing and desertification (Bailey 1993). In 1990, Harmon (as cited in Bailey 1993) estimated that an average of 50% of available leopard habitat in Africa has been lost, with the greatest losses in savannah grassland and forest habitats, which are known to be prime leopard habitat. Bailey (1993) suggested that the only remaining secure leopard habitat could be found on the Kalahari Plateau and in the wet region of the Zaire River Basin. The African leopard population continues to diminish outside protected areas in East and southern Africa. According to the estimates of Ray et al. (2005), leopards have disappeared from approximately 37% of their historical range in Africa, showing South Africa to be one of the regions with the most profound losses.

Since African leopards are Africa's most widespread big cat and inhabit the widest range of habitats, there is an inclination to believe that they are abundant creatures in southern Africa (Daly et al. 2005). It has been recognized that estimates of South African leopard numbers are mostly erroneous, highlighting the need to undertake detailed leopard research as well as prioritizing the species in terms of conservation focus, making it a matter of importance (Martins et al. 2005). In 2005 it became evident that the population may be much smaller and more patchy than formerly predicted, rendering informed decisions regarding applicable conservation strategies difficult (Martins et al. 2005). The IUCN assessment of the *Panthera pardus* by Henschel et al. (2008) moved leopards from Least Concern to Near Threatened. In 2016, Stein et al. (2016) reclassified *P. pardus* as Vulnerable, meeting the A2cd criterion (IUCN Redlist accessed 07/04/2022) (Table 1.4). Their basis for this reclassification included the exploitation of leopards, loss of habitat and prey and a declining population, the causes of which currently poorly understood and continuing. Stein et al. (2016) anticipates a future decline in global leopard populations unless effective conservation measures are implemented soon. During their 2019 reassessment, Stein et al. (2020) kept the classification of *P. pardus* as Vulnerable, Vulnerable A2cd ver 3.1. (IUCN Redlist accessed 07/04/2022). Leopards, a currently listed Appendix I species by the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), is a species that is currently threatened with extinction (CITES checklist of CITES species accessed 10/04/2022).

1.5.2 South African conservation

Specific threats to leopards in South Africa

Worldwide, leopards face the same conservation pressures as cheetahs, lions and tigers due to similar threats. Conversely, unlike the other big cats, the leopard is a comparatively overlooked species when viewed from a conservation perspective. The key threats to African leopards in South Africa and the rest of Africa can be divided into four main categories: habitat loss and fragmentation (Nowell & Jackson 1996), lack of prey (Datta et al. 2008, Qi et al. 2015), poaching and the illegal trade in leopard parts (Oswell 2010, Raza et al. 2012), and mismanagement of trophy hunting and captive breeding (Packer et al. 2011, Harvey 2020). Land across much of the leopard's range has been changed for agricultural purposes, resulting in habitat loss and fragmentation which is a key contributor to the decline in leopard populations (Nowell & Jackson 1996). Habitat is also lost to prey species, lowering their populations and making it more difficult for leopards to survive in a given area. It is very possible that, in the upcoming decades, this threat will become increasingly important for leopards in Africa, where it is expected that economies will grow, human populations will increase and land use patterns will change (Ahlers et al. 2014). In Africa's rainforests, competition between humans and leopards for bush meat culminates in localized extinctions, of both leopard and prey species (Henschel et al. 2011). Expanding anthropomorphic activities in Africa intensifies this situation, and Jacobson et al. (2016) suggested this to be a driving force of diminishing ranges across Africa's savannahs, the Sahara desert and the Middle East.

Table 1.4 IUCN evaluations of leopards from 1986 to 2020 (from Stein et al. 2020).

Year of assessment	Conservation status
1986	Vulnerable
1988	Threatened
1990	Threatened
1996	Least Concern
2002	Least Concern
2008	Near Threatened
2016	Vulnerable
2019	Vulnerable

Lack of prey and habitat loss does not seem to affect leopards in South Africa as much as their counterparts in other areas of the world. Lindsey et al. (2009) attributed this to the growth of commercial game ranching in South Africa, which led to a subsequent increase of potential prey and thus allowed leopards to successfully persist in areas outside formally protected areas. On the other hand, this expansion of game-ranching areas also led to an increase in direct competition for wild ungulates between humans and leopards (Lindsey et al. 2009). Leopards are also often killed in livestock conflict situations throughout their South African range (Balme et al. 2009).

Leopards are illegally traded on a global market (Ray et al. 2005) and body parts are highly valued in central and west Africa for use in traditional rituals (Henschel & Ray 2003). This continues to be a major threat throughout large parts of their range, globally and in Africa (Myers 1976, Hamilton 1981, Ray et al. 2005), with a number of markets that have leopards as the most regularly trafficked big cat (Oswell 2010). Four Paws (2022) identified illegal hunting of captive leopards to be a problem in the Western Cape. African locals in public tourist

marketplaces sell vast amounts of African leopard parts on an annual basis (Künzel et al. 2000, Shipp 2002) and this illegal trade and poaching of leopards throughout Africa pass mostly unnoticed by authorities (Balme 2009). Illicit trade of African leopard parts in southern Africa is a rarer occurrence than in the rest of the continent, but nevertheless takes place. The illegal smuggling of leopard skins for cultural regalia poses a threat to populations in South Africa (Hunter et al. 2013), especially in KwaZulu-Natal, where 58 leopard skins destined for export to international markets, were seized in 2004 (Pitman 2012, Hunter et al. 2013). It is the accepted custom for South Africa's Zulu royalty to wear animal skins, particularly leopard pelts, while taking part in traditional ceremonies (Associated Foreign Press 2016). According to Guy Balme, South Africa also have immense religious groups that use leopard pelts as symbols of status, and one such church is thought to possess more than 15 000 skins (as cited by Visser 2016).

Unsustainable trophy hunting is usually limited to countries where it is legal to hunt leopards, such as South Africa. The source of this unsustainability in these countries is two-fold: on one hand hunting regulations are not sufficiently enforced and on the other, the current legal levels of off-take are not set at a sustainable level (Balme et al. 2010b). Balme et al. (2010a) and Lindsey et al. (2011) agree that the impacts of leopard trophy hunting have not been comprehensively researched, despite its popularity, and Balme et al. (2010a) additionally argue that none of the countries where this activity is legal possesses detailed information on the population status of leopards. Balme et al. (2010a) and Lindsey et al. (2011) have a valid point, as ample evidence exists that trophy hunting can adversely affect leopard populations, especially by disrupting social and spatial dynamics (see for example the reports by Balme et al. 2009, Packer et al. 2011). Furthermore, distinguishing between trophy hunting impacts and the number of so-called 'problem animals' killed every year can prove highly problematic and therefore it is probable that a larger number of leopards are killed than the allocated quota (Balme et al. 2010a). Due to these published recommendations, and following the IUCN's reclassification from Near Threatened to Vulnerable, South Africa's Department of Environmental Affairs imposed a total ban on hunting leopards in 2016, a decision that was extended to 2017 (Associated Foreign Press 2016, IUCN Species surivial commission (SSC) Cat specialist group 2017). Even though the 2015 IUCN reclassification indicated a decline in the leopard population, the hunting ban was lifted again in 2018 (Avery 2018), just to be reinstated in 2019 for one year (Pinnock 2022). It was announced in 2022 that, despite an ongoing population decline, South Africa has issued a quota of 10 leopards for the trophy hunting industry, rolling forward its 2021 quota (Pinnock 2022). Conservationists found this decision puzzling, as a great and mostly unknown number of leopards are killed every year due to conflict with humans or as a means to supply highly sought-after skins to traditional churches (Swanepoel et al. 2015, Pinnock 2022).

Quotas and trades

The online CITES trade database showed South African leopard exports from 2013 (the start of the current study) to 2022 mostly include trophies (n=665), skulls (n=174), skins (n=26), bodies (n=18), specimens (n=3), one garment and a number of bones, claws, teeth and so-called

derivatives (CITES trade database, https://trade.cites.org/, accessed 7 April 2022) (CITES 2022). Notably, a number of trophies, skulls, skins, bodies and rugs were exported all over the world during 2016, the year when leopard hunting was banned in South Africa. The majority of lion bones traded from South Africa are exported to countries in southeast Asia (Williams et al. 2017). Unlike large commercial trade in lion and tiger parts with Asian countries (Williams et al. 2017, Williams & Sas-Rolfes 2019, Four Paws 2022), the largest number of trade exports of leopards and associated parts went to the United States of America (n=71), followed by Russia (n=29) (CITES trade database, https://trade.cites.org/, accessed 7 April 2022) (CITES 2022). Additionally, live leopards were also exported from South Africa to several countries all over the world, as can be seen in Table 1.5. Worryingly, the number of live leopards exported does not always correspond with the number of leopards imported (Table 1.5), which may indicate either poor record keeping or the loss of live animals in transit. One extreme example is a trade record of live leopards in 2018 between South Africa as exporter and China as importer. For this record, South Africa reported the export of 12 leopards, while China reported the import of 24 leopards. The reason for these discrepancies is unclear (https://trade.cites.org/, accessed 7 April 2022) (CITES 2022).

National conservation

South Africa's wildlife, globally considered of diverse and high quality, is today acknowledged as one of this country's greatest natural resources (Flack et al. 2011). This has always been the case, however, South African wildlife has not always been utilized in a sustainable manner and experienced the greatest depletion ever witnessed on the African continent between the years 1800 and 1950. During this time the elimination of indigenous wildlife was extensive, especially in the case of so-called vermin species such as leopards, which needed to be eradicated to make way for cattle and other livestock (Flack et al. 2011).

In the early years of Sabie Game Reserve, one of the oldest conserved areas in South Africa, James Stevenson-Hamilton (1906) noted that larger antelopes have all but been eradicated from the area, and he attributed this to the presence of too many carnivores (as cited by Joubert 2007). As the conservation focus in those early years was on large antelopes, carnivores were "almost seen as a threat to the conservation effort" since they "slowed the growth of the prey populations" (Joubert 2007). As a consequence, large predators such as leopards were shot on first sight for the most part of the first half of the 20th century (Joubert 2007).

In the 1986 and 2004 publications of the National Red Data Book of Mammals of South Africa (Smithers 1986; Daly & Friedmann 2004), it was reported that leopard populations were expanding in the Western Cape and overall populations were stable in protected areas. As a result, the leopard was classified as of Least Concern during both publications (Friedmann & Daly 2004) (Table 1.4). According to Daly et al. (2005) the "fundamental to the effective management and conservation of any species is a reliable estimation of population size, distribution and trends". The lack of information on this species has, however, not prevented

frequent decision-making regarding their management, and since 2004 an increase in CITES quotas for leopards as hunted trophy animals has taken effect in Namibia and South Africa (Martins et al. 2005). Friedmann & Traylor-Holzer (2008) reported that, until 2005, there were no national plans for leopard conservation in South Africa in existence. The Leopard Population and Habitat Viability Assessment rectified this by drawing up a current, relevant national leopard conservation management strategy and established the South African Leopard Forum (Daly et al. 2005).

Recent increases in game ranching practices in South Africa may have allowed leopards to inhabit larger areas, but it also simultaneously created more frequent conflict opportunities between game ranchers and leopards (Lindsey et al. 2009). To erect fences around conservation areas is common practice in South Africa and Hayward et al. (2009) investigated the constraint effect of fences on large predators in Addo Elephant National Park and concluded that it has not affected the natural behaviour of the predators. Nevertheless, even though it may seem that more habitat has become available to leopards, Daly et al. (2005) and Swanepoel et al. (2013) found that suitable leopard habitat in South Africa was severely fragmented by land-use activities unsuited to leopard inhabitation, with the majority of potential habitat occurring outside protected areas. Daly et al. (2005) suspected that this consequentially disturbs leopard movement patterns and the authors recommended that leopard conservation practices should focus much more on non-protected areas.

Table 1.5 International trade of live leopards in South Africa from 2013 to 2022. (CITES trade database (https://trade.cites.org/) accessed 7 April 2022) (CITES 2022).

Country of import	Number of leopards exported from South Africa	Number of leopards imported	Origin of leopards	Reason for export
Egypt	3	No data	Captive bred	Zoo
Gabon	2	No data	Captive bred	Zoo
Canada	18	12 (No data for some records)	Captive bred Wild	Zoo Hunting
Pakistan	4	2 (No data for some records)	Captive bred Wild	Zoo
Bahrain	1	Not stated	Captive bred	Breeding in captivity
Cote d'Ivoire	1	1	Captive bred	Zoo
Chile	3	1	Captive bred	Zoo
Philippines	8	4 (No data for some records)	Captive bred Wild	Zoo
Vietnam	12	9 (No data for some records)	Captive bred	Zoo
China	16	24 (No data for some records)	Captive bred	Zoo
Italy	2	1 (No data for some records)	Captive bred	Zoo
Mozambique	5	No data	Wild	Hunting

The model provided by Daly et al. (2005) predicted that the leopard metapopulation in South Africa is likely to persist over the next 100 years, but also showed a small risk of extinction (< 5%) for the Orange River, Northern Limpopo, Waterberg/Mpumalanga, Wild Coast, Eastern Cape valley and Eastern Cape Mountain populations (see Fig. 1.3). Therefore, due to largely uncollaborative research on leopards (see Martins et al. 2005), sound management and conservation decisions regarding their fate currently seems far from possible. Captive populations may also become more important in terms of conserving the species. Eleven years ago, Uphyrkina & O'Brien (2003) already suggested the use of captive Amur leopards *Panthera pardus orientalis* (Schlegel, 1857) to diversify the genetic characteristics of their wild populations. The practice of rehabilitating and releasing orphaned felids is also relatively common, and the study by Houser et al. (2011) illustrated great success with the proper pre-release preparation of these animals. However, Houser et al. (2011) emphasized the lack of attention given to monitoring rehabilitated felids after they have been released, suggesting that more attention to these individuals is needed.

Ex-situ conservation

Non-domestic carnivore species have been and still are kept in captivity for purposes such as education, recreation, research and captive breeding programmes (Williams & Thorne 1996). Common misconceptions up until the late 1990s, as identified by Law et al. (1997) included the notion that captive large felids do not contribute to the conservation of big cats in the wild. However, many large felids, including Indian leopards *Panthera pardus fusca* (Singh 1982), have been born and raised in captivity and has been successfully reintroduced into the wild (Adamson 1966, Singh 1982, Caro 1994). A well-known account of this process involving an African leopard *Panthera pardus pardus* female, was recorded by Joy Adamson who wrote about an orphaned leopard cub she raised and released into a wildlife reserve upon maturity (Adamson 1980). Another common misconception is that prepared diets provide felids with all the required nutrients (Law et al. 1997), but these types of diets have been associated with dental health problems (Bond & Lindburg 1990) and are usually not nutritionally effective (Williams 1993). Keepers believed that cats are inherently lazy and that it is close to impossible to stimulate hunting and associated feeding behaviour in a captive environment (Law et al. 1997).

A captive breeding ex-situ facility is defined in the Threatened or Protected Species (TOPS) regulations of South Africa as "one where listed threatened or protected" fauna are bred in a "controlled environment for conservation or commercial purposes" (Four Paws 2022). Harvey (2020) did a cost-benefit analysis of the captive predator breeding industry in South Africa. This publication estimated that there are more than 300 captive predator breeding facilities in South Africa. Harvey (2020) concluded that the market for human interactions with big cats may generate approximately $180 million annually in South Africa. Justification of captive breeding programs often stems from the idea that these programs support wildlife conservation (Williams & Sas-Rolfes 2019, Li 2021). Some parties motivate that these breeding

programs serve to meet consumer demands while lifting pressure from natural wild populations (Williams & Sas-Rolfes 2019, Li 2021), but, contrarily, Harvey (2020) concluded that there is no sound scientific evidence to support conservation value of captive breeding programs, especially not from those that focus on human-big cat interactions.

As seen previously in this chapter, a big motivational factor for captive breeding programs is their economical contribution towards conservation. Mossaz et al. (2015) researched the extent of ecotourism contributions towards big cat conservation in Africa and found that some tourism enterprises successfully contribute in this regard, while others do not. They concluded that the extent of successful conservation contribution depends on the specifics of each conservation programme, its marketing and community involvement, and that commercial ecotourism may create successful funding for big cat conservation in Africa.

Preceding the onset of the environmental enrichment concept (Markowitz 1975), the majority of captive environments were structurally simple and did not take the behaviour and well-being of its inhabitants into consideration. Captive environments lacked the provision of interactive opportunities for animals with their surroundings, which discouraged the display of species-typical behaviours. However, modern animal welfare movements have had a substantial role in changing the nature of these artificial environments. Since the late 1970s, efforts have been undertaken worldwide to modify and adapt the complexity, configuration and interactivity of captive environments to the behaviour and needs of its inhabitants (e.g. Markowitz & Spinelli 1986, Shepherdson et al. 1998, Baumans 2005). These efforts mainly included the enhancement of several abiotic factors such as introducing components of natural elements to improve ecological relevance to captive animals (Hutchins et al. 1984). Captive animals usually have no control over several key aspects of their lives, such as hiding from potential threats, choosing breeding and social partners, what kind of food they eat, when they feed and how they raise their young. The absolute predictability of an ex-situ environment, in several aspects, also proves detrimental to most animals, which is far removed from the variability and unpredictability offered by natural environments (Sambrook & Buchanan-Smith 1997).

The main recognized stressor to captive animals is the interference of their innate behavioural processes (Morgan & Tromborg 2007), which seems to be conducive to an animal's well-being (Friend 1989). Research on the management of animals in captivity during the last few decades, showed that enriched captive environments can preserve these innate specific behaviours (Rabin 2003) and current global enrichment programs for carnivores are often focused on stimulate these behaviours (McPhee 2002). One of the chief stressors to captive animals is the constraint of movements due to inadequate sizes of enclosures (Hediger 1955, 1964). This could lead to a lack of exercise, which in turn can result in overweight and obese captive animals. A large proportion of an animal's time is spent searching for and consuming food, referred to as hunting success in carnivores (Hayward et al. 2006). However, in a captive situation, food is regularly provided, leaving the animals no choice in diet and no energy spent to obtain food. The type of food offered is usually also considerably different from, and more artificial than the animal's natural diet. Ex-situ diets may differ from natural diets in having

higher protein contents and highly concentrated nutrients, lower fibre content, artificial textures, and food is usually consumed much faster than natural food (Morgan & Tromborg 2007). Predictability of always having food at certain scheduled times can therefore prove detrimental to some captive animals (Friend 1999, Vickery & Mason 2005). Therefore, various modern enrichment activities include enticing captive animals to work for their food (see for example Morgan & Tromborg 2007).

Large carnivores have comparatively high metabolic needs and subsequent large energetic constraints, and therefore require adequate amounts of prey resources (Carbone et al. 1999). These animals are consequently sensitive to small changes in resource levels (Arthur 2014), which often result in weight gain when over fed. One of the main conflicts encountered by ex-situ facilities such as zoos is finding a balance between offering paying visitors a fair chance to see the animals, and keeping the animal's best interests in mind. Unfortunately, forced human-animal interactions often result from a facility's attempt to promote empathy and improve attitudes with regard to conservation. Usually, at facilities with big carnivores, regular feeding times in the presence of tourists, can lead to overfeeding and an overall drop in activity of the animals. This was observed, for example, in zoo-housed Indian leopards (Mallapur & Chellam 2002). Well-managed ex-situ facilities such as wildlife rehabilitation centres are in demand in South Africa (Wimberger et al. 2010), however, due to lack of funding and poorly unregulated management protocols, the current value of these facilities as a conservation effort is questionable (Wimberger et al. 2010).

According to Four Paws (2022), there exists a lack of effective regulation and record-keeping of big cat husbandry in South Africa. Transparency in terms of big cat conservation management seems to be an issue among ex-situ facilities in South Africa. Hundreds of private facilities in South Africa are intensively breeding lions, leopards and tigers for commercial trade, and the commercial global trade in leopard parts is quite extensive (CITES 2022, Four Paws 2022). A recent report by Four Paws (2022) stated that countries such as Thailand, Vietnam and China serve as hotspots for big cat farming and illegal trade. South Africa breeds a significant proportion of the big cats traded to these countries. There are currently an unknown number of leopards in captivity in South Africa, as illustrated by the difficulty Four Paws (2022) had to obtain information from provincial authorities in this regard. Their report was only able to report captive leopard numbers from the Western Cape (n=50), Gauteng (n=6) and Limpopo (n=31), and even this data is questionable and unrepresentative.

Little attention is given to the current status of captive leopards, globally and also in South Africa. Even detailed reports, such as the recent report by Four Paws (2022), focus on tigers or lions, and only briefly mention leopards. As Four Paws (2022) report has shown, no official statistics exist to confirm how many captive leopards there currently are in South Africa. Ex-situ facilities are also very selective in sharing their information and tend to be uncooperative with conservation researchers (Williams & Sas-Rolfes 2019, Four Paws 2022). Li (2021) reported that South Africa's Ministry of Forestry, Fisheries and the Environment officially recommended putting a stop to the captive breeding of big cats as a means to curb this issue.

1.6 Challenges with leopard research in South Africa

Since the onset of research on leopards, several topics have been extensively covered, but the majority of research is conducted independently and uncollaboratively. Research elucidating their behaviour (Bothma & le Riche 1984, 1995, Bailey 1993, de Ruiter & Berger 2001, Steyn & Funston 2009, Balme et al. 2012, Balme & Hunter 2013) social interactions on an intra- as well as interspecific level (Sunquist 1983, Balme & Hunter 2004, Odden & Wegge 2005, Odden et al. 2010), home range sizes, territoriality, daily activity budgets and circadian rhythms (Bothma & le Riche 1984, Bailey 1993, Bertram 1999, Hunter et al. 2003, Bothma & Coertze 2004, Marker & Dickman 2005, Odden & Wegge 2005, Bothma & Bothma 2006, Stein et al. 2011), morphological variations in different geographical regions (Meijaard 2004), habitat requirements (Monod 1965, Stuart & Stuart 1989, Nowell & Jackson 1996, Sunquist & Sunquist 2002, Simcharoen et al. 2008, Munoz 2013) and hunting strategies have been widely reported and analysed. Perhaps the most studied aspect of leopards is their feeding ecology (Mills & Hes 1997, Ray & Sunquist 2001, Rödel 2004, Schwarz & Fischer 2006, Hayward et al. 2006, Andheria et al. 2007, Balme et al. 2007, Owen-Smith & Mills 2008, Wang & MacDonald 2009, Martins et al. 2011, Fröhlich et al. 2012), with the majority of research focusing on leopards inside formally protected areas. A few studies have addressed captive leopards worldwide, especially from a behavioural and dietary viewpoint (Barbiers et al. 1982, Jakob et al. 1997, Sinha et al. 2000, Upadhye & Dhoot 2002, Karanis et al. 2007, Earle 2008, Sandberg 2012, Kelly et al. 2013).

Many early descriptions of leopards provide little factual information on their ecology, but for two exceptions, which provided more detailed accounts (Corbett 1947, Stevenson-Hamilton 1947). In the late 1960s, Turnbull-Kemp (1967) summarized the existing knowledge on leopards at that point in time in his book 'The Leopard'. Up to the early 1970s, this big cat remained relatively unstudied in the wild, with observations often in the form of a few comments from studies focusing on other species such as lions *Panthera leo* (Turnbull-Kemp 1967, Eisenberg & Lockhart 1972, Schaller 1972, Muckenhirn & Eisenberg 1973, Guggisberg 1975). The first intensive study on wild African leopards was undertaken by Hamilton (1976) in Tsavo National Park, Kenya, where he studied their movements using radio telemetry. Onwards from the 1980s to the present day, numerous researchers studied this cat from a biological perspective, mostly inside conserved areas where encounters are a likelihood (Le Roux 1984, Bothma & le Riche 1984, 1986, 1995, Norton & Lawson 1985, Norton & Henley 1987, Le Roux & Skinner 1989, Hes 1991, Bailey 1993, Mills & Biggs 1993, Miththapala et al. 1996, Chauhan & Goyal 2000, Hancock 2000, Henschel 2001, Henschel & Ray 2003, Hunter et al. 2003, Uphyrkina & O'Brien 2003, Balme & Hunter 2004, Santiapillai & Jayewardene 2004, Bothma 2005, Maheshwari 2006, Schwarz & Fischer 2006, Bothma & Bothma 2006, Hayward et al. 2006, Balme et al. 2009). The African leopard was also studied as a taphonomic agent in dolomitic caves of South Africa (de Ruiter & Berger 2001).

Its elusive nature renders the leopard a difficult subject to study and new avenues of observing leopards indirectly have been explored since the late 1990s. Results from Stander (1998) in the Kaudom Game Reserve and Tsumkwe District, Namibia, indicated that leopard spoor density can be a function of true density. Subsequently, based on their prior leopard research in Gabon and Zaire, Henschel & Ray (2003) wrote a handbook on applicable techniques for monitoring leopards in African rainforests. Six years later, Wang & Macdonald (2009) illustrated the effectiveness of camera traps as a tool for assessing population sizes of leopards, and Khorozyan (2003) and Maputla et al. (2013) showed that camera traps can serve as an unbiased sampling tool if used correctly.

1.6.1 The history of African leopard research

Detailed leopard research in South Africa started about 30 years ago, in the Kruger National Park and the Kalahari Desert (Bothma & le Riche 1984, Bailey 1993). These early studies focused on leopard ecology and behaviour such as hunting efficiency (Bothma & le Riche 1984, Bothma & Coertze 2004). This research quickly spread to the mountain leopards in South Africa's Cape Province, where Norton & Lawson (1985) reported on home range sizes and how they were influenced by prey abundance. During this same time, scientific interest in central African, Sri Lankan and far-east leopards was sparked, with authors such as Hoppedominik (1984), Santiapillai et al. (1982) and Pikunov & Korkishko (1985) reporting on dietary preferences and population status. During the mid 1980s research with regards to genetics (Roychoudhury & Acharjyo 1984), physiology (Abbasi & Braunitzer 1985, Ahmed et al. 1988) and the status of captive leopards in the zoos of the world surfaced (Shoemaker 1985).

By the end of the 1980s an African leopard population model had been published (Martin & de Meulenaer 1988) and detailed research with a behavioural ecology focus has been conducted in the Orange River Basin and private game reserves (Le Roux & Skinner 1989, Stuart & Stuart 1989). It was not long before severe criticism of Martin & de Meulenaer (1988)'s population model has been published (see Norton 1990, Jenny 1996), making this one of the most controversial leopard population models to date. The 1990s proved to be a time where leopard research flourished, with reports on prey preference and feeding ecology throughout its range (Grimbeek 1992, Cavallo 1993, Johnson et al. 1993, Stuart & Stuart 1993), as well as an increase in veterinary research addressing topics such as physiology (Macdonald & Johnstone 1995, Ray et al. 1996, 1997, Stander 1997), parasitology (Patton & Rabinowitz 1994, Pozio et al. 1997), canine distemper (Appel et al. 1994, Harder et al. 1996), genetics (Miththapala et al. 1996) and bacteriology (Thorel et al. 1998).

1.6.2 A geographic and applied challenge

There seems to be disparity between where and on which topics South African leopard research is carried out, and the research and geographic areas that need research focus. This overlap in research topics added detailed knowledge to certain conservation aspects

concerning leopards but simultaneously left large gaps with respect to others. According to Balme et al. (2013), South African leopard research conducted by Non-governmental Organisations (NGOs) and government organizations had a much larger applied theme than studies published by academics. They found this to be the inverse of the leopard research trends in other parts of the world. In addition to the majority of studies only investigating leopards in formally protected areas, most of the studies to date have been conducted in easily accessible forest and savannah ecosystems (e.g. Bertram 1974 in the Serengeti National Park, Balme et al. 2007 and Chapman & Balme 2010 in Phinda Private Game Reserve), the desert areas of southern Africa (e.g. Bothma & le Riche 1986 and Bothma & Bothma 2006 in the Kalahari desert) and equatorial Africa (see for example Jenny & Zuberbühler 2005 and Henschel et al. 2008). Research on leopards in rough, mountainous landscapes is lacking (Martins et al. 2011).

Another challenge identified by Balme et al. (2013) was the tunnel-vision-like research conducted on South African leopards, with studies being very site-specific and mostly conducted inside protected areas, in prime leopard habitat (as classified by Swanepoel et al. 2013). Even though leopard research from South Africa accounts for a large proportion of the available peer-reviewed publications, these publications repetitively focus on ecological matters and rarely address, and seem to avoid concerning areas such as conservation management and cross-disciplinary studies. This is inconsistent with the report by du Toit & Broomhall (2000), who showed that the most common focus of publications concerning South African mammals essentially addresses conservation. Somehow, this conservation focus has been left unaddressed for leopards.

1.6.3 Challenges with non-scientific research projects

The start-up of enduring leopard research projects in different areas of the world (e.g. The Cape Leopard Trust; Mun-Ya-Wana Leopard project; the Panthera Foundation) sparked scientific attention to these cats by the start of the new millennium. Subsequently, the past decade brought on a rise in behavioural (de Ruiter & Berger 2001), conservation (Al-Johany 2007, Balme et al. 2009, Caro & Riggio 2014), genetic (Kawanishi et al. 2010) and evolutionary research (Zuberbühler & Jenny 2002). Human-leopard conflict was also addressed worldwide from a mitigatory and management angle (see Schiess-Meier et al. 2007, Sangay & Vernes 2008, Weilenmann et al. 2010, Athreya et al. 2011, Van As 2012) and the availability of remote-sensor camera traps and GPS tracking systems aided researchers in studying leopards (Balme et al. 2009, Swanepoel et al. 2010, Martins et al. 2011, Van As 2012).

Publically operated, non-scientific leopard projects are quite widespread in South Africa (e.g. www.ingweleopard.com and www.leopardcon.co.za) and Balme et al. (2013) stated that a total of 39 South African leopard projects were running from the onset of the millennium until 2012. Although the majority of local research projects generate some unique insights, the tendency of these projects not to contribute to well-established peer-reviewed publications (Pitman 2012, Balme et al. 2013) (see for example the short note by Pirie et al. 2014) exacerbates the

gaps in knowledge of leopards (Balme et al. 2013) and usually excludes academic endeavours in the areas where these groups operate.

1.6.4 Current gaps in knowledge

A complicated, emerging problem with regards to the viability of leopard populations in South Africa is the uneven distribution of these cats throughout their range. While the largest part of the population occurs outside protected areas (Grey et al. 2013), populations within protected areas do not seem to meet their carrying capacity (Chapman & Balme 2010). Link this with seemingly diminishing genetic variability in the larger population outside protected areas (see reports on increasing erythrism by Spong et al. 2000, Pirie et al. 2014), and the problem becomes very intricate and needs elucidation. It does seem that, for the South African leopard, the concept of 'safety in numbers' may not apply on the long run.

There is a great need for cross-disciplinary, applied research on leopards in South Africa (see Pitman 2012, Balme et al. 2013), both on in-situ and ex-situ populations. For example, little is known about the role played by leopards in the carnivore guild of which they form part. Ray et al. (2005) pointed out that particular gaps exist in knowing how the carnivore guild responds to the exacerbated loss of apex predators such as leopards. Balme et al. (2013) also pointed out that the role of infectious diseases in regulating South African leopard populations need to be investigated if these cats are to be successfully conserved. Add to this the shortcoming of not having exact knowledge on the size of South Africa's leopard population, and the mix of these gaps can become quite a concern in the future management of these cats.

The impression still prevails that leopards are one of the most widespread, resilient carnivores in the world. Nevertheless, the range loss (63 – 75%) calculated by Jacobson et al. (2016) exceeds the average range loss calculated for other large carnivores in the world (see Ripple et al. (2014) by almost 40%. These figures waylay the abovementioned idea and emphasize the need for a better conservation management plan for this species. A total ban on hunting leopards may help to stabilize the South African population, as was the result in Tanzania (see Packer et al. 2011), but research is needed on the duration of this measure for it to be effective. The total lack of geographically comprehensive genetic analyses, especially within Africa, is also of great concern and needs urgent attention from research projects.

1.5. Thesis problem statement

Pitman (2012) reported an increasing trend in leopard research from 1970 to 2010, however, less than 10% of publications address issues such as genetics, heredity and parasitology. According to Pitman (2012), only 2% of available publications address biochemistry and molecular aspects of leopards. Knowledge regarding conservation management and threats of disease to leopards are severely lacking (see Balme et al. 2013). More studies, such as that by

Castro-Prieto et al. (2011) on "the relationship between parasite load and the composition of the major histocompatibility complex, a major component of the mammalian immune system", is needed for the African leopard. Genetics-related studies on South African leopards are also very few compared to similar studies on leopards elsewhere. Since only 32% of suitable leopard habitat in South Africa occurs within the confines of protected areas (Swanepoel et al. 2013), most leopard research is done within protected areas (Balme et al. 2013), therefore a huge gap in knowledge about the leopard in the majority of its distributional range in south Africa has been created.

Currently, no specific guidelines for managing ex-situ (captive) leopard populations exist in South Africa. A successful conservation strategy promotes the awareness of the importance of leopard conservation, which includes an increased emphasis on ex-situ conservation. The assumption in many regions that leopards are 'safe from extinction' may be unwarranted as this species is elusive, solitary and largely nocturnal, making it a very difficult subject on which to collect empirical data, especially outside of protected areas (Hunter et al. 2003). Their conservation status is often assumed and overestimated on the basis that they are able to persist where other large felids cannot (Hunter et al. 2003). Dalerum et al. (2008) studied the potential of using large carnivores to promote biodiversity conservation in Africa and concluded that the global appeal of large carnivores, such as the leopard, render them important flagship species and as such should be regarded as umbrella species. In 2004, the Northern Ireland Environmental Agency produced a guidance booklet on the keeping of leopards, pumas and jaguars in captivity (NIEA 2004). This type of knowledge, from a South African perspective, would be highly useful and should be available to South African ex-situ managers.

Blood parasites have been reported to cause mortality in susceptible large carnivores (see for example East et al. 2008). There usually exists a balance between host and parasite in wild free-ranging populations. Hosts and parasites have evolved together and the presence of parasites in natural conditions is usually of limited clinical significance (Penzhorn & Chaparro 1994). However, stressful conditions such as living in captivity, can disturb the host-parasite balance, resulting in an increasing parasite burden, leading to the development of clinical symptoms in the host (Penzhorn & Chaparro 1994). Therefore the role of molecular genetics in the conservation of endangered taxa, in terms of identifying potential pathogens, is widely recognized (Uphyrkina et al. 2001) and this type of analysis provides important insights on the current health and taxonomic status of endangered species.

The ecological and behavioural characteristics of species play a significant role in potential exposure to contaminants and foraging habits are important determinants of an animal's ecology and biology. Contaminant exposure differs among herbivores, omnivores and carnivores due to the paths followed by contaminants through the food web (Smith et al. 2007). The accumulation of harmful chemicals in terrestrial food chains is far less studied than in aquatic ecosystems, mostly because exposure tend to be less in terrestrial predators than in their aquatic counterparts (Larsson et al. 1990, Mason & Wren 2001). However, various carnivores, such as leopards, are highly opportunistic predators and by eating what is most

readily available, may find themselves the apex predators of an aquatic food chain (Mason & Wren 2001). Ecotoxicology is a multidisciplinary field integrating ecology and toxicology by studying the effects of toxic chemicals on biological organisms. It integrates the effects of stressors across all levels of biological organisation, from molecular level to whole ecosystems. The ultimate goal of this type of study is to have the ability to predict effects of pollution in order to identify most efficient/effective action to prevent/remediate detrimental effects.

There is a complete gap in available information on African leopard health, both ex-situ and in-situ leopards. Parameters to monitor the health of wild individual animals, with the option of resampling, are severely lacking. Studies on captive African leopards are less common than that of wild leopards and are usually limited to a few captive individuals at a time. It is therefore evident that an increasing need for applied knowledge and multi-disciplinary approaches to South African leopard research exists.

Thus, the following challenges have been identified concerning current knowledge of leopards in South Africa and hence their conservation management:

1. No ecotoxicological data for South African leopards exist, and genetic information on South African leopards is scanty and limited.
2. The majority of leopard research in South Africa has focused on free-ranging leopards inside formally protected areas (in-situ populations), where these leopards are habituated to human presence and relatively easily observed, as compared to research on populations outside formally protected areas.
3. No standard procedure for managing leopards in ex-situ facilities exists.
4. Very limited information on the current health status of free-ranging and captive leopards is available for leopards in South Africa.

1.6. About this study

Taking into account the gaps in knowledge highlighted above, this study aims to elucidate the current status of leopard health in South Africa by using a certain set of parameters. Even though similar studies have been carried out on other organisms in other parts of the world (Dove et al. 2012), this study is not only the first of its kind on leopards but provides a basis for work on leopards around the world.

Overall, this study aims to use a multi-disciplinary approach that integrates ecotoxicology, haematology, blood parasites and other physical factors, to establish a workable, repeatable, holistic approach to the health assessment of South African leopards, the one aspect of this cat that has remained largely unstudied. This will be the first study of its kind on African leopards. This study will therefore focus on comprehensive evaluation of blood samples, genetic and molecular samples, toxicological material and additional clinical information such as body condition (BC) and diet.

1.6.1 Research question

Gaps in the current knowledge of South African leopards as identified earlier in this chapter led to the main research question: What is the current health status of leopards in South Africa? How can ecotoxicology, haematology, haemoparasitology and clinical data be integrated to indicate the health of a leopard? What haemoparasite and ectoparasite associations do leopards have and is there a difference between that of captive and wild leopards? What toxicological characteristics do leopards have, if at all traceable? Keeping in mind the aforementioned parameters: are ex-situ leopards in better overall health than in-situ leopards? How does the leopard immune system differentially respond to internal factors (e.g. haemoparasites) and external factors (e.g. Organochlorine pesticide (OCP) concentration levels), if at all?

1.6.2 Hypotheses, aims and objectives

This study aims to assess the health of leopards and validate ecotoxicology, haematology, haemoparasites and clinical factors as integrated leopard health parameters. The overall hypothesis is that wild leopards will be in better overall health than captive leopards, due to several interacting factors such as ex-situ and in-situ environments and its subsequent effects on these cats. The research questions as stated above in section 1.6.1 led to the formation of the more specific hypotheses as outlined in this section below. Subsequent aims to test these hypotheses and specific objectives have been established for the successful execution of this study.

The following hypotheses will be researched and addressed in this study:

1) Haematological characteristics of captive and wild leopards will differ from each other as well as from other wild felids.
2) All leopards will be infected by at least one species of intraleukocytic parasite and prevalence and parasitaemia will be higher among wild leopards.
3) Higher infestations of haematophagous arthropods will be encountered on wild leopards, and possible developmental stages of intraleukocytic parasites may be found in engorged ticks collected from infected leopards.
4) Using ecotoxicology as a health assessment tool has never before been applied to leopards, and this study will be investigating this parameter as an option for leopard health assessment.
5) It is hypothesized that the serum-concentrations of OCPs will be higher in the blood of ex-situ leopards than in-situ leopards.
6) It is hypothesized that all ex-situ facilities will manage their leopards in a different manner and that some facilities will fare better at managing these animals than others.
7) Integrated statistical analysis of the identified health parameters will give detailed base line information on the health status of captive and wild leopards in South Africa.
8) Different leopard populations will be affected differently by OCP concentrations and

haemoparasite prevalence and parasitaemias.

Subsequently, the following aims and objectives will be employed in Chapters 3 to 7 to address the abovementioned hypotheses:

The aim of Chapter 3 is to provide a detailed baseline guide for morphological characterization of blood cells and haematological characteristics observable under a light microscope in captive and wild African leopards. This will be achieved by screening thin blood smears to collect relevant haematological data for description, differential cell counts and comparisons.

Chapter 4 follows with the aims to investigate whether captive and wild leopards in South Africa are infected with species of *Hepatozoon*, and if these infections would cause clinical symptoms. This study also aims to specifically identify the haemogregarines infecting the leopards from this study. This will be achieved by screening for and identifying hepatozoan infections by means of gamont morphology observed in peripheral blood smears and molecular analysis of a fragment of the 18S rRNA gene. All potential clinical symptoms of infected leopards will also be noted.

The aim of Chapter 5 is to explore the role of ticks as potential vectors of a species of *Hepatozoon* infecting African leopards in South Africa. To achieve this, engorged haematophagous vectors will be collected directly from infected leopards, dissected and screened under a light microscope for possible life cycle stages.

Furthermore, Chapter 6 aims to establish baseline concentrations of OCPs in African leopards in South Africa, compare differences between captive and wild leopards and do a preliminary assessment of individual and group category differences to account for anticipated life history effects of sex, age class, phenotypes and conservation management approaches. Collection of enough blood from each individual leopard to maximize analysis possibilities of serum will be vital. OCPs have to be identified and quantified for the purpose of comparison across individuals, ages, gender, localities and in-situ and ex-situ leopards.

Chapter 7 follows with the aim to validate haematology, haemoparasitology, OCP concentrations and differing conservation management approaches as parameters to assess leopard health. Relevant details concerning ex-situ management need to be obtained from each facility where leopards have been sampled. These details will include general management and clinical history of subjects. A standardized questionnaire will be used to interview ex-situ managers with regards to how their leopards are kept. This questionnaire will be drafted based on what is known and unknown of captive management approaches from available literature. This chapter aims to show how the parameters of the previous chapters integrate/interact when thrown together. By doing an integrated analysis of the abovementioned parameters, this chapter will show how these parameters interact and will provide valuable baseline information on the health of captive and wild leopards in South Africa.

1.7 Expected outcomes

This study will clarify the current status of leopard health in South Africa and will also elucidate the typical intraleukocytic parasites found in both wild and captive leopards. By determining the physical health of an ex-situ population and comparing it to that of an in-situ population, knowledge on how OCPs and haematology interrelate as health parameters can aid immensely in formulating a more holistic conservation management approach. Chapter 1 gives a literature review on the African leopard as well as the general aims and objectives of this study. Following this chapter is Chapter 2, which addresses the general field- and lab-based methodology of this study. Chapter 3 concerns the haematological characteristics of African leopards, followed by Chapters 4 and 5 where haemoparasites and associated life cycles and vectors are addressed. Chapter 6 is about OCP analysis of blood serum from in-situ and ex-situ leopards. Finally this thesis will end with Chapter 7's inclusive discussion on the health aspects, integrated parameters and captive management of leopards in South Africa.

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CHAPTER 2
General materials and methods

To achieve the aims of this study as outlined in Chapter 1, the study involved capturing, and researching various biological and ecological aspects of wild leopards occurring in in-situ as well as captive leopards in ex-situ conservation management areas in South Africa (Fig. 2.1 a and b). Care had been taken to ensure that individuals used in the study were representative of both aforementioned populations. For the purpose of data collection various trained wildlife veterinarians, with each in-situ and ex-situ facility choosing its own vet, sedated all leopards.

2.1 Study sites

Leopards at three ex-situ facilities and three in-situ locations were sampled during this study (Fig. 2.2, 2.3 and 2.4).

2.1.1 Ex-situ facilities with captive leopards

Guidelines and research on captive felids in South Africa are relatively rare in comparison with the rest of the world. Captive felid management includes an intricate process involving major aspects of animal behaviour such as social organization, mating systems, territoriality, degree of and need for parental care, spatio-structural requirements, interspecific interactions and foraging behaviour of the target species (see Wielebnowski 1999). For the purpose of this study, leopards have been classified as 'captive' when they have been born in captivity or raised in captivity from a very early age (< 1 month old). The age of captive leopards were obtained from their ex-situ managers and they were allocated an age-category as described in Table 2.1.

Ex-situ Site1 is a big cat ex-situ facility situated on the outskirts of Bloemfontein, Free State, South Africa, and was established in 2007 with one hectare of land (Fig. 2.1 b blue triangle). The initial focus of this facility involved a captive breeding program for cheetahs *Acinonyx jubatus* (Schreber, 1775) with the aim to rehabilitate these animals in the wild, but they have expanded

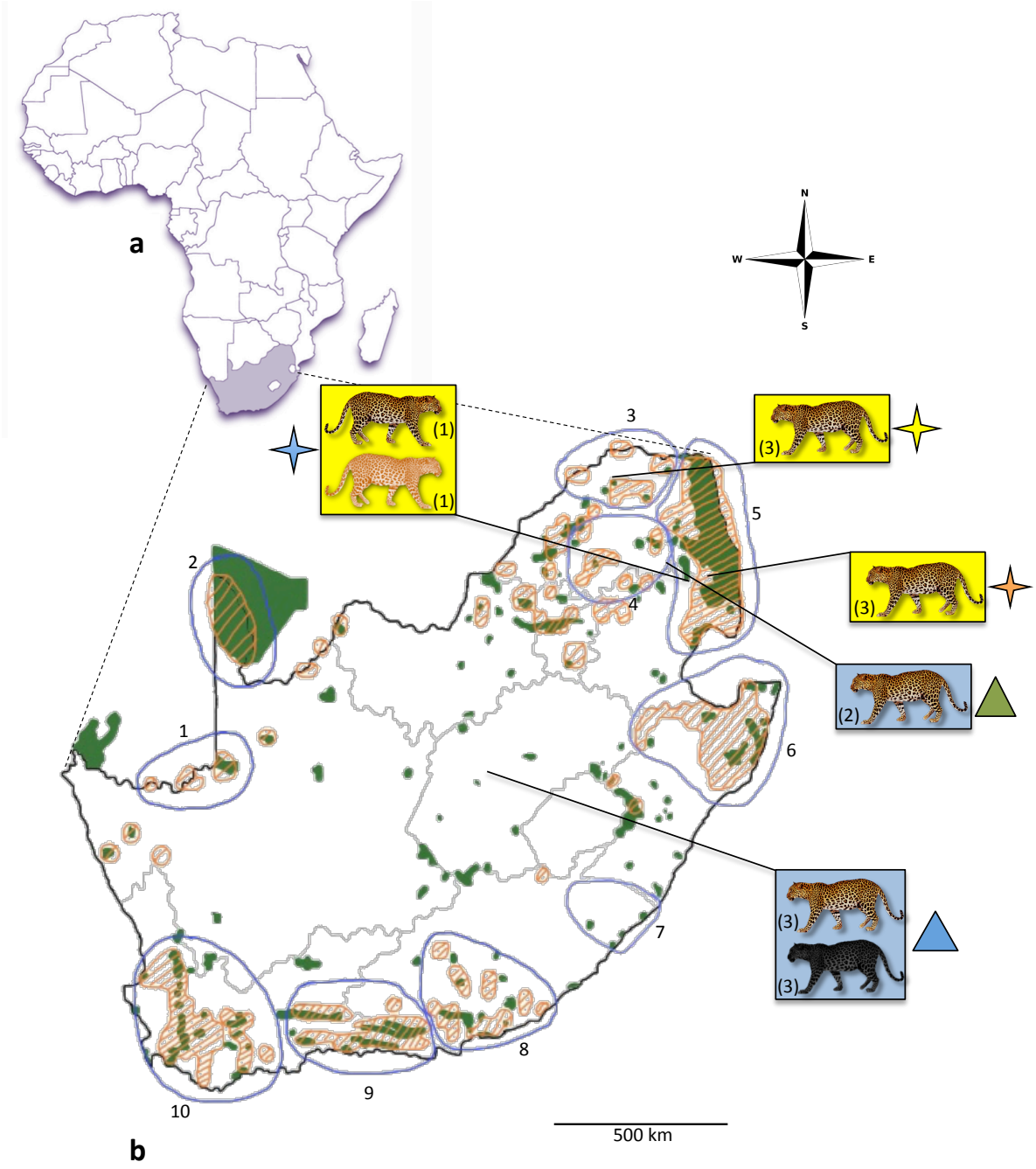


Figure 2.1 a & b Digitally redrawn maps of the African continent, with a detailed focus on South Africa, showing localities of leopards sampled during this study. Dashed lines from the grey area in **a** indicates the magnified map area shown in **b**. Orange striped polygons - leopard observations; Green polygons - conserved areas of South Africa. Number indicates core leopard populations, encircled in blue lines: **1** – Orange river; **2** – Kalahari; **3** – Northern Limpopo; **4** – Waterberg/Mpumalanga; **5** – Greater Kruger; **6** – Northern KwaZulu-Natal; **7** – Wild Coast; **8** – Eastern Cape Valley; **9** – Eastern Cape Mountain; **10** – Western Cape. Yellow squares – wild population; Blue squares – captive population. Colour variations of leopards: brown coloured leopard- normal leopard; black coloured leopard - melanistic leopard; red coloured leopard - erythristic leopard. Numbers in brackets indicates number of each type of leopard sampled. Blue triangle – Ex-situ Site1 and Ex-situ Site2; Green triangle – Ex-situ Site3; Yellow star – In-situ Site1; Green star – In-situ Site2; Orange star – In-situ Site3. Redrawn from Daly *et al.* (2005) and SA-Venues.com (2013) with Autodesk® Sketchbook®, Autodesk Inc. ©2015.

to house several other species such as lions, leopards and caracals *Caracal caracal* (Schreber, 1776). Enclosures were kind of small (CF2 and CM2 shared an enclosure of ~ 625 m^2 and CF3's enclosure was ~ 165 m^2) and these animals did not have access to whole enclosure areas at all times. Although these enclosures had wire fencing all around, even at the top, they were separated from each other by footpaths of about 5 – 6 m wide, giving neighbouring animals some degree of separation. Enclosures were semi-artificial with lots of structures made from natural materials such as wood stumps, but also concrete sleeping cages and tyre/rubber swings. All leopards were fed from stainless steel bowls or wooden boards. Ex-situ Site1 was the only facility where people were forced to sanitize hands, arms and shoe soles before entering an enclosure.

Of all three facilities in this study, ex-situ Site1 did the most enrichment activities with their leopards out of all the ex-situ facilities of this study (Fig. 2.3). The photographic images presented in Figure 2.3 is a public record of all enrichment activities of the specific leopards sampled at this facility, sourced from this facility's social media pages for the sake of non-bias and transparency from the author. The main focus of enrichment activities was of a behavioural nature. Enrichment was meant to be stimulating to the leopards, and most likely was/is as none of them showed stereotypical behaviours such as aimless pacing. Small cubs are given stuffed toy animals and plastic pet-specific toys as a form of enrichment (Fig. 2.3 a and b). Adults are given old vehicle tyres (Fig. 2.3 d and i), cardboard boxes (Fig 2.3 e and f), small wildfowl species (Fig. 2.3 g), and discarded animal skins, sheep wool and tails (Fig. 7.1 h – k), and fruit (Fig. 2.3 k and l).

Figure 2.2 An obese leopard male housed in a walled enclosure at Ex-situ Site2.

Ex-situ Site2 is a zoo in Bloemfontein (Fig 2.1 blue triangle), Free State, South Africa, which was established in 1906 and pioneered the breeding of ligers (a cross between a lion and a tiger) in the 1930s. This is also one of the oldest zoos and animal sanctuaries in South Africa and covers an area of 15 ha. It is situated across the Loch Logan Island and houses more than 100 species. Bloemfontein is situated on the southern periphery of the South African Highveld at 1 400 m above sea level. It borders the semi-arid Karoo region with Highveld grassland habitat. This area experiences a semi-arid climate, with summer maximums of up to 32 ^{0}C, with recurrent afternoon thunderstorms and is mostly flat with intermittent hills. Winters are usually dry and cool, with lows reaching -3 ^{0}C, with frequent frost, and it may snow infrequently. Captive female CF1 was housed in a small enclosure (~ 490 m^2) that was highly artificial, with solidly built brick walls and few other structures in the enclosure (Figure 2.2). Two trees at the back of the enclosure provided the only source of shade. This facility was the most artificial of all three, with separate, cement-floored feeding cages and no enrichment strategy for these leopards in place.

Figure 2.3 Enrichment activites of leopards sampled at Ex-situ Site1. **a** Taken for walks on leash and harnass, stuffed toys (CF3 2014). **b** CM1 with plastic pet chew toy (2014). **c** CF2 with plastic soda bottle filled with rocks (2014). **d** CM2 with car tyre (2014). **e** CF2 with old pizza box filled with pooh from other animals (2014). **f** CM2 playing with old cardboard box (2016). **g** CF2 with fresh guineafowl (2015). **h** CF3 tail enrichment (2015). **i** CM1 playing with cow skin in car tyre (2014). **j** CM2 cow skin (2014). **k** CF2 and CM2 playing with soaked sheep's wool and fruit in splash pool (2013). **l** CF2 fruit enrichment (2013). All images were sourced from this Ex-situ Site1's public Facebook page.

Ex-situ Site3 is a wildlife rehabilitation centre, situated near the town of Hoedspruit, Mpumalanga (Fig. 2.1 b green triangle), South Africa, and was established in 1991. This highly regarded contributor to conservation in South Africa houses various injured, abandoned, poisoned and permanent wildlife species and has expanded remarkably over the last decade. The mission of Ex-situ Site3 is to return rehabilitated animals to the wild whenever possible and facilitate a number of successful breeding programs. This area usually receives around 410 mm of rain annually, normally during the hot mid-summer months. Average summer temperatures are higher than 30 ^{0}C, with winter low-temperature averages ranging around 7 ^{0}C. Frost is an infrequent event.

Ex-situ Site3 had the most natural, largest enclosures (~ 1720 m^{2}) with natural vegetation and wire fencing (Fig. 2.4 a and d) separating neighbouring animals. Having only wire fencing

between them seemed to cause some stress in adjoining animals, as seen in Figure 2.4 c where a male lion (Fig. 2.4 c yellow arrow) immediately took an aggressive stance as soon as he saw CF5 trapped in her feeding cage. This caused CF5 to feel threatened, as can be seen in her posture with the ears pulled back (Fig. 2.4 c black arrow). Leopards were fed in feeding cages (see Fig. 2.4 b) and food items were taken to the enclosures in buckets. Due to the natural character of these leopard enclosures and no sign of stereotypic behaviour, their managers did not feel the need for enrichment activities so none can be reported.

Figure 2.4 The environment of Ex-situ Site3. **a** Enclosures are large and mostly natural with little artificial elements. **b** Leopards have specific feeding cages where their food is placed. Here is CF5 in her feeding cage, tame enough to be sedated by hand. **c** Enclosures of different species are separated only by wire fences. Note the aggressive stance of the next-door male lion (yellow arrow) as soon as he realized CF5 is trapped in her feeding cage and note CF5's posture with ears pulled back (black arrow), indicating she feels threatened. **d** All food items are provided in buckets directly to enclosures. **e** Screen grab of a volunteer's video blog at Ex-situ Site3 in 2019, showing how meat is stored in a cold-room (Nika Pozun 2019).

2.1.2 Wild leopards in-situ sampling sites

For the purpose of this study, leopards have been classified as 'wild' when they have been born in the wild, was caught at an adult stage in the wild or when they were at rehabilitation centres for less than 6 months.

In-situ Site1, a conservancy and research centre, located in the Soutpansberg Mountains of northern South Africa's Limpopo Province (Fig. 2.1 b yellow star), is well known for its pristine wilderness and floristic diversity. It forms part of the Luvhondo Nature reserve within the United Nations Educational, Scientific and Cultural Organization (UNESCO) Vhembe Biosphere Reserve and has been declared a Natural Heritage Site. The area does not present classic African savannah but is characterized by a variety of veld types including montane grasslands, mistbelt forests, woodland and thicket and has strong altitudinal gradients. This area was a source of samples for three wild leopards in this study (see Fig. 2.1 b yellow star and Table 2.4).

In-situ Site2, the Lydenburg area in Mpumalanga, uniquely lies in the ecotone of the Sekhukhuneland and Lydenburg Centers of Plant Endemism (Fig. 2.1 b green star). The Afromontane flora of the Lydenburg Centre of Plant Endemism links it southwards to the southern Drakensberg mountain range and northwards to the Zimbabwean Highlands (Emery et al. 2002). Situated in the rainfall shadow of the Drakensberg escarpment, the Sekhukhuneland Centre of Plant Endemism is arider than the areas to the east, with the vegetation correspondingly adapted. The study area contains combinations of several habitat types such as Lydenburg Montane Grassland, Sekhukhune Montane Grassland and Lydenburg Thornveld. Remnant Afromontane forest patches and shrub-like thickets (Emery et al. 2002). These patches are usually relatively species-poor and occur at altitudes ranging between 1 100 and 1 900 m above sea level. Mountain slopes are characterised by dense, sour grassland with scattered thickets of trees and shrubs. Two wild leopards, one of which an erythristic female, were sampled from this area (see Figure 2.1 b green star and Table 2.4).

Three wild leopards were sampled at In-situ Site3, the southwestern part of the Greater Kruger Conservation Area (Fig. 2.1 b orange star and Table 2.4). This is an area of South Africa's Mpumalanga Province where the Big Five still roams freely and lies at an altitude of 350–450 m above sea level, with gradually undulating terrain and a rise to the west. The climate in the GKCA varies from humid and hot in summer to mild and dry in winter (June to August). Rainfall varies throughout the region and the wet season is predominantly from November to March (Venter & Gertenbach 1986, Bunting et al. 2016). Average rainfall in the southwestern parts of GKCA is around 600 mm per year and the area has an arid steppe climate with average summer maximum temperatures reaching 32 ^{0}C in mid-summer months (Venter & Gertenbach 1986). Average minimum temperatures during the dry winter months range around 5 ^{0}C. The vegetation in this area is characterized as deciduous savannah, with relatively dense woodland in the southwestern parts (Venter & Gertenbach 1986, Venter et al. 2003, Bunting et al. 2016).

2.2 Reasoning for using whole blood as the main source of data in this study

Blood contains various cell types, nutrients, metabolites and electrolytes, circulates the whole vascular system (Weiss & Wardrop 2011) and provides a diversity of potentially handy variables for the monitoring of health in wildlife (Maceda-Veiga et al. 2015). Authors such as Singh et al. (1999) and Du Plessis (2009) have shown that basic knowledge of the haematological values and structure and function of blood elements may indicate potential underlying clinical conditions. Peripheral blood reflects the functioning of an organism as a whole, thus being the most informative, non-lethal tissue available to researchers of animal health (Weiss & Wardrop 2011, Kumar et al. 2014).

Specific methods of haematological, haemoparasite and ecotoxicologial data sampling and analyses are presented in Chapters 4 to 6 of this manuscript.

2.3 Capture and sedation of leopards

All leopards were sampled while under sedation and under the supervision and with the aid of several trained, registered veterinarians. This study received ethical approval (North West University ethics approval no. NWU-00255-17-A5). Wild leopards were free-darted to minimise the effects of stress on sample quality (Kreeger & Armeno 2007, Maceda-Veiga et al. 2015). The author always made sure to work with veterinarians that are approved by and known to the ex-situ managers and who have previous working experience with their leopards. The pharmaceuticals used to induce sedation was unique for each veterinarian throughout the study and mostly constituted the use of a solution of ketamine (Anaket-V; Centaur labs) (20 – 25 mg) and Domitor® (Zoetis; South Africa) (dose 80 – 90 μg/kg), diluted with saline. Each leopard was sedated with a solution uniquely made up according to the estimated or known weight of each. Weights for wild leopards were mostly estimated and the solution made up accordingly, occasionally requiring the need for a sedative top-up while working with the animal. Sedatives were mostly administered with the use of a dart gun, but with some of the tame leopards a regular injection could be used. As soon as the dart or the needle penetrated the skin the time was noted, as well as when the sampling procedure began once the leopard was fully sedated. Noting the starting and ending times of sampling was important so that I could aim to finish sampling the animal without having to add time to the period of sedation in order to minimize stress. All leopards were handled with sterile gloves and it took an average of 20 minutes to collect samples. After all samples have been collected, leopards were injected with Antisedan® (Zoetis; South Africa) and all leopards were carefully monitored until they were fully awake and alert.

2.3.1 Captive (ex-situ) leopards sampling and sedation

All captive leopards were sampled during the daytime hours and were sedated in their enclosures by resident veterinarians familiar with these animals. Some leopards were tame

enough to inject by hand, while others had to be darted with either a dart gun or a blowpipe. Once fully sedated, the leopard would be removed from its enclosure and carried on a tarpaulin to a nearby shady spot for data collection. It was always ensured that time working with the animal is limited as much as possible. After sampling the leopard would be placed back in a shady spot in its enclosure and was continuously monitored until fully awake.

2.3.2 Wild leopards (in-situ) sampling and sedation

All wild leopards were sampled at night and were caught by using bait, as well as free-darting. At one sampling location, baited loop-traps were set out, fitted with a trigger plate for the leopards to step on. Each trap was equipped with a trigger sensor coupled to a radio, so that when the trap is set off immediate notification is received. These baited traps were also deactivated during the day, but left in place to be activated for the following night. Traps were also checked every three hours in case the trigger sensor failed to notify that something has been caught. Once a leopard has been caught, the data collection team was notified and went out to the trap. The veterinarian would cautiously approach the animal and dart it from his/her vehicle. Once the leopard was fully sedated all relevant data was collected as efficiently as possible. When all data has been collected the leopard would be taken to an open area where it was continuously monitored until fully awake. Three leopards were caught with these baited traps.

In other cases, bait was tied to a tree situated in an open area with minimal vegetative cover. These sites were usually adjacent to a dirt road, where the bait have been dragged up and down for 2 km to leave a scent trail on the ground. Bait was set up half an hour before sunset and monitored continuously by the research team for any predator activity. The monitoring team would wait within darting distance from the bait with all vehicle lights turned off and only using soft voices when speaking if needs be. Additionally, free-darting was used once as the opportunity arose during a sampling trip. This refers to darting an animal on sight from a vehicle if the vegetation allows for fast recovery. Once it was fully sedated, the leopard was moved to the data collection team.

A data collection team accompanied by another veterinarian was situated approximately 2 km from the baited area to ensure that researchers could work safely without interference from surrounding predators attracted to the bait and without disturbing the area close to the bait. If a leopard were detected on the bait, the veterinarian would dart it, visually aided by a red-filtered spotlight. Once the animal was asleep the veterinarian would cautiously approach it before the rest of the team brought a tarpaulin to carry the leopard to the nearby vehicle. The vehicle would then drive to the data collection team, with the vet staying by the sedated animal to monitor it at all times. Upon arrival with the data collection team, the animal was off-loaded and relevant data was collected as efficiently as possible. When all data has been collected the leopard would be taken to an open area, approximately 2 km from the data collection team in the opposite direction from the baited area, off-loaded and injected with the component

needed for it to wake up. All leopards were guarded against possible attacks by surrounding predators until fully alert and self-sufficient. Three leopards were sampled with this method.

2.4 Field-based onsite data collection

Age classes were allocated to all leopards sampled during this study according to the stipulations used by Fattebert et al. (2015) (Table 2.1). Wild leopards were aged according to the nature of tooth wear (as described by Stander 1997) and a combination of morphological cues (as described by Balme et al. 2012) (see Fig. 2.5 a – d). Captive leopards were aged according to the information provided by their ex-situ managers. This information is presented in Tables 2.3 and 2.4 of this chapter.

Table 2.1 Leopard age classes used during this study (as stipulated by Fattebert et al. 2015).

Age (actual or estimated)	Age class assigned
< 1 year	Cub
1–3 years	Subadult
> 3 years	Adult

Taking morphological measurements with a measuring tape and calliper (Fig. 2.6 a, b & e) and recording it on pre-designed data sheets followed the process of blood collection (Fig. 2.6 c & d). These measurements included the leopard's unique identification code age (estimated or known), date, physical condition, weight (estimated or known), body length, tail length, shoulder height, neck circumference, breast circumference, muzzle dimensions, cranial dimensions, canine length and paw sizes (see Fig. 2.5). Additional information, such as the presence of wounds or vein collapses, was also recorded on these data sheets.

2.4.1 Standardised questionnaires for ex-situ managers

Predesigned questionnaires were used to obtain information on how the leopards were managed at each ex-situ facility. Questions were carefully constructed from investigating the standard needs of leopards held in captivity as identified by Law et al. (1997), Shoemaker et al. (1993) and Wielebnowski (1999). Interviews were held with the persons responsible for managing the leopards, and using the questionnaires as a guideline the following aspects of keeping leopards were addressed:

- Information on the leopard's gender, age and name
- The origin of the leopard, its parents, location of origin, and whether it was born in captivity
- Clinical history and previous illnesses
- Ectoparasite treatment regime
- Diet, feeding schedule and method of providing food
- Information on the use of nutritional supplements
- Characteristics of the leopard's enclosure and cohabitation, if any
- Environmental enrichment

- Any additional information the manager thinks may be applicable (e.g. behavioral discrepancies).

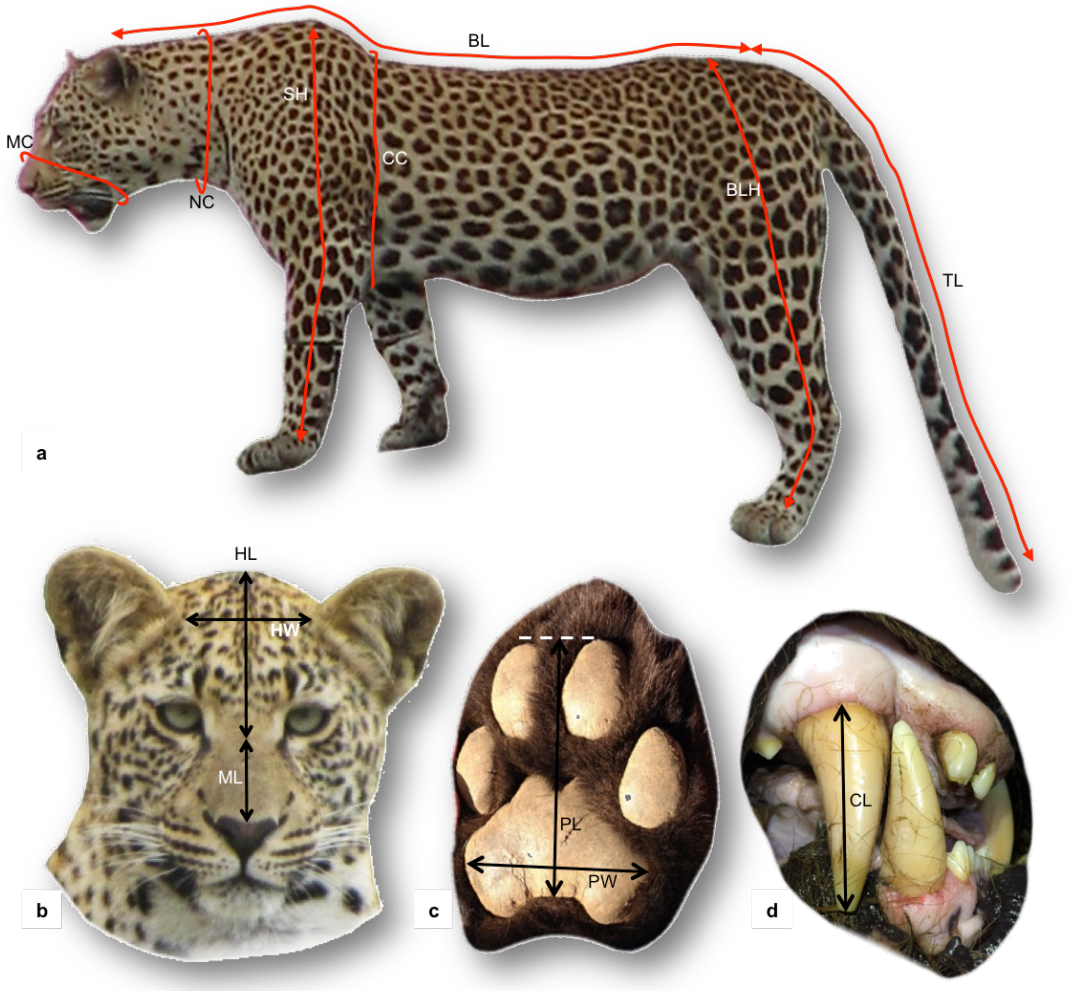


Figure 2.5 Annotated photographs of leopards showing their measurements (in coloured lines). Abbreviations: **a** MC – muzzle circumference where muzzle joins cranium; NC – neck circumference; CC – chest circumference immediately behind scapula; SH – shoulder height from tip of scapula to where toe phalanges begin; BLH – back leg height from tip of pelvis to where toe phalanges begin; BL – body length from knob of cranium to base of tail; TL – from base to tip of tail. **b** ML – muzzle length from tip of nose to where muzzle joins cranium; HL – head length from where muzzle joins cranium to knob of cranium; HW – head width between bases of ears. **c** PL – paw length; PW – paw width. **d** CL – canine length from where canine meets gingival margin to tip of canine.

2.4.2 Body condition score design

Maintaining an ideal body mass index (BMI) or body weight (BW) is an important part of good feline health (Bjornvad et al. 2011). Although BMI is a precise and objective measurement (Bjornvad et al. 2011), it is not always possible under strenuous field conditions in the African bush. It also fails to provide information on body condition (BC) and therefore lacks value as a comparative tool across populations (Bjornvad et al. 2011).

Rationale for using a newly designed body condition score (BCS) system

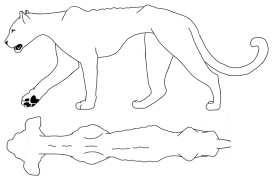
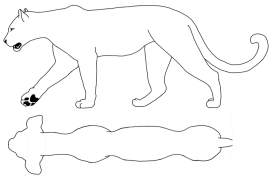
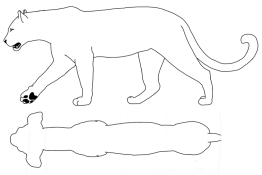
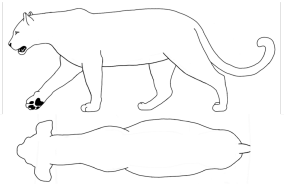
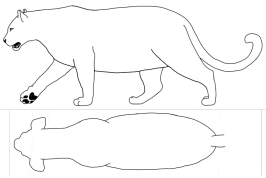
BC scoring is a way to visually quantify a cat's body condition (BC) and it is a method of 'judging the overall health' of felines (Laflamme et al. 1994; Scott et al. 2002). The BCS is a value allocated to an animal to indicate whether it is emaciated, underweight, ideal, overweight or obese. BC scoring is a reliable tool used by veterinarians to determine the BC of domestic cats (Bjornvad et al. 2011), but the current domestic cat model is not completely applicable to big cats. Therefore, several literature resources were combined to create a newly designed 5-point scale in this study – a visual BCS system specific to big cats, based on certain morphological characteristics visible upon first sight of the animals (see Table 2.2). This method of quantifying body condition in live animals is more reliable as a data tool than the body mass index (BMI), where weight and physical measurements are used in conjunction (Bjornvad et al. 2011). Weight and measurements, as taken during this study and described in this chapter, can be accurate, but it often happens that the necessary equipment is not readily available or the opportunity to sample a leopard is of such a short duration, that the author found the BCS system to be a more efficient, reliable way to quantify BC of leopards. Another advantage of the BCS system is that it allows the researcher to score the leopard on sight, even before sedation, which leaves more time for other data gathering under strenuous field conditions.

As described earlier in this chapter, BW and standardised sets of measurements were taken in conjunction as a means of indicating BMIs. Additionally, by using the novel BCS system, the BC of leopards was quantified based on certain morphological characteristics visible upon first sight of the animals. All leopards were evaluated and given a BC score (1=emaciated; 2=underweight; 3=ideal; 4=overweight; 5=obese) onsite while collecting samples in the field (Table 2.2). A detailed description of each allocated score is specified in Table 2.2 and individual BCSs are presented in Tables 2.3 and 2.4.

2.4.3 Blood and ectoparasite collection

Before collecting blood samples, the dense pelt over the identified area of collection was shaved with a clipper and wiped with an alcohol-soaked cotton ball (see Fig. 2.6 c & d), which helped to remove superficial skin contaminants and improved visualization of the vein. Peripheral blood was usually collected from the jugular (Fig. 2.6 c) or the cephalic vein (Fig. 2.6 d) by venipuncture with the use of a Vacutainer system, in BD Vacutainer® (Franklin Lakes, USA) CAT (Clot Activator Tubes) and Vacutainer® Ethylenediaminetetraacetic acid (EDTA) tubes for molecular use. Thin blood smears were made on site (Fig. 2.6 f). Drawing blood from some individuals, such as CF2, proved a challenge due to the collapsing of veins during sedation. In these cases, the method of blood collection shifted to the use of a regular syringe, which usually proved successful after a few attempts. Attention was given to prevent haemolysis during blood collection as far as possible.

Table 2.2 Body condition score (BCS) system constructed to allocate body condition (BC) scores to the leopards from this study (Compiled from Laflamme 1997, Russell et al. 2000, Laflamme et al. 2001, Scott et al. 2002, Bjornvad et al. 2011).

Status	BCS	Description	Visual example
Emaciated	1	• No detectable fat. • Wings of ilia easily visible. • Lumbar vertebrae easily visible. • Ribs highly visible. • Severely narrow waist when viewed from above. • Highly conspicuous abdominal tuck.	
Underweight	2	• Minimal detectable fat. • Lumbar vertebrae visible. • Ribs visible with thin fat layer. • Marked, narrow waist behind ribs when viewed from above. • Pronounced abdominal tuck.	
Ideal	3	• Well-proportioned. • Ribs not visible, but felt when touched lightly. • Waist visible behind ribs when viewed from above. • Minimal abdominal tuck. • No/minimal abdominal fat pad.	
Overweight	4	• Ribs not visible. • Moderate fat layer over face, limbs and lumbar area. • Ribs covered with moderate fat layer and not easily felt. • Waist barely visible behind ribs when viewed from above. • Visible abdominal rounding. • No abdominal tuck. • Moderate abdominal fat pad.	
Obese	5	• Ribs not visible and can't be felt under heavy fat layer. • Heavy fat layer over face, limbs and lumbar area. • No waist when viewed from above. • Marked distention of abdomen. • Extensive abdominal fat pad.	

Adult and nymph tick specimens were collected by hand from the skin of sedated leopards with the aid of a forceps and identified with the help of available literature such as Walker et al. (2003). Sub-samples of the ticks were sent to ARC-OVI at Onderstepoort Veterinary Institute for identification. 75% of all ectoparasites were stored in 70% EtOH contained in Eppendorff tubes and 25% were stored without added preservative in a -20 ^{0}C freezer. Tick specimens representing four genera were used to make squash preparations. These preparations were made by opening a tick with sharp forceps and squashing and smearing its contents on a

microscope slide. A single tick was squashed per slide and if it was a large engorged specimen, two slides were used to avoid smears that were too thick. Tick smears were subsequently fixed with absolute methanol for one minute and left to air dry.

Rationale for using thin smears of peripheral blood for haematological and haemoparasite characterisation

Thin blood smears have a certain advantage over automated blood counters in terms of providing useful information on morphological characteristics and arrangements of blood elements, including a visual record of potential haemoparasites and other blood cell abnormalities (see Chapter 3). Pathogens such as haemoparasites can be directly observed in thin blood smears, giving the researcher a good first impression of infections and parasitaemias (see Chapter 4). This gives the researcher a solid approximation of that animal's immune status and aerobic ability (Gillooly & Zenil-Ferguson, 2014) and is advantageous as most researchers are unlikely to have quick access to fully equipped laboratories while collecting samples and data in the field. Another benefit is that blood smears are easily stored at the field site for later processing in the laboratory, unlike fresh blood that needs to be taken to the lab for automated haematological analysis (Pendl 2013 as cited in a review article by Maceda-Veiga et al. 2015). Differential white blood cell (WBC) counts and neutrophil-to-lymphocyte ratios (NLRs) are useful markers to indicate stress or innate immune responses (Davis et al. 2008; Pavlova et al. 2015, Maceda-Veiga et al. 2015).

Thin peripheral blood smears

Small blood droplets (enough to provide three to four blood smears) were placed onto clean, pre-labelled microscope slides for the purpose of making thin blood smears. Blood smears were left to air dry and packed away in dust-free boxes for further processing in the lab. On the day of blood collection, blood smears were fixed with absolute methanol for one minute and left to air dry. Thin blood smears were used to obtain haematological (see Chapter 3) and haemoparasite data (see Chapters 4 and 5), and variable specific methods are represented in Chapters 3, 4 and 5, respectively.

Rationale for using peripheral blood as a means of OCP concentration determination

Since blood functions as the body's transport system, blood serum screening for contaminants such as OCPs is an informative way to gather data on possible toxicants in an animal's system (Maceda-Veiga et al. 2015). The possibility of resampling an individual after a certain period of time also becomes possible, providing the researcher with the possibility for long-term health tracking and assessment of individuals in captivity and the wild.

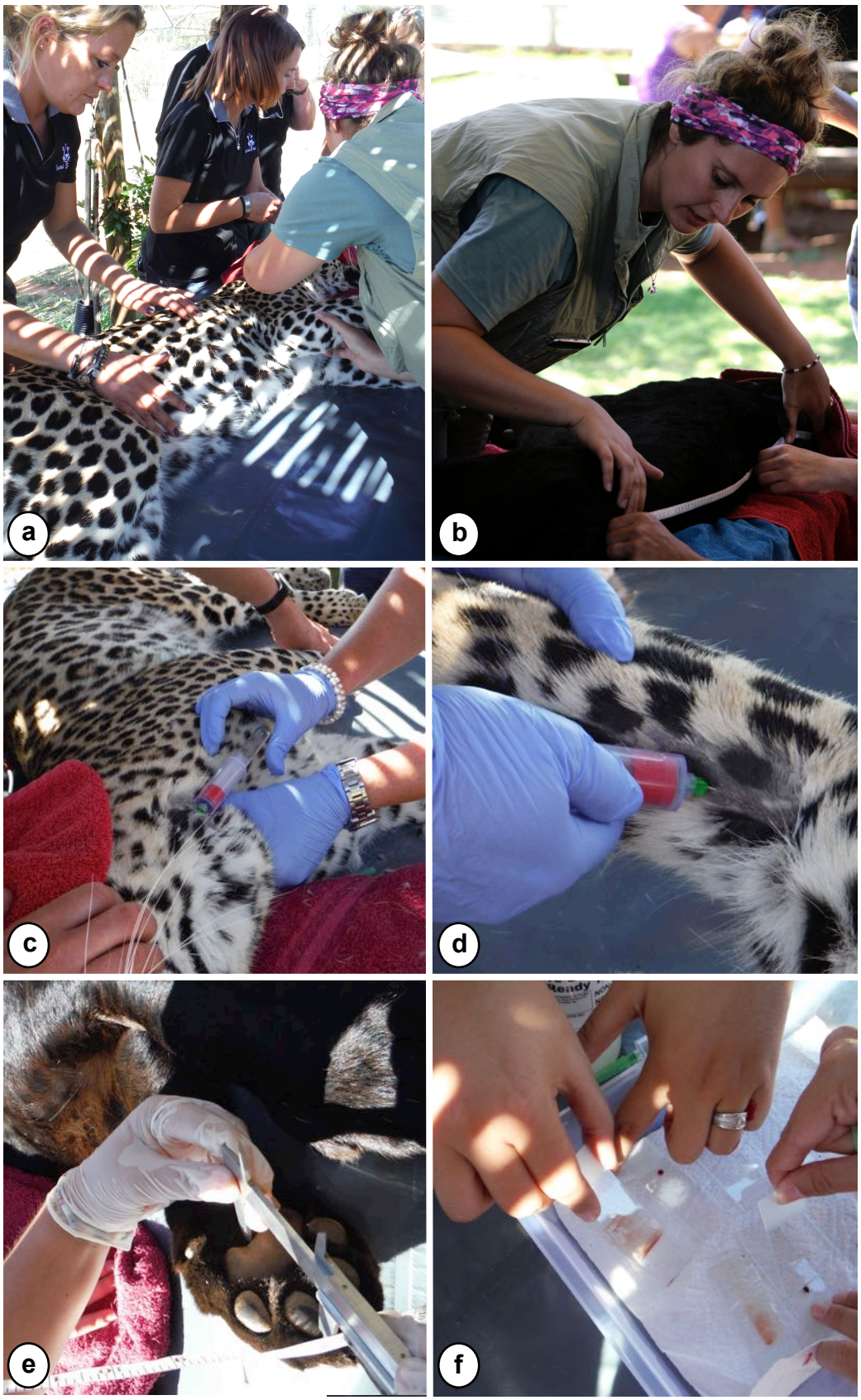


Figure 2.6 Blood collection, blood smear preparation and morphometrical measurements taken from leopards. **a & b** The author taking morphometrical measurements with a tape measure from **a** a regular coloured and **b** melanistic leopard with assistants in the background continuously monitoring heart rate and breathing regularity of the subject. **c** Blood samples collected by means of a Vacutainer system from the carotid artery in the neck or **d** the cephalic vein of the front leg. **e** Measuring front and back paw sizes with a metal caliper. **f** The author and assistants making thin blood smears on clean microscope slides.

Specific descriptions of the handling of whole blood and blood serum samples in the field and the laboratory are detailed in Chapter 6 of this study. As reported in Chapter 6, this method of blood and subsequent serum collection is not dependent upon high-tech facilities, as the serum samples only need to be kept at -20 °C (possible in any standard, commercially available freezer) until processing can take place back at the lab. This simplicity can be a great advantage to researchers in the field, where conditions can quickly turn unpredictable. Additionally, the advantages of using blood serum samples are that necropsied animals are redundant for data intake, and stress inducement on study subjects is less due to its lower invasiveness.

2.5 Make-up of sampled leopard population

Various types of samples were obtained from 17 individual leopards, including males, females, cubs, subadults and adults representing all three phenotypes (as addressed in Chapter 1) (Tables 2.3 and 2.4).

2.5.1 Captive leopards sampled during this study

As described in Chapter 1, it is currently unknown how many captive leopards are kept in ex-situ facilities in South Africa. This study also encountered similar problems of uncooperative captive breeders in terms of participating in research projects, coinciding with the report by Four Paws (2022), which encountered the same issue. At the onset of this study, 20 facilities around South Africa and representatives of all provinces were contacted with a detailed project proposal and request for collaboration. This study offered these facilities the option of remaining anonymous in the process, if it would make them more comfortable in joining. Some facilities never responded after several attempts at establishing communication, while others responded quite aggressively that “nobody will get close” to studying their cats. Several facilities throughout the Eastern and Western Cape were initially keen to be part of this study but withdrew 2 weeks before sampling would have taken place, without specific explanations as to why they withdrew. Hence the low number of captive breeding facilities included in this study.

From January 2013 to December 2015, over a period of four days, a total of nine captive leopards were sampled, three of which were sampled twice throughout the study (Table 2.3). Re-sampling of these three individuals was conducted in different years at two ex-situ facilities, with at least one year going by between the first and second sample. Samples were obtained from captive leopards representative of two colour variations, six had the normal colour variation (Fig. 1.4 a) and three leopards were melanistic (Fig. 1.4 b) (also see Table 2.3). Four captive males and five females were used for this study, and several age classes, as defined by Fattebert et al. (2015) were represented (Table 2.1 and Table 2.3).

Table 2.3 Captive leopards sampled during this study from January 2013 to December 2015.

Leopard	Gender	Phenotype	Age class	Location	Number of times sampled	Body Condition Score (BCS)	Blood smears	Blood samples for PCR of intraleukocytic haemoparasites	Blood serum for OCP analysis	Ticks collected
CF1	Female	Normal	Adult	ES Site2	1	5	Yes	Yes	Yes	None found
CF2	Female	Normal	Adult	ES Site1	2	4; 4	Yes	Yes	Yes	None found
CF3	Female	Melanistic	Subadult	ES Site1	1	3	Yes	Yes	Yes	None found
CF4	Female	Melanistic	Adult	ES Site1	1	3	Yes	Yes	Yes	None found
CF5	Female	Normal	Adult	ES Site3	2	3	Yes	Yes	Yes	Yes
CM1	Male	Normal	Subadult	ES Site1	1	3	Yes	Yes	Yes	None found
CM2	Male	Melanistic	Adult	ES Site1	2	4; 4	Yes	Yes	Yes	None found
CM3	Male	Normal	Adult	ES Site1	1	4	Not collected (sample donated)	Yes	Yes	Not collected (sample donated)
CM4	Male	Normal	Subadult	ES Site3	1	3	Yes	Yes	No (sample was not adequate for analysis)	None found

ES Site1 (Ex-situ Site1)	29°04'02.5"S, 26°09'27.2"E
ES Site2 (Ex-situ Site2)	29°0 6' 44.57" S, 26° 12' 22.58" E
ES Site3 (Ex-situ Site3)	24°30’52.44”S, 30°54’8.82”E

2.5.2 Wild leopards sampled during this study

Due to the nature of this study, fresh blood and ectoparasite samples needed to be obtained in the field. A total of 16 days were spent in the field to collect samples from wild leopards. When compared to the success rate of sampling captive leopards, it can be seen that much more time is needed to successfully obtain a full set of data from a wild leopard. The difficulty of obtaining samples from wild big cats is the main reason why the majority of research focuses on captive animals (Maceda-Veiga et al. 2015, Rodríguez-Jorquera et al. 2017).

From January 2013 to December 2015 a total of eight wild leopards were sampled in the Limpopo and Mpumalanga Provinces (Table 2.4 and Fig. 2.1 b). Samples were obtained from wild leopards representative of two colour variations, one leopard was erythristic and seven had the normal colour variation (Fig. 1.4 a and c). Five wild males and three females were used for this study, and only two age classes, as classified by Fattebert et al. (2015) (see Table 2.1) were represented.

2.6 Canonical Correspondence analysis (CANOCO)

Canonical Correspondence analysis (CANOCO) using CANOCO v5.2 statistical software (Ter Braak 1994) was used in this study to analyse a variety of internal and external health parameters, specified in Chapter 7 of this study. CANOCO has an emphasis on dimension reduction, restrained ordination and regression analysis, which is a combination of the two. This type of multivariate data analysis and visualisation allows for the assessment of complementary physiological, environmental and biological variables of parasite hosts, with measurable statistical significance obtainable (Ter Braak & Smilauer 2018). Identical or similar values were located close together while samples that were far apart represented those samples with the most different variable associations. Explanatory data is represented by arrows pointing in the direction of higher values, where correlations among the variables occurred (Ter Braak & Smilauer 2018). In this study, the derivative of principle component analyses (PCA), a constrained redundancy analysis (RDA), was used (Ter Braak 1994). The leopards are presented in the diagram as points at the location of the values on the latent variables.

The variables of this study were broadly divided into internal/health and environmental factors. Internal factors included the differential cell counts of leukocytes, lymphocytes, neutrophils and eosinophils, as well as the neutrophil-to-lymphocyte ratio per ~ 1 000 erythrocytes (see Chapter 3). Internal factors also included a BCS allocated for each leopard as indicated earlier in this chapter. OCP concentrations were plotted as environmental factors, the values of which are presented in Chapter 6, and supplementary data referred to the leopards' diet, which is termed 'wild diet' or 'captive diet' and haemogregarine parasitaemias with the characterisation of infected, uninfected and moderately infected leopard hosts.

Multivariate statistical analysis was performed on 14 samples where a constrained redundancy analysis (RDA) was realised to assess OCPs concentrations associated with health factors (BCS, NLR, lymphocyte counts, leukocyte counts, neutrophil counts, eosinophil counts) of individual leopards, with their respective diets (captive or wild) and parasite infections (uninfected, infected, moderately infected) overlayed as supplementary variables. The triplot explains 83.2% of the variation, with 55.9% on the first axis and 27.3% on the second axis. The explanatory variables of this analysis included 13 environmental variables (DF = 12), and the supplementary variables included five functional traits (DF = 3); pseudo-F = 0.3; p = 0.99.

Table 2.4 Wild leopards sampled during this study from January 2013 to December 2015.

Leopard	Gender	Phenotype	Age class	Location	Number of times sampled	Body Condition Score (BCS)	Blood smears	Blood samples for PCR of intraleukocytic haemoparasites	Blood serum for OCP analysis	Ticks collected
WF1	Female	Normal	Adult	In-situ Site2	1	2	Yes	Yes	Yes	Yes
WM2	Male	Normal	Adult	In-situ Site1	1	3	No (sample donated)	Yes	Yes	Not collected (sample donated)
WM1	Male	Normal	Adult	In-situ Site1	1	2	Yes	Yes	Yes	Not collected (sample donated)
WM3	Male	Normal	Adult	In-situ Site1	1	3	No (sample donated)	Yes	No (sample not adequate for analysis)	Not collected (sample donated)
WM4	Male	Normal	Adult	In-situ Site3	1	3	Yes	Yes	Yes	Yes
WM5	Male	Normal	Adult	In-situ Site3	1	3	Yes	Yes	Yes	Yes
WF3	Female	Normal	Subadult	In-situ Site3	1	3	Yes	Yes	Yes	Yes
WF2	Female	Erythrystic	Adult	In-situ Site2	1	3	No (only dried blood scabs collected from frozen leopard)	Yes (only dried blood scabs collected from frozen leopard)	No (only dried blood scabs collected from frozen leopard)	Yes

In-situ Site1	23°02’17.1”S, 29°26’26.5”E
In-situ Site2	25°9’51.31”S, 30°26’55.46”E
In-situ Site3	24°34’43.50”S, 31°25’46.48”E

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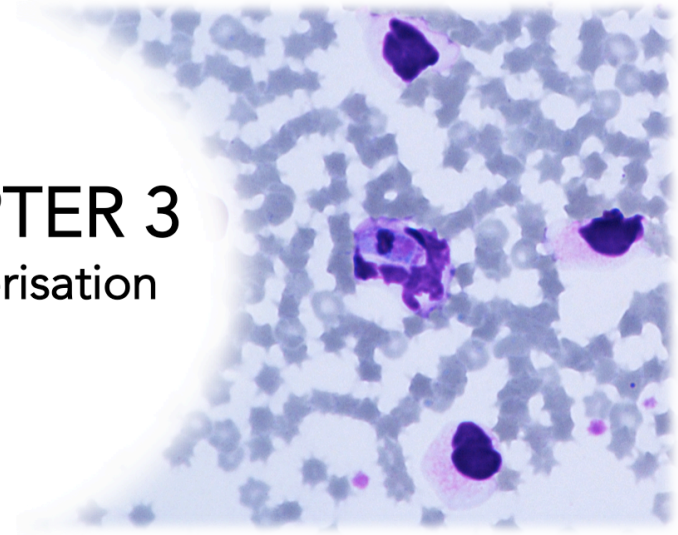
CHAPTER 3
Haematological characterisation of leopards



3.1 Introduction

Blood, the plasmic fluid containing various cell types, nutrients, metabolites and electrolytes, circulates the whole vascular system (Weiss & Wardrop 2011) and provides a diversity of potentially handy variables for the monitoring of health in wildlife (Maceda-Veiga et al. 2015). In vertebrates, peripheral blood can reflect the functioning of a whole organism (Weiss & Wardrop 2011), making it the most informative, non-lethally sampled tissue available to researchers for the assessment of animal health (Kumar et al. 2014). Singh et al. (1999) stated that "the determination of hematological values are useful for understanding the state of animal health" and Du Plessis (2009) added that basic knowledge on the structure and function of blood elements may serve to alert researchers of underlying clinical conditions when parameters are outside normal ranges. Systematic build-up of information on basic haematological values in wildlife populations may in future serve as a valuable conservation tool, serving as early indicators of threatened populations (Maceda-Veiga et al. 2015). Therefore, as pointed out by Hawkey et al. (1990) and Maceda-Veiga et al. (2015), it is important to establish the normal ranges of blood cells in mammals, even though haematology is still an obscure science for wildlife researchers (Maceda-Veiga et al. 2015). Another complication, as stated by Maceda-Veiga et al. (2015), is that the apparent norms for domestic animal haematology are not always applicable to wildlife species.

Haematology generally serves as a tool to assess the ability to fight infections, and by implication, general health and, being species-specific, the baseline haematological values are unknown for the majority of mammalian species, especially rare species (Pavlova et al. 2015). Haematological values can potentially indicate pathological conditions associated with stress (Pavlova et al. 2015), for example according to Maceda-Veiga et al. (2015), an animal's stress can be measured indirectly by looking at the white blood cell (WBC) counts. Although individual traits may influence physiological responses to contaminants (Evans 2008, Cabarcas-Montalvo

et al. 2012), the similarities in vertebrate metabolic pathways make the prediction of how health is affected in single taxa applicable to a broader spectrum (Maceda-Veiga et al. 2015).

Haematological values can be influenced by factors such as sex, age, individual differences and seasons (Lumsden 1998, Touma & Palme 2005). By studying blood smears, the morphology of red blood cells (RBCs) and their dimensions can be determined, providing information on the presence and effect of intraerythrocytic parasites, metabolic rate and exposure to contaminants (Llacuna et al. 1996, Gregory 2001, Davis 2008). Differential WBC counts or leukograms are a popular blood marker used as a measure of stress or innate immune responses (Pavlova et al. 2015, Maceda-Veiga et al. 2015). This measure is usually defined as the relative proportion of neutrophils to lymphocytes, called the N:L ratio (NLR) (Davis et al. 2008, Maceda-Veiga et al. 2015), as the effects of stress can cause an opposite effect in the numbers of neutrophils and lymphocytes within an individual (Pavlova et al. 2015). According to Pavlova et al. (2015), a high NLR typically coincides with elevated stress, immunosuppression and disease caused by endoparasites in animals.

Even though clinical haematology has a central role in health assessment of domestic animals, the lack of haematological baseline values for many wild vertebrate species is a key limiting factor in using it to assess the health of wildlife (Maceda-Veiga et al. 2015). Discrepancies also exist in how haematological data is gathered and reported, further complicating and hampering comparisons to be made from literature (see Davis et al. 2008). The haematology of domestic cats *Felis catus* Linnaeus, 1758 has been well reported (see e.g. colour atlases by Valli 2007, Weiss & Wardrop 2011, and Valenciano et al. 2014), but there are only a few reports on the haematology of wild cat species (Jain 1993, Pothiwong et al. 2006). Reports from wild cat species include haematological values in leopards (Singh et al. 1999, Sabapara et al. 2008, Salakij et al. 2009, Khoshnegah et al. 2012, Gupta & Kumar 2013, Mohapatra et al. 2014, Shanmugam et al. 2017, Shrivastav et al. 2019) and cheetahs (Hawkey & Hart 1986, Caro et al. 1987, Du Plessis et al. 2004), Snow leopards (Kumar et al. 2014, Hussain et al. 2016), tigers (Fowler 1986, Seal et al. 1987, Singh et al. 1999, Chandranaik et al. 2006, Sajjad et al. 2012, Boon et al. 2019, Proverbio et al. 2021), Canadian lynx *Lynx canadensis* Kerr, 1792 (Weaver & Johnson 1995), cougars (pumas) *Puma concolor* (Currier & Russel 1982, Dunbar et al. 1997), bobcats *Lynx rufus* (Schreber, 1777) (Miller et al. 1999), jaguars (Hawkey & Hart 1986, Deem 2004, Mussart et al. 2009, Widmer et al. 2012, Perez & Paredes 2013), leopard cats *Prionailurus bengalensis* (Kerr, 1792) (Salakij et al. 2010), clouded leopards *Neofelis nebulosa* (Griffith, 1821) (Singh et al. 1999, Salakij et al. 2008a), jungle cats *Felis chaus* Schreber, 1777 (Salakij et al. 2011), Pallas cats *Otocolobus manul* (Pallas, 1776) (Pavlova et al. 2015), Bengal tigers *Panthera tigris tigris* (Singh et al. 1999, Shrivastav & Singh 2012), Siberian tigers *Panthera tigris altaica* (Linnaeus, 1758) (Larsson et al. 2015, Liu et al. 2021) and lions (Du Plessis 2009, Maas et al. 2013, Larsson et al. 2015). Most of these reports are from captive animals in ex-situ facilities.

Morphologic characteristics, such as cell characteristics and populations of blood cells from members of the family Felidae are heterogenous (Salakij et al. 2009). Variations in cell characteristics can be found between species within the Felidae, and according to Anderson et

al. (1971), the size and number of RBCs vary even within one species (domestic cats) according to the age of the individual. Shrivastav et al. (2012) found similar trends by reporting minor differences when they compared the blood pictures of wild and captive Bengal tigers (Seal et al. 1987). However, an interesting study done by Du Plessis (2009) found the platelet morphology in African lions to be highly similar to that of cheetahs, with only a slight size variance (Du Plessis et al. 2004). In some wild cat species, such as cougars (Currier & Russel 1982) and Canadian lynx (Weaver & Johnson 1995), no haematological differences can be seen between males and females. However, in other species such as bobcats (Miller et al. 1999), lions and Siberian tigers (Larsson et al. 2015), differences in leukograms can be easily seen between the genders. In some cases it also seems that these values are the same in young individuals and adults (e.g. in Canadian lynx) (Weaver & Johnson 1995).

In the late 1990s, Singh et al. (1999) were the first to conduct a basic, baseline study of the normal haematological values of captive leopards from a tropical region (Sanjay Gandhi Biological Park, India). Due to the limited knowledge on the ultrastructural features of blood cells in leopards (see haematological reference created for Indian leopards *Panthera pardus fusca* (Meyer, 1794) by Sabapara et al. 2008), Salakij et al. (2009) carried out a baseline study on the haematological and ultrastructural characteristics of blood cells of clinically healthy captive leopards in the Khao Kheaw Open Zoo in Thailand. Kumar et al. (2014) reported baseline haematological ranges from captive Snow leopards in India.

Singh et al. (1999)'s study focused on providing baseline haematological values for tigers, leopards and clouded leopards in captivity, and they found optimum concentration of total erythrocyte count in tigers and female leopards of different age groups, but all after the age of puberty. During their study on Indian leopards, Sabapara et al. (2008) found no significant difference in haematological values of captive males and females, and they found the haematological values of the Indian leopard comparable to that of other leopard subspecies, stating that it could be viewed as a guideline to detect possible pathological cases. Salakij et al. (2009) showed the erythrocytes of leopards to be slightly anisocytosic, containing Heinz bodies and with blunt end crenation, features that are similar to the erythrocytes of clouded leopards (Salakij et al. 2008a), flat-headed cats *Prionailurus planiceps* (Vigors & Horsfield, 1827) (Salakij et al. 2008a, 2008b), fishing cats *Prionailurus viverrinus* (Bennett, 1833) (Prihirunkit et al. 2007) and domestic cats (Jain 1993). During their study, Salakij et al. (2009) found that the packed cell volumes (PCVs) and absolute eosinophil numbers were significantly higher in male leopards than females and during differential leukocyte counts, Salakij et al. (2009) found the most numerous circulating leukocyte to be neutrophils, followed by lymphocytes, eosinophils and monocytes. This is a similar trend to that of domestic cats (Latimer et al. 2003).

This chapter aims to provide a detailed guide for morphological characterization of blood cells and haematological characteristics observable under a light microscope in captive and wild African leopards, and it is the first study in South Africa to do so.

3.2 Materials and Methods

3.2.1 Data collected in the Field

Capture and sedation of leopards, as well as basic field data collection and processing took place as described in Chapter 2 of this thesis.

Thin blood smears

A Giemsa (Sigma-Aldrich®) solution was prepared with distilled water with a ratio of 9:1 in a 50 ml staining container. Air-dried, thin blood smears were stained in the Giemsa solution for 20 minutes (see Van As et al. 2020), rinsed with a slow stream of distilled water and again left to air dry. Smears were then stored away in dust free boxes until they could be individually scanned for infections.

3.2.2 Data collected in the lab

Screening of blood smears and haematological data

Stained smears were completely examined under the 100x oil immersion objective of a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, The Netherlands) and photographs taken with an attached Nikon DS-Fi1 digital camera and accompanying software. Photographs of blood smears were calibrated according to the guidelines stipulated by the ImageJ Image Processing and Analysis software (Rasband 2014) and the length, width and areas of blood components and parasites were subsequently measured. Blood elements were morphologically characterized according to specifications of Valenciano et al. (2014). Measurements were obtained using the ImageJ version 1.47 software program (Wayne Rasband National Industries of Health, USA) (http://imagej.nih.gov/ij) from micrographs, taken with a 100x oil immersion objective, from both infected and uninfected erythrocytes and white blood cells, as well as their accompanying blood parasites (see Fig. 3.1). All measurement data was recorded in Microsoft® Excel spreadsheets and the average, standard deviation, minimum and maximum values were calculated for each cell and parasite type.

Only the monolayer regions of blood smears were considered for data collection in relation to differential cell counts (DCCs) and 10 fields for each leopard under the 40x objective of a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, The Netherlands) were photographed as described above. Thick areas of the blood smear and feathered edges were ignored for this type of data collection, as these areas on the slide will not give an accurate representation of cell counts (Valenciano et al. 2014). Blood cells were counted on the microphotographs, using the ImageJ version 1.47 software program (Wayne Rasband National Industries of Health, USA)

(http://imagej.nih.gov/ij). Differential cell counts and NLRs were determined by counting at least 200 leukocytes from each leopard (Salakij et al. 2009).

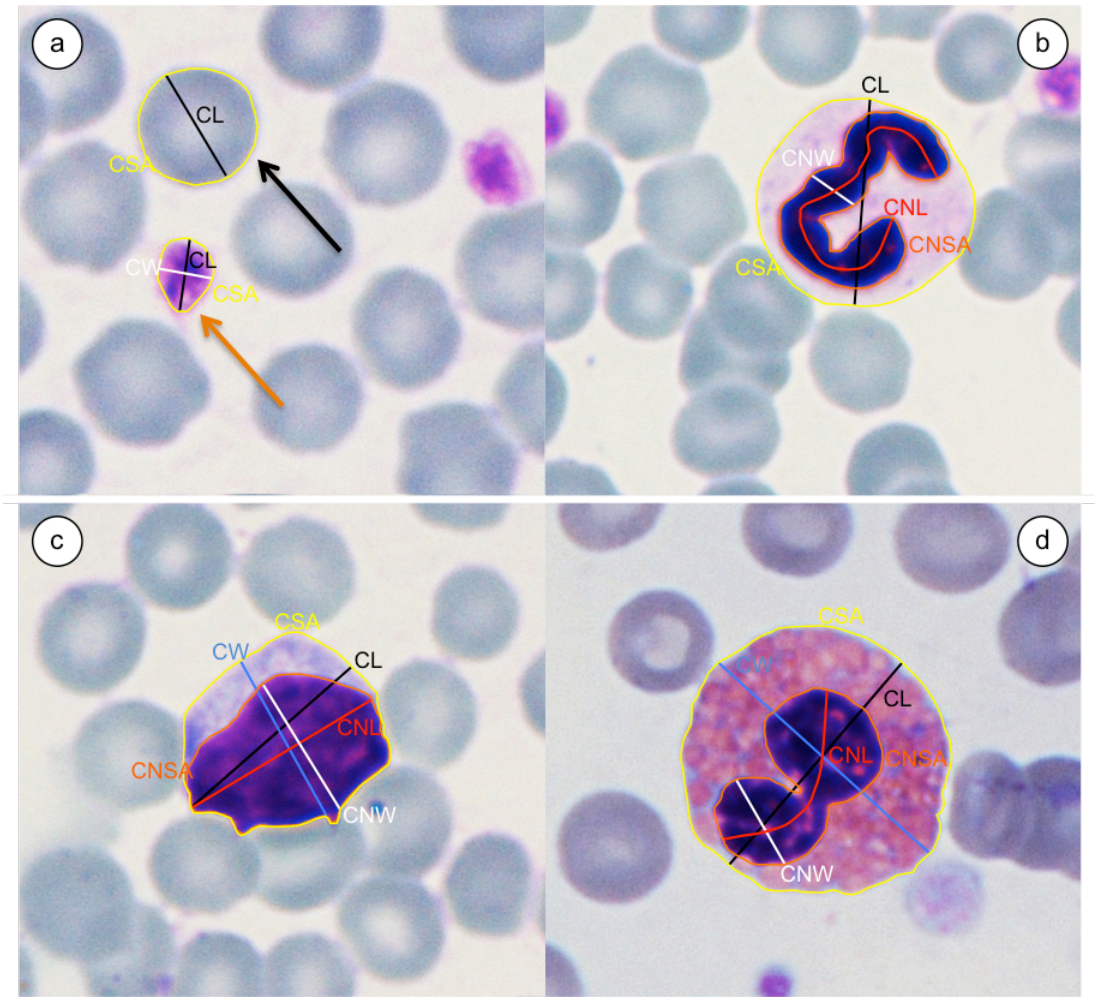


Figure 3.1 Annotated micrographs of Giemsa stained blood smears showing healthy leopard blood cells and their measurements (in colored lines). Healthy blood cells include **a** erythrocytes (black arrow), platelets (orange arrow), **b** neutrophils, **c** lymphocytes and **d** eosinophils. CL – cell length; CW – cell width; CSA – cell surface area; CNL – cell nucleus length; CNW – cell nucleus width; CNSA – cell nucleus surface area.

3.3 Results

Refer to Chapter 2, Tables 2.3 and 2.4 to see which samples were obtained for which captive and wild leopards in this study.

3.3.1 Morphological characteristics of blood elements

Erythrocytes

Figure 3.2 a – l

Anucleate, cup-shaped cells, lacking central pallor (Fig. 3.2 a – c). Erythrocytes measured 5.47 ± 0.60 (4.28–7.02) μm in diameter, with an area of 22.77 ± 4.54 (14.41–36.25) μm^2, stained uniformly and were homogenous in colour (Table 3.1). Erythrocyte diameter was similar in females and males, even though the area of female RBCs was slightly smaller than that of males ($p > 0.05$). Erythrocyte diameter and area were similar in wild and captive individuals ($p > 0.05$) (Table 3.1). The only cub sampled during this study (CM1), a male, had the smallest RBC diameter and area of all leopards from this study. Erythrocyte diameter and area was similar among melanistic and regular leopards ($p > 0.05$).

Rouleaux formations, groups of RBCs forming linear stacks (Fig. 3.2 a), and agglutination (Fig. 3.2 b) were observed in the monolayer of some leopards. The blood smears of three leopards (CM2 and CF3 and WF3) revealed hypochromic RBCs, which were pale mature cells with notable central pallor, changing to light red at the periphery of the cells (Fig. 3.2 c). Several leopards also showed signs of anisocytosis (Fig. 3.2 e – i), with some macrocytes (Fig. 3.2 e), microcytes (Fig. 3.2 e) and poikilocytes (Fig. 3.2 f – i) observed. Keratocytes, or blister cells (Fig. 3.2 g – h), as well as acanthocytes (Fig. 3.2 i – j), was observed in some individuals. Most leopards had Howell-Jolly bodies present in their RBCs (Fig. 3.2 d, j), and some individuals also had Heinz bodies, sometimes seen as pale areas within the RBCs (Fig. 3.2 g, l). Basophilic stippling was only seen in two wild males (WM1 and WM5) (Fig. 3.2 j), and no nucleated RBCs were observed. Some individuals had cytoplasmic holes in their RBCs (Fig. 3.2 k - l).

Leukocytes

Figure 3.3 a – l

Only characteristics of neutrophils, lymphocytes and eosinophils are reported, as there were too few basophils and other leukocyte elements detected to form accurate descriptions.

Neutrophils

Figure 3.3 a – g

Neutrophils were the most prevalent WBC and had a diameter of 10.91 ± 1.10 (8.60–13.60) μm and an area of 92.07 ± 18.23 (53.35–132.89) μm^2. These round cells had clear cytoplasm, with some cells showing slightly pink distinct cytoplasm granules and coiled, multi-lobed and rare mono-lobed nuclei (Fig. 3.3 a – g). The nuclei were highly condensed and measured 20.94 ± 3.87 (11.03–29.70) μm long by 2.76 ± 0.64 (1.77–5.25) μm wide, with an area of 43.84 ± 7.41 (28.05–60.73) μm^2 (see Table 3.1). Multi-lobed nuclei generally had two to four lobes, and in some cases faint bluish-pink granules could be seen in the cytoplasm. Nuclear lobes were often connected by either a thin strand of chromatin (Fig. 3.3 a, f), or by a simple narrowing of the chromatin between the lobes (Fig. 3.3 b, d). The diameter and area of neutrophils was smaller in females than males ($p > 0.05$). Wild leopards had slightly smaller neutrophils than captive leopards ($p > 0.05$). The cub's neutrophils had the largest variation in diameter and area. There were no significant differences between the neutrophil measurements of regular and

melanistic leopards ($p > 0.05$). Even though the neutrophil diameter of regular leopards were slightly larger than that of melanistic leopards, the neutrophil area of regular leopards were slightly smaller than that of melanistic leopards ($p > 0.05$). The neutrophil nuclei of females were slightly longer and narrower than that of males ($p > 0.05$), with a slightly larger area ($p > 0.05$). There was no significant difference in the measurements of nuclei of wild and captive leopards ($p > 0.05$). The neutrophil nuclei of wild leopards were shorter and slightly wider than that of captive leopards (Table 3.1). The nuclei of wild leopards were also slightly smaller in area ($p > 0.05$). All neutrophil nuclei parameters were the smallest in subadults and the cub had the longest, widest nuclei, with the largest areas. The neutrophil nuclei of regular leopards were slightly longer and narrower, with a larger area than that of melanistic leopards ($p > 0.05$).

Band neutrophils were seen in the blood smears of most leopards (Fig. 3.3 c), and Barr bodies were observed in the neutrophils of most females (Fig. 3.3 d). Foamy cytoplasm was only seen in WM4 and hyper-segmented neutrophils were observed in CF1, CM2 and WM5 (Fig. 3.3 f). All the neutrophils of CM2 were hyper-segmented, with the nuclei lying at the periphery of the cell. Giant neutrophils were present in the blood smears of two individuals (CM2 and CF4), both melanistic and from the same facility (Fig. 3.3 g).

Lymphocytes

Figure 3.3 d; h – k

Lymphocytes were the second most prevalent WBC and well differentiated, measuring 10.40 ± 0.9 (9.6–12.7) μm in diameter, with an area of 83.61 ± 27.25 (40.17–152.86) μm^2. The nuclei were densely chromaticised and stained a dark purple, and usually longer 9.64 ± 1.87 (6.53–15.12) μm than they were wide 7.42 ± 1.23 (4.68–10.41) μm (diameter of 8.53 ± 1.33 (6.71–11.12) μm), with an area of 57.06 ± 14.57 (29.63–91.45) μm^2 (see Table 3.1) (Fig. 3.3 d, h). Small and large lymphocytes were observed, with some lymphocytes containing azurophilic granules in their cytoplasm (Fig. 3.3 i). The diameter of lymphocytes was slightly smaller among females ($p > 0.05$). However, the area of lymphocytes was slightly larger among males ($p > 0.05$). The diameter and area of lymphocytes of wild leopards was slightly smaller than that of captive leopards ($p > 0.05$). Overall, the diameter and area of lymphocytes were the smallest among subadults, followed by that of the cub and then adults. There was no significant differences between the lymphocyte dimensions of regular and melanistic leopards ($p > 0.05$) (see Table 3.1), however the area of lymphocytes was larger, in regular leopards than in melanistic leopards ($p > 0.05$). The lymphocyte nuclei of females were slightly shorter, but just as wide as that of males. The area of nuclei of females were slightly larger than that of males ($p > 0.05$). The length, width and area of lymphocyte nuclei was slightly smaller in wild leopards than in captive leopards ($p > 0.05$). Subadults had nuclei with the smallest area ($p > 0.05$), with that of the cub longer but narrower, and a larger area than that of adults. The lymphocyte nuclei of regular leopards were longer, wider and with a slightly larger area than that of melanistic leopards ($p > 0.05$).

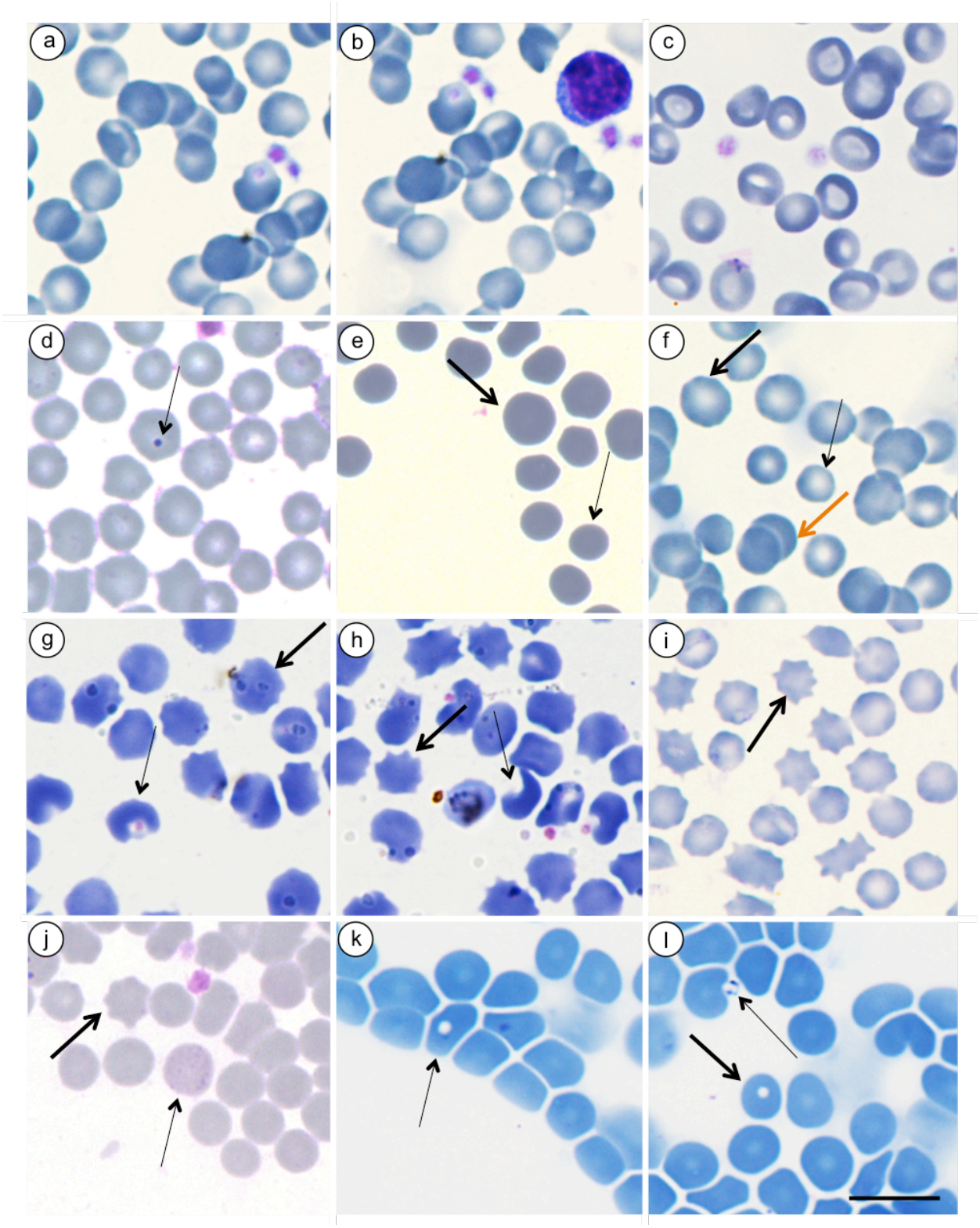


Figure 3.2 Morphological characteristics of erythrocytes of leopards. Erythrocytes presented as cup-shaped anucleate, disc-like cells. **a** Rouleaux formations of erythrocytes. **b** Agglutination of erythrocytes. **c** Hypochromic erythrocytes. **d** Howell-Jolly body (thin black arrow). **e** Anisocytosis of erythrocytes showing macrocytes (thick black arrow) and microcytes (thin black arrow). **f** Slightly hypochromic erythrocytes with anisocytosis (macrocytes – thick black arrow; microcytes (thin black arrow); regular erythrocyte (orange arrow)). **g** Heinz body (thin black arrow) and poikilocytes (thick black arrow). **h** Keratocytes (blister cells) (thin black arrow) and poikilocytes (thick black arrow). **i** Poikilocytes (thick black arrow) of various shapes and sizes. **j** Basophilic stippling (thin black arrow) and poikilocytes (thick black arrow). **k** Artifacts/cytoplasmic holes (thin black arrow) in erythrocytes. **l** Heinz-body (thin black arrow) and cytoplasmic holes (thick black arrow). Scale bar = 10 μm.

Granular lymphocytes, with pinkish purple intracytoplasmic granules dispersed throughout the cytoplasm, were detected in most leopards (Fig. 3.3 j – k). Granules were either dispersed throughout the cytoplasm or concentrated in a perinuclear region. Macrophages were only observed in CF3 and WM1.

Eosinophils

Figure 3.3 l

Eosinophils had distinct round bright pink granules which usually filled the entire cytoplasm and measured 13.17 ± 1.74 (10.02–17.25) μm in diameter, with an area of 131.85 ± 32.66 (79.04–187.80) μm^2. The cytoplasm usually stained pale blue, faintly basophilic, or was clear. Eosinophil nuclei were less lobulated and chromatised than that of neutrophils, but still slightly lobed, often divided into two distinct lobules (Fig. 3.3 l). Nuclei were generally longer than they were wide and measured 15.39 ± 3.87 (8.98–21.80) μm long by wide 4.62 ± 1.41 (3.06–8.08) μm, with an area of 52.00 ± 13.28 (32.12–78.35) μm^2 (see Table 3.1). Eosinophils were observed in the blood smears of all leopards. The eosinophils of females were slightly smaller in diameter and area than that of males ($p > 0.05$) (see Table 3.1). Wild leopards had eosinophils with smaller diameter and area than captive leopards ($p > 0.05$) (see Table 3.1). The cub (CM1) had the largest eosinophils with the most variation in diameter and area. The area of eosinophils of subadults were the smaller than that of adults ($p > 0.05$). Eosinophil diameter and area was larger in regular leopards than in melanistic leopards, ($p > 0.05$) (see Table 3.1). Eosinophil nuclei of females were slightly narrower, shorter and with a slightly smaller area than that of males ($p > 0.05$) (Table 3.1). Eosinophil nuclei of wild leopards were significantly shorter ($p < 0.05$), slightly narrower and with a smaller area than that of captive leopards ($p > 0.05$). Eosinophil nuclei area was smallest among subadults, followed by adults and then the cub. Variation in area was largest among subadults. The length, width and area of eosinophil nuclei was slightly smaller among melanistic leopards ($p > 0.05$) (see Table 3.1).

Platelets

Figure 3.4 a – g

Platelets were usually distributed in small to large clumps (Fig. 3.4 a), with pleomorphic individual platelets (Fig 3.4 b – e). Platelets were anucleated, round to oval in shape and were elongated to rounded (Figure 3.4 a – g). They measured 3.07 ± 0.61 (2.01–4.60) μm long (maximum projection) by 2.61 ± 0.43 (1.75–3.62) μm wide (minimum projection), with an area of 6.35 ± 2.13 (2.94–11.90) μm^2 (see Table 3.1). They stained light blue to light pink and had prominent reddish purple granules. This was the smallest element observed in the peripheral blood of leopards. There were significant differences in platelet morphology of males and females. The platelets of male leopards measured significantly longer, wider and with a larger area than that of females ($p < 0.05$). The length of platelets was the same among wild and captive leopards, but the width and area of that of captive leopards was slightly larger than that of wild leopards ($p > 0.05$) (see Table 3.1). The smallest platelets were observed among adults,

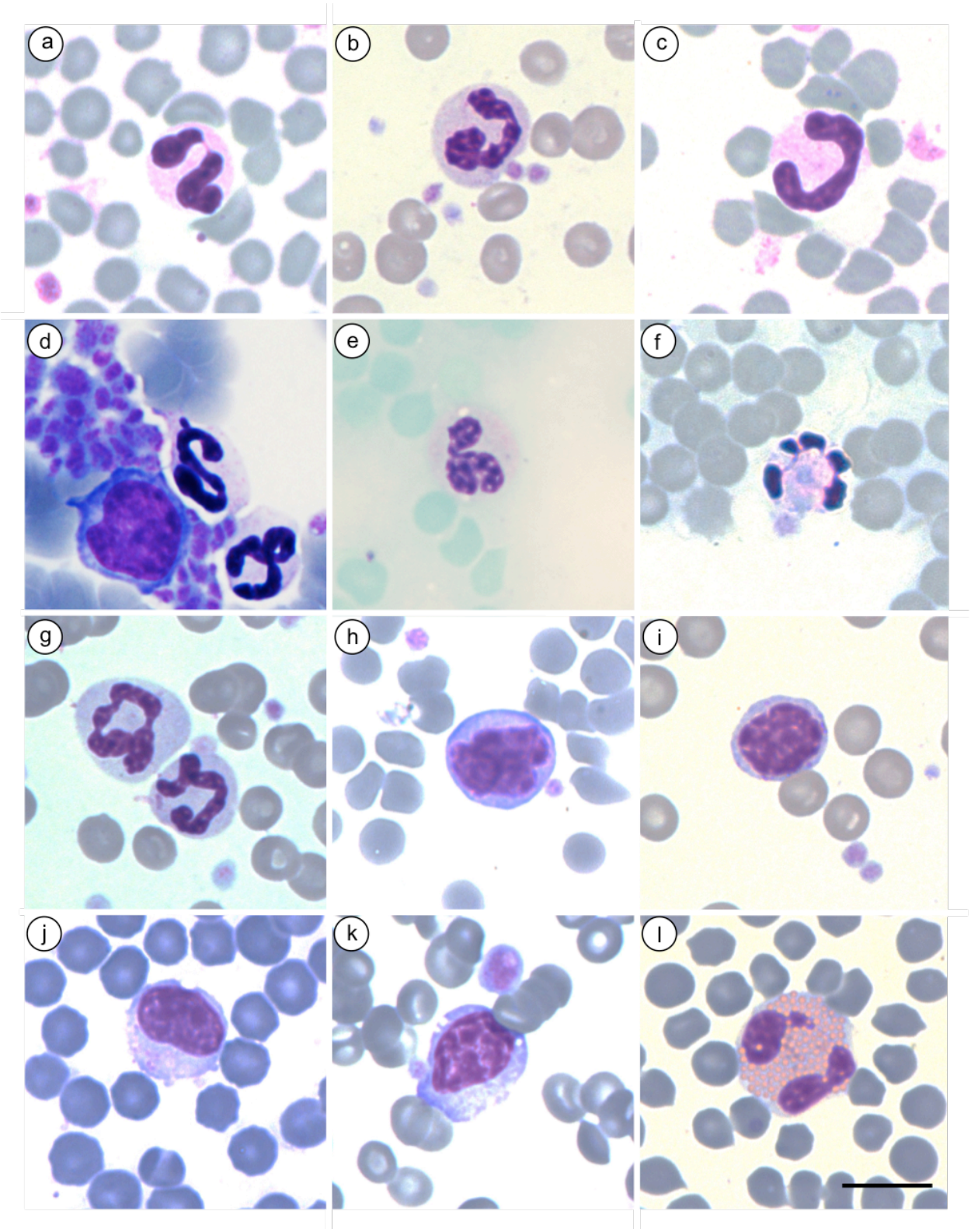


Figure 3.3 Morphological characteristics of leukocytes of leopards. **a – g** Variety of neutrophils seen in peripheral blood. **a** Nuclear lobes connected by a thin strand of chromatin. **b** Nuclear lobes connected by a narrowing of chromatin between the lobes. **c** Band neutrophil. **d** Neutrophil with barr body; regular mature lymphocyte. **e** Neutrophil with concentrated lobes. **f** Hypersegmented neutrophil. **g** Nuclei lobe in giant neutrophil extremity touch to form a circle; regular mature neutrophil. **h – k** Variety of lymphocytes in peripheral blood. **h** Reactive Lymphocyte. **i** Lymphocyte with azurophilic granules in cytoplasm. **j** Granular lymphocyte. **k** Granular lymphocyte. **l** Eosinophil. Scale bar = 10 μm.

followed by subadults and the cub ($p > 0.05$). Platelet area of regular leopards were slightly larger than that of melanistic leopards ($p > 0.05$) (Table 3.1). Clumps of platelets were seen in the blood smears of six leopards. Megathrombocytes (Fig. 3.4 a – f), some of which activated (Fig. 3.4 e – g), were seen in some individuals, both wild and captive, in both males and females.

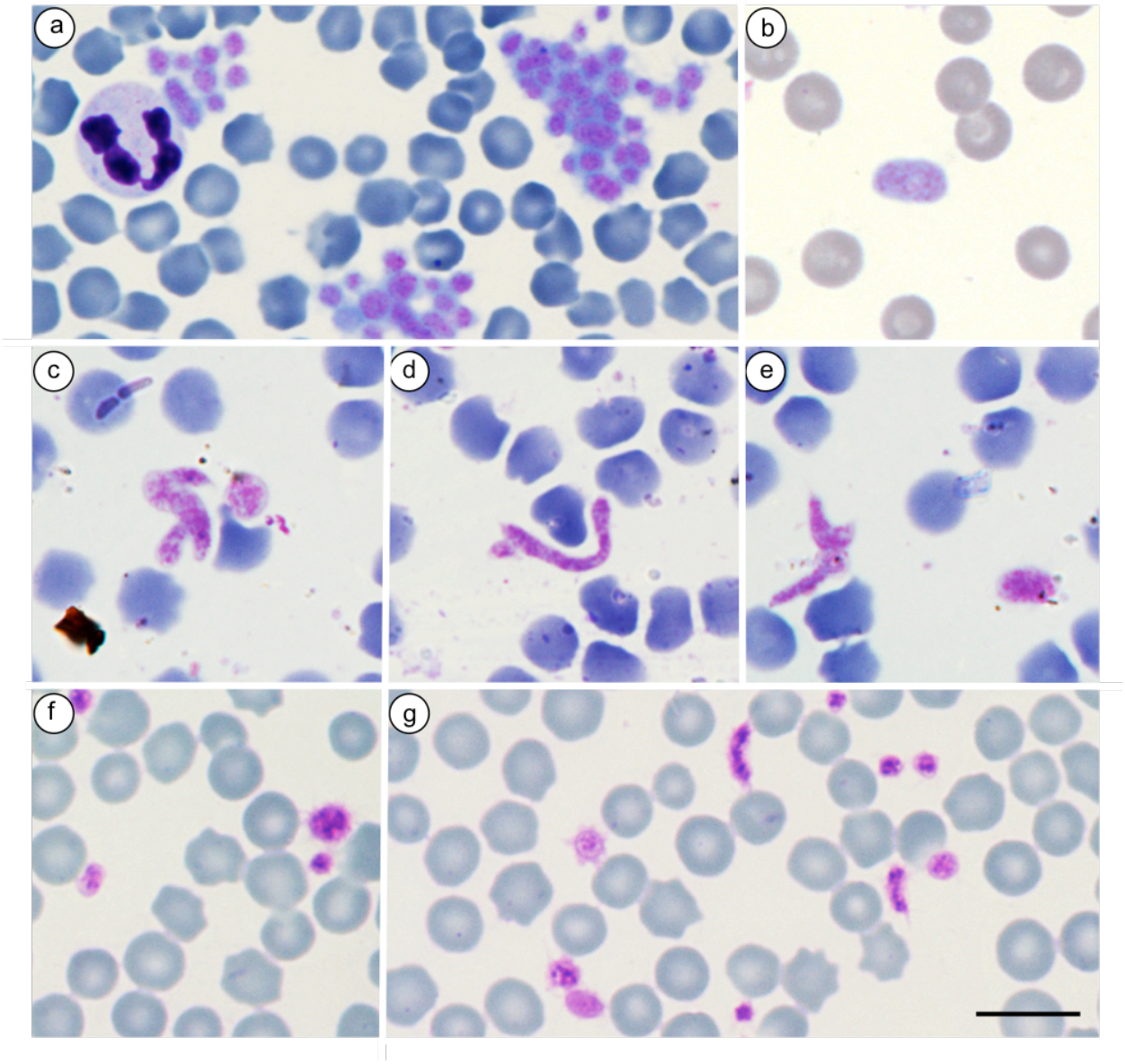


Figure 3.4 Morphological characteristics of platelets of leopards. **a** Clumped group of several distinct platelets. **b** Single platelet. **c** Macroplatelet in between regular platelets. **d** Macroplatelet with regular platelet lying at its extremity. **e** Two overlapping macroplatelets and a regular platelet. **f** Activated platelets with thin cytoplasmic projections. **g** Activated platelets and macroplatelets with thin cytoplasmic projections. Scale bar = 10 μm.

Table 3.1 Morphologicall characteristics of leopard blood cells. Dimensions are expressed as (Average ± Standard deviation (range)) in μm (diameter, length, width) and μm^2 (area). Numbers in brackets indicate number of samples analyzed, including resampling of three captive leopards. Values in bold font indicate significant differences ($p < 0.05$) in tested category.

		All leopards (16)	Wild (5)	Captive (11)	Males (7)	Females (9)	Regular (12)	Melanistic (4)
Erytrhocytes								
	Diameter	5.47±0.60 (4.28-7.02)	5.40±0.31 (5.0-5.9)	5.43±0.31 (4.74-6.12)	5.42±0.42 (4.73-6.11)	5.42±0.30 (4.81-5.90)	5.42±0.44 (4.73-6.12)	5.42±0.11 (5.32-5.51)
	Area	22.77±4.54 (14.41-36.25)	22.43±4.25 (13.54-32.59)	22.95±4.69 (14.89-38.28)	22.87±4.25 (14.13-35.34)	22.68±4.75 (14.62-36.93)	22.83±4.79 (13.98-37.52)	22.54±3.60 (15.98-31.57)
Leukocytes								
Neutrophils								
Cell	Diameter	10.91±1.10 (8.60-13.60)	10.78±1.34 (8.62-12.41)	10.99±1.01 (9.24-13.63)	11.31±0.62 (10.43-12.43)	10.61±1.32 (8.63-13.60)	10.92±1.24 (8.61-13.62)	10.92±0.70 (9.80-11.91)
	Area	92.07±18.23 (53.35-132.89)	89.81±17.56 (59.21-124.73)	93.32±18.60 (50.10-137.42)	98.01±17.84 (55.09-129.98)	87.61±18.52 (52.05-135.07)	91.98±17.56 (55.24-129.18)	92.41±20.70 (46.42-146.48)
Nuclei	Length	20.94±3.87 (11.03-29.70)	19.96±3.86 (10.20-27.12)	21.48±3.88 (11.49-31.14)	20.09±3.93 (10.04-28.99)	20.09±3.93 (10.04-28.99)	21.06±3.98 (10.87-29.64)	20.48±3.45 (11.62-29.94)
	Width	2.76±0.64 (1.77-5.25)	2.77±0.59 (2.01-4.75)	2.76±0.67 (1.66-5.53)	2.79±0.53 (2.01-4.46)	2.79±0.53 (2.01-4.46)	2.75±0.57 (1.80-4.72)	2.80±0.88 (1.74-7.17)
	Area	43.84±7.41 (28.05-60.73)	43.74±6.75 (31.93-56.62)	43.90±7.78 (25.90-63.01)	45.05±7.47 (29.02-59.15)	45.05±7.47 (29.02-59.15)	44.30±7.42 (28.68-59.99)	42.17±7.38 (25.76-63.44)
Lymphocytes								
Cell	Diameter	10.40±0.94 (9.63-12.71)	10.24±0.93 (8.62-11.52)	10.49±0.91 (9.01-12.73)	10.44±0.78 (9.57-11.63)	10.37±1.14 (8.46-12.72)	10.39±1.03 (8.62-12.71)	10.45±0.83 (9.42-11.63)
	Area	83.61±27.25 (40.17-152.86)	81.98±28.61 (44.22-158.21)	84.51±26.50 (37.92-149.88)	84.24±28.73 (38.89-161.86)	83.14±26.15 (41.12-146.11)	83.63±26.63 (44.14-149.15)	83.53±29.55 (25.60-166.44)
Nuclei	Diameter	8.53±1.33 (6.71-11.12)	8.34±1.24 (6.84-9.93)	8.64±1.32 (6.72-11.14)	8.59±1.31 (7.31-11.14)	8.49±1.33 (6.71-10.63)	8.56±1.24 (6.74-11.13)	8.45±1.30 (7.21-10.01)
	Area	57.06±14.57 (29.63-91.45)	55.04±14.76 (30.45-89.56)	58.19±14.46 (29.17-92.50)	56.83±15.61 (27.86-95.97)	57.24±13.80 (30.95-88.05)	57.24±14.37 (32.06-90.32)	56.40±15.29 (20.73-95.59)
Eosinophils								
Cell	Diameter	13.17±1.74 (10.02-17.25)	12.40±1.33 (10.02-14.33)	13.59±1.84 (11.13-17.23)	13.32±1.63 (11.12-17.23)	11.22±5.52 (1.63-21.11)	13.24±1.72 (10.01-17.21)	12.91±1.91 (11.12-16.43)
	Area	131.85±32.66 (79.04-187.80)	118.74±30.98 (70.71-174.31)	139.13±33.60 (83.66-195.29)	137.19±36.20 (82.33-193.15)	127.84±30.01 (76.57-183.79)	133.01±31.79 (79.89-189.03)	127.58±35.86 (75.91-183.30)
Nuclei	Length	15.39±3.87 (8.98-21.80)	**13.65±3.77 (7.98-19.79)**	**16.36±3.92 (9.53-22.91)**	14.37±3.65 (9.06-20.50)	16.15±4.03 (8.92-22.77)	15.60±3.93 (8.76-21.77)	14.63±3.63 (9.75-21.91)
	Width	4.62±1.41 (3.06-8.08)	4.59±1.44 (2.98-7.75)	4.63±1.39 (3.11-8.25)	4.83±1.43 (3.25-7.82)	4.46±1.39 (2.92-8.26)	4.67±1.49 (3.05-8.32)	4.44±1.13 (3.09-7.20)
	Area	52.00±13.28 (32.12-78.35)	46.31±10.84 (30.76-69.74)	55.17±14.63 (32.87-83.14)	52.75±12.03 (35.21-73.40)	51.45±14.22 (29.80-82.06)	52.87±13.19 (32.28-78.64)	48.84±13.60 (31.52-77.30)
Platelets								
Cell	Maximum projection	3.07±0.61 (2.01-4.60)	3.05±0.66 (1.94-4.54)	3.09±0.58 (2.04-4.63)	**3.53±0.72 (2.29-5.50)**	**2.75±0.52 (1.81-3.95)**	3.09±0.62 (2.05-4.64)	3.02±0.57 (1.90-4.47)
	Minimum projection	2.61±0.43 (1.75-3.62)	2.59±0.49 (1.65-3.70)	2.62±0.39 (1.80-3.59)	**2.98±0.50 (2.09-4.06)**	**2.34±0.38 (1.52-3.31)**	2.58±0.40 (1.79-3.52)	2.68±0.49 (1.64-3.95)
	Area	6.35±2.13 (2.94-11.90)	6.10±2.35 (2.72-11.64)	6.48±2.03 (3.05-12.02)	**8.25±2.77 (3.59-15.03)**	**5.00±1.68 (2.48-9.66)**	6.41±2.09 (3.05-11.58)	6.19±2.26 (2.61-12.85)

3.3.2 Differential blood cell counts per 1 000 erythrocytes

Differential cell counts of leopards were quite variable. The differential leukocyte count (DLC) for all leopards were 4.9 ± 3.6 (1.8–11.8), and wild leopards had a higher DLC (7.6 ± 3.9 (3.2–11.6)) than captive leopards (3.7 ± 2.8 (1.8–11.8)) ($p > 0.05$) (Table 3.2). Males had a higher DLC (5.3 ± 3.6 (1.8–11.8)) than females (4.6 ± 3.8 (1.9–11.6)), while melanistic leopards had a higher DLC (5.2 ± 4.4 (2.2–11.8)) than regular leopards (4.8 ± 3.5 (1.8–11.6)) ($p>0.05$) (Table 3.2).

The neutrophil count for all leopards were 2.9 ± 2.1 (0.9–8.7) per ~ 1 000 erythrocytes. The DCCs of lymphocytes and eosinophils were 1.2 ± 1.0 (0.2–3.0) and 0.6 ± 0.9 (0.0–2.9) respectively, and the number of platelets averaged at 40.1 ± 43.0 (0.0–178.5) (see Table 3.2). Females had higher platelet counts, while males had more neutrophils and eosinophils ($p > 0.05$) (Table 3.2). Males and females had a very similar lymphocyte count ($p > 0.05$) (Table 3.2). Wild leopards had a significantly higher lymphocyte count ($p < 0.05$) and also had higher platelet, neutrophil and eosinophil counts than captive leopards ($p > 0.05$) (Table 3.2). No significant differences in DCCs across age groups were found ($p > 0.05$). Adult leopards had lower platelet, lymphocyte and eosinophil counts than subadults, but subadults had slightly higher neutrophil counts ($p > 0.05$). The only significant difference in DCCs between regular and melanistic leopards, was that regular leopards had significantly higher leukocyte counts than melanistic individuals ($p < 0.05$). Melanistic leopards had higher counts of neutrophils, eosinophils and platelets, but lower counts of lymphocytes than regular leopards ($p > 0.05$).

DLCs revealed that neutrophils were the most common type of WBC (62%), followed by lymphocytes (27%) and eosinophils (11%). No basophils were observed. The average NLR for all leopards were 5.1 ± 10.0 (0.7–41.0), and the NLR for females (3.2 ± 1.8 (0.7–7.0)) was lower than that of males (7.6 ± 15.2 (1.3–42.0)). The NLR of captive leopards (6.7 ± 11.8 (0.7–42.0)) were higher than that of wild leopards (1.8 ± 0.6 (1.3-2.8)), and that of melanistic leopards (12.8 ± 19.5 (2.7–42.0)) were much higher than that of regular individuals (2.6 ± 1.8 (0.7–7.0)) (Table 3.2).

3.4 Discussion

Few holistic publications on blood cell morphology in big cats are presently available in literature, and the majority of reports are from captive animals. A common theme from current existing literature is that there can be intra- and interspecific variation on blood cell morphology in felids. Reports on blood cell morphologies from wild African felids are few and far between, with the closest reports on the descriptions of platelets in captive cheetahs (Du Plessis et al. 2004) and lions (Du Plessis 2009). Other publications, such as that on wild lions by Maas et al. (2013), does not address morphological characteristics at all. This is the first report on the haematological characteristics of the African leopard, including information on both captive and wild populations.

Table 3.2 Differential cell counts of leopards, expressed as number of cells per ~ 1000 RBCs (Average ± Standard deviation (range)). Numbers in brackets indicate number of samples analyzed, including resampling of three captive leopards. Values in bold font indicate significant differences ($p < 0.05$) in tested category.

		All leopards (16)	Wild (5)	Captive (11)	Males (7)	Females (9)	Regular (12)	Melanistic (4)
Leukocytes		4.9±3.6 (1.8-11.8)	7.6±3.9 (3.2-11.6)	3.7±2.8 (1.8-11.8)	5.3±3.6 (1.8-11.8)	4.6±3.8 (1.9-11.6)	4.8±3.5 (1.8-11.6)	5.2±4.4 (2.2-11.8)
	Neutrophils	2.9±2.1 (0.9-8.7)	3.9±1.9 (1.7-6.1)	2.4±2.1 (0.9-8.7)	3.5±2.8 (0.9-8.7)	2.4±1.4 (1.3-4.9)	1.6±0.5 (0.9-2.3)	3.7±3.3 (1.5-8.7)
	Lymphocytes	1.2±1.0 (0.2-3.0)	**2.2±0.8 (1.2-3.0)**	**0.7±0.7 (0.2-2.4)**	1.2±0.7 (0.2-2.2)	1.2±1.2 (0.3-3.0)	0.8±0.8 (0.3-2.4)	0.6±0.3 (0.2-1.0)
	Eosinophils	0.6±0.9 (0.0-2.9)	0.8±1.0 (0.0-2.6)	0.5±0.8 (0.0-2.9)	0.7±1.0 (0.0-2.9)	0.6±0.8 (0.0-2.6)	0.3±0.1 (0.0-0.5)	0.9±1.3 (0.2-2.9)
	NLR	5.1±10.0 (0.7-41.0)	1.8±0.6 (1.3-2.8)	6.7±11.8 (0.7-42.0)	7.6±15.2 (1.3-42.0)	3.2±1.8 (0.7-7.0)	2.6±1.8 (0.7-7.0)	12.8±19.5 (2.7-42.0)
Platelets		40.1±43.0 (0.0-178.5)	62.4±73.0 (1.4-178.5)	29.9±16.7 (0.0-50.3)	31.2±21.4 (1.4-51.6)	46.9±54.8 (0.0-178.5)	23.1±17.3 (0.0-42.8)	41.7±6.2 (36.3-50.3)

3.4.1 Morphological characteristics as observed by light microscopy

The morphology and staining properties of erythrocytes, platelets, neutrophils and lymphocytes were not significantly different from those of other wild and domestic cats, as will be seen in the discussion below. This is, however, not the case for leopard eosinophils.

Erythrocyte morphologies

Erythrocytes were morphologically similar to that of other felids from the genus *Panthera*, leopards (Salakij et al. 2009), Bengal tigers (Chandranaik et al. 2006, Shrivastav & Singh 2012), as well as other members of the family Felidae (domestic cats (Jain 1993), fishing cats (Prihirunkit et al. 2007), flat-headed cats (Salakij et al. 2008b) and clouded leopards (Salakij et al. 2008a). Slightly hypochromic erythrocytes were seen in some leopards from this study. Other reports of hypochromic erythrocytes in wild feline species include that of leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011). Hypochromic RBCs are slightly haemoglobin deficient, which can indicate an iron deficiency and/or anaemia (Valenciano et al. 2014, Maceda-Veiga et al. 2015).

Erythrocyte diameter of leopards from the current study was similar to that reported for other felids. Interestingly, erythrocytes from captive leopards in Thailand (Salakij et al. 2009) were larger than those from the current study. Leopard RBC diameter was most similar to that of flat-headed cats (Salakij et al. 2008b), but was smaller than records from domestic cats (Cowell & Decker 2000), fishing cats (Salakij et al. 2008a), clouded leopards (Salakij et al. 2008a), leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011). Larger erythrocytes amongst the males from the current study may indicate that African leopards have a larger genome size amongst males (Gregory 2001) and that males may have better aerobic fitness than females (Gillooly & Zenil-Ferguson 2014). The relatively small red blood cells of the cub from this study may also underscribe this notion, but further investigation with more leopard cubs are needed in support of this statement. Anisocytosis, or the variable size of erythrocytes within an individual, is considered normal in felids (Valenciano et al. 2014). However, increased numbers of differently sized red blood cells may indicate some form of impairment of red blood cell formation (Gillooly & Zenil-Ferguson 2014) or metabolic dysfunction (Maceda-Veiga et al. 2015). Anisocytosis have been reported from other members of the genus *Panthera*. Chandranaik et al. (2006) and Shrivastav & Singh (2012) observed slight anisocytosis in captive Bengal tigers, and it was also reported in captive leopards and jungle cats (Salakij et al. 2009, 2011) . According to Gillooly & Zenil-Ferguson (2014), erythrocyte size may be used as a way to measure an organism's aerobic ability. When one keeps this in mind, the small cell size seen in the leopard cub from this study may make sense.

Rouleaux formations are often seen in healthy feline blood smears, but can also indicate possible inflammatory conditions (Valenciano et al. 2014, Maceda-Veiga et al. 2015). Jain (1986) associated this formation with erythrocyte sedimentation in domestic cats. Amongst

members of the genus *Panthera*, Bengal tigers were reported to commonly have rouleaux formations (Chandranaik et al. 2006, Shrivastav & Singh 2012) and Salakij et al. (2009) regularly observed it in the blood smears of captive leopards (Thailand). Smaller wild felids, such as flat-headed cats (Salakij et al. 2008b) and clouded leopards (Salakij et al. 2008a), are also reported to have rouleaux formations. Interestingly and similar to our findings, Salakij et al. (2008b) reported rouleaux formations from flat-headed cats with hepatozoonosis. This may underscribe the possibility that these formations may be associated with the feline body's responses to inflammation (Valenciano et al. 2014).

The presence of Heinz bodies in red blood cells is a unique and commonly observed feature to the family Felidae (Jain 1986, Valenciano et al. 2014). Heinz bodies have been observed in members of the genus *Panthera* such as Bengal tigers (Chandranaik et al. 2006, Shrivastav & Singh 2012), captive leopards (Thailand) (Salakij et al. 2009), as well as in leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011). These bodies are thought to be the result of oxidative injury (Valenciano et al. 2014), or it may indicate diabetes (Winzelberg Olson & Hohenhaus 2019) or enzymatic dysfunction (Maceda-Veiga et al. 2015). Howell-Jolly bodies, on the other hand, are fragments of red blood cell nuclei that is retained in the cell after expulsion of the nucleus upon maturation (Valenciano et al. 2014). Salakij et al. (2010) reported these bodies from red blood cells of captive leopard cats in Thailand. Howell-Jolly bodies may be indicative of abnormalities in nuclear division in erythrocytes (Maceda-Veiga et al. 2015).

Poikilocytes, or abnormally shaped erythrocytes, can occur in various forms that have various causes – such as red blood cell fragmentation or abnormalities of the red blood cell membrane (Valenciano et al. 2014). Different forms of poikilocytes have been reported from flat-headed cats that had hepatozoonosis (Salakij et al. 2008b), leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011), and the presence of poikilocytes may indicate a form of anaemia (Valenciano et al. 2014). The current study is among very few that report keratocytes and acanthocytes in feline peripheral blood smears. Keratocytes are formed from damaged erythrocytes and can be associated with disorders concerning erythrocyte abnormalities (Valenciano et al. 2014). Acanthocytes may be observed in cats with liver, bone marrow or renal disorders (Valenciano et al. 2014, Winzelberg Olson & Hohenhaus 2019). Basophilic stippling is also rarely reported, but it was observed in leopards from this study. Basophilic stippling may occur with regenerative anaemias, caused by a myriad of factors such as iron deficiency, lead poisoning (Valenciano et al. 2014, Maceda-Veiga et al. 2015) or prolonged nutritional deficiencies (Shrivastav & Singh 2012).

Leukocyte morphologies

Neutrophils

Detailed reports on wild cat neutrophil morphology and staining properties are scarce in existing literature, especially with reference to nuclei dimensions. The neutrophil morphology

from the leopards of this study, including nucleus morphology and staining properties, were similar to that of other members of the genus *Panthera* (Bengal tigers (Chandranaik et al. 2006, Shrivastav & Singh 2012); captive leopards in Thailand (Salakij et al. 2009)), as well as that of domestic cats (Valenciano et al. 2014), flat-headed cats (Salakij et al. 2008b), clouded leopards (Salakij et al. 2008a), and leopard cats (Salakij et al. 2010). The neutrophil diameter of the current study's leopards was smaller than that of clouded leopards (Salakij et al. 2008a), but larger than that of jungle cats (Salakij et al. 2011), leopard cats (Salakij et al. 2010), flat-headed cats (Salakij et al. 2008b) and captive leopards in Thailand (Salakij et al. 2009).

Band neutrophils are the most common young neutrophil released into feline peripheral blood (Valenciano et al. 2014). Similar to those from the leopards from this study and in members of the genus *Panthera*, band neutrophils have been reported from captive Siberian tigers, but were not observed in captive lions from that same study (Larsson et al. 2015). Band neutrophils have also been reported from leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011), but Salakij et al. (2008b) specifically mentions the absence of these cells in flat-headed cats. Therefore, it seems that the presence of band neutrophils are not to be expected in the blood smears of all felids, even though Valenciano et al. (2014) indicated that these cells are normally present in low numbers in healthy felids. The presence of Barr bodies in neutrophils can possibly indicate the inactive X chromosome in females (Valenciano et al. 2014) and was observed in most female leopards from this study. Barr bodies have also been documented in the neutrophils of flat-headed cats that were infected with a *Hepatozoon* spp. (Apicomplexa: Adeleorina: Hepatozoidae) (Salakij et al. 2008b), and in captive leopards (Thailand) (Salakij et al. 2009), and does not have any diagnostic significance (Valenciano et al. 2014).

The presence of hyper-segmented neutrophils, such as those observed in the leopards from this study, may indicate anaemic conditions caused by Vitamin B12 deficiency (Singh et al. 2017), which can result in erythrocyte defects leading to an undersupply of oxygen to the body. According to Valenciano et al. (2014), hyper-segmented neutrophils can be an inherited aging-related change in felids. Segmented neutrophils were reported in the peripheral blood of jungle cats (Salakij et al. 2011). Giant neutrophils were seen in some leopards from this study, but other reports of these cells in wild felids are scarce in literature. Giant neutrophils are cells that skipped cell division during rapid neutrophil production, and Valenciano et al. (2014) associates giant neutrophils with other toxic changes in the blood.

Lymphocytes

Lymphocytes of this study's leopards were morphologically similar to that of flat-headed cats (Salakij et al. 2008b), clouded leopards (Salakij et al. 2008a) and Bengal tigers (Shrivastav & Singh 2012). Lymphocyte sizes from the current study were within the range of that of clouded leopards (Salakij et al. 2008a) and captive leopards in Thailand (Salakij et al. 2009), but were generally larger than that of flat-headed cats (Salakij et al. 2008b) and jungle cats (Salakij et al. 2011).

Granular lymphocytes (lymphocytes containing cytoplasmic azurophilic granules) are normally present in low numbers in healthy felids, but increased numbers may indicate a chronic immune response (Valenciano et al. 2014). Similar to that of the current study's leopards, lymphocytic cytoplasmic azurophilic granules were observed in flat-headed cats that were also infected by a *Hepatozoon* spp. (Salakij et al. 2008b), captive leopards (Thailand) (Salakij et al. 2009) and captive Bengal tigers (Shrivastav & Singh 2012).

According to Gori et al. (2021), few reports on NLRs in felines can currently be found in literature. Differential leukocyte counts and NLR can serve as a diagnostic and prognostic tool to assess feline health, especially as an indicator of severity of inflammatory responses (Chiti et al. 2021, Gori et al. 2021, Petrucci et al. 2021). Gori et al. (2021) reported that there is a positive correlation between NLR, degree of sickness and mortality in domestic cats. The current study's leopards had a higher NLR than that of healthy domestic cats (Meyer & Harvey 2004, Gori et al. 2021) and Pallas cats (Pavlova et al. 2015), but much lower than NLRs of Siberian tigers, Amur leopards *Panthera pardus orientalis* (Schlegel, 1857) (Pavlova et al. 2015) and sick domestic cats (Gori et al. 2021). Captive leopards had a higher NLR than wild leopards from the current study, and males almost double the NLR than females. Interestingly, black leopards had an NLR almost six times higher than their regular counterparts from this study.

Eosinophils

Morphologically, leopard eosinophils from this study were similar to that reported from lions (Jain 1986), captive leopards (Thailand) (Salakij et al. 2009), clouded leopards (Salakij et al. 2008a) and Bengal tigers (Shrivastav & Singh 2012), especially the round shape of the cytoplasmic granules. Interestingly, these eosinophilic granules were dissimilar to the rod-shaped granules of domestic cats (Jain 1986, Reagan et al. 1998, Latimer et al. 2003), cheetahs (Jain 1986), fishing cats (Prihirunkit et al. 2007), flat-headed cats (Salakij et al. 2008b), leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011). Eosinophil diameter from the leopards from this study was larger than that of flat-headed cats (Salakij et al. 2008b), clouded leopards (Salakij et al. 2008a), captive leopards (Salakij et al. 2009) and leopard cats (Salakij et al. 2010), and was most similar to that of jungle cats (Salakij et al. 2011). Few observations on nuclear morphology of feline eosinophils can be found in current literature, which highlights the differences in size between the tested groups from this study.

Platelet morphologies

Leopard platelets were morphologically similar to that of cheetahs (Du Plessis et al. 2004), flat-headed cats (Salakij et al. 2008b), clouded leopards (Salakij et al. 2008a), wild lions (Limpopo and KZN provinces, South Africa) (Du Plessis 2009), captive leopards (Thailand) (Salakij et al. 2009), leopard cats (Salakij et al. 2010) and jungle cats (Salakij et al. 2011). This study's platelets were highly variant in shape among individual leopards, an interesting correspondence with Du

Plessis (2009)'s finding of intraspecific shape variation between two wild lion populations in different regions of South Africa.

Feline platelets tend to be large and heterogenous in size, often equal in size to red blood cells (Zelmanovic & Hetherington 1998, Du Plessis 2009). The size of leopard platelets from this study was most similar to that of wild lions (Du Plessis 2009) and leopard cats (Salakij et al. 2010), and within the range of measurements reported from flat-headed cats (Salakij et al. 2008b) and jungle cats (Salakij et al. 2011). Leopard platelets from this study were much larger than that of cheetahs from South Africa (Du Plessis et al. 2004). Maximum projection, minimum projection and area of male leopard platelets were significantly larger than that of females from this study, and this was also the only significant morphological difference found among all blood cells tested among all groups of leopards from this study (Table 3.1). Similar reports from felids are rare and not commonly found in literature. Platelet clumping is not uncommon in felids (Norman et al. 2001, Valenciano et al. 2014) and have been observed in flat-headed cats (Salakij et al. 2008b), clouded leopards (Salakij et al. 2008a), wild lions (Limpopo and KZN provinces, South Africa (Du Plessis 2009), captive leopards (Salakij et al. 2009) and Bengal tigers (Shrivastav & Singh 2012). Interestingly, Du Plessis et al. (2004) did not report platelet clumping from captive cheetahs in South Africa, showing that clumping should not be expected in all felids.

Megathrombocytes are generally considered to be large, young, granular platelets (Valenciano et al. 2014) and it is relatively common to find platelets with poorly defined cytoplasmic margins and surface projections or pseudopodia in felids (Norman et al. 2001, Valenciano et al. 2014). It is normal to see low numbers of megathrombocytes in healthy feline peripheral blood smears (Valenciano et al. 2014), but it can also be observed in animals recovering from thrombocytopenia due to infections (Jain 1993, Jordan et al. 1993), and in felids with hereditary platelet function defects (Valenciano et al. 2014). Salakij et al. (2008b) reported megathrombocytes in peripheral blood of flat-headed cats that were infected with a *Hepatozoon* sp. Miller, 1908. Du Plessis et al. (2004) observed large, rod-shaped platelets, morphologically similar to what this study defines as megathromobocytes (Fig. 3.4 c – e), in a captive king-cheetah, but not in the regular captive cheetahs from their study and they could not pinpoint the reason for this abnormality. The king-cheetah's phenotypic expression, as well as the phenotypically black leopards from this study, is the result of the expression of a recessive gene that needs to be inherited from both parents in order to be expressed. Therefore, it could have been argued that megathrombocytes could be related to recessive gene inheritance, but since the current study also observed it in regular leopards, this argument needs further investigation.

Activated thrombocytes, as seen by the central condensation of the cytoplasmic granules and the presence of pseudopodia, are often seen in feline blood smears since feline platelets activate quite easily (Valenciano et al. 2014). Few studies on blood cell characterization in felids report on the presence of activated platelets that can be observed under a light microscope (see Salakij et al. 2008a, 2008b, 2009, 2010, 2011). Du Plessis (2009) reported activated

platelets from wild lions in South Africa, with a morphology similar to that of the leopards from this study.

3.4.2 Differential cell counts

Differential cell counts obtained by examination of conventionally stained, thin blood smears can provide a researcher a comparative tool to assess differences in the number of circulating blood cells per blood volume in an individual. Haematological parameters, such as DCCs are variable among members of the genus *Panthera* (Du Plessis 2009, Salakij et al. 2009, Shrivastav & Singh 2012, Maas et al. 2013, Larsson et al. 2015, Pavlova et al. 2015). Similarly, the DCCs of leopards from the current study proved to be quite variable amongst the tested groups from this study, as well as when compared with current reports from other felids.

Differential leukocyte counts

In comparison with current existing data of other members of the genus *Panthera*, the differential leukocyte counts of leopards from this study were amongst the DLCs of other big cats. This study's leopard DLCs were lower than the range described by Singh (2005) for tigers, as well as the DLCs reported for Indian leopards (Sabapara et al. 2008), jaguars (Widmer et al. 2012) and Persian leopards *Panthera pardus tulliana* (Valenciennes, 1856) (Khoshnegah et al. 2012). Leopards from this study had higher DLCs than that of captive leopards (Salakij et al. 2009), snow leopards (Hussain et al. 2016), captive and wild Indian leopards (Shanmugam et al. 2017), jaguars (Hawkey & Hart 1986, Deem 2004, Perez & Paredes 2013), Bengal tigers (Chandranaik et al. 2006, Shrivastav & Singh 2012, Boon et al. 2019, Proverbio et al. 2021), Siberian tiger (Larsson et al. 2015), Amur tiger (Liu et al. 2021) and captive African lions (Larsson et al. 2015). Clouded leopards from Thailand were also reported to have lower DLCs than this study's leopards (Salakij et al. 2008a).

Diverse reports exist on the DLC differences between males and females of a feline species. As suggested by Larsson et al. (2015), these variations could be related to physiological differences between the sexes of a species. Studies, like that of Maas et al. (2013) on wild lions (South Africa), report finding significant haematological differences between males and females in their study. Both male and female DLCs from the current study's leopards were lower than that of captive male and female Indian leopards (Sabapara et al. 2008), but higher than that of wild jaguar males and females (Widmer et al. 2012). The current study's leopard males had higher DLCs than Indian leopard males (Shanmugam et al. 2017), but lower than that of wild Persian leopard males (Khoshnegah et al. 2012). Female leopards from the current study had lower DLCs than males, seemingly following the same sex-based trend as some other members of the genus *Panthera*. This sex-based trend was also reported from other captive male and female leopards (Salakij et al. 2009), Indian leopards (Shanmugam et al. 2017), jaguars (Widmer et al. 2012), Siberian tigers (Larsson et al. 2015), as well as from other smaller felids such as bobcats *Felis rufus* (Miller et al. 1999) and clouded leopards (Salakij et al. 2008a). Causes for this

intraspecific difference between males and females still need further investigation, and Larsson et al. (2015) ascribed this trend in Siberian tigers to the possibility that males may experience higher levels of stress than females during sampling. On the other hand, Sabapara et al. (2008) reported Indian leopard males to have lower DLCs than the females in their study, and Larsson et al. (2015) reported captive African lion males and females to have very similar DLCs. Thus, Larsson et al. (2015)'s suggestion of the effects of stress on males may not always be applicable to all members of the genus *Panthera*. Other smaller felids, such as pumas *Puma concolor* (Currier & Russel 1982) and Canadian lynxes *Lynx canadensis* (Weaver & Johnson 1995) also had no significant haematological variation between the sexes.

Wild leopards had higher DLCs than captive leopards in the current study, thus having the same trend as that of wild and captive African lions from South Africa (Maas et al. 2013). In comparison with records of other wild big cats, wild leopards' DLCs from this study were higher than that of wild lions that were sampled in the same region of South Africa (Maas et al. 2013), wild Bengal tigers (Shrivastav & Singh 2012) and snow leopards (Hussain et al. 2016). However, wild leopards had lower DLCs than that of wild jaguars (Widmer et al. 2012) and wild Persian leopards that were infected with a *Hepatozoon* sp. (Khoshnegah et al. 2012), confirming variation among wild/free-ranging members of the genus *Panthera*. Captive leopards from this study had lower DLCs than captive Indian leopards (Sabapara et al. 2008), but higher DLCs than other captive big cats such as captive leopards in Thailand (Salakij et al. 2009), Bengal tigers (Chandranaik et al. 2006, Shrivastav & Singh 2012, Boon et al. 2019, Proverbio et al. 2021), Amur tigers (Liu et al. 2021), Siberian tigers (Larsson et al. 2015), African lions (Maas et al. 2013, Larsson et al. 2015), and jaguars (Perez & Paredes 2013). Although the difference was not significant, melanistic leopards had higher DLCs than regular leopards from this study, which may confirm findings that melanism may cause intraspecific physiological variations in vertebrates (Ducrest et al. 2008, Roulin & Ducrest 2011).

Neutrophil counts

Neutrophil counts from all leopards of this study was within the range of that was observed in captive snow leopards and tigers (Kumar et al. 2014). Neutrophil counts of this study's leopards were most similar to that of jaguars (Widmer et al. 2012) and high in comparison to that of other members of the genus *Panthera* (jaguars (Deem 2004, Perez & Paredes 2013), leopards (Salakij et al. 2009), Bengal tigers (Proverbio et al. 2021), snow leopards (Hussain et al. 2016)) and smaller cats (clouded leopards (Salakij et al. 2008a)). This study's leopards' neutrophil counts were lower than that reported for only Persian leopards (Khoshnegah et al. 2012).

Overall, male leopards from the current study had more circulating neutrophils per blood volume than females. This trend was also observed in other members of the genus *Panthera* (captive leopards in Thailand (Salakij et al. 2009), jaguars (Widmer et al. 2012), captive lions and Siberian tigers (Larsson et al. 2015)). Neutrophil counts from the current study's wild leopards were higher than that of captive leopards. Interestingly, wild leopards from this study, some of

which were infected with a *Hepatozoon* species, had lower neutrophil counts than that of a wild, *Hepatozoon*-infected Persian leopard (Khoshnegah et al. 2012). Notwithstanding, wild leopard neutrophil counts were higher than that of wild jaguars (Widmer et al. 2012) and a wild snow leopard (Hussain et al. 2016). Neutrophil counts of captive black leopards from this study were higher than that of captive regular leopards and all captive leopard neutrophil counts were higher than that of captive jaguars (Perez & Paredes 2013), captive clouded leopards and leopards (Thailand) (Salakij et al. 2008a, 2009) and captive Bengal tigers (Proverbio et al. 2021).

Lymphocyte counts

Current available literature on wild leopards and other wild big cats to compare the lymphocyte counts from the current study to, is limited. Lymphocyte counts from all leopards of the current study fell within the upper ranges reported by Kumar et al. (2014) for captive leopards and tigers, but were much higher than observations in other members of the genus *Panthera* (captive jaguars (Widmer et al. 2012, Perez & Paredes 2013), Bengal tigers (Proverbio et al. 2021), captive leopards (Thailand) (Salakij et al. 2009)). However, counts of leopards from this study, some of which were infected with a *Hepatozoon* sp., were lower than that reported for *Hepatozoon*-infected Persian leopards (Khoshnegah et al. 2012).

Wild leopard lymphocyte counts were higher than captive leopard counts in this study, and wild leopards had more circulating lymphocytes than wild jaguars (Widmer et al. 2012) and wild snow leopards (Hussain et al. 2016). This study's captive leopards had higher lymphocyte counts than other captive big cats (captive leopards (Thailand) (Salakij et al. 2009); Bengal tigers (Proverbio et al. 2021); jaguars (Deem 2004, Perez & Paredes 2013)) and clouded leopards (Salakij et al. 2008a). Male leopards from the current study had higher lymphocyte counts than females, which have also been found among male and female jaguars (Widmer et al. 2012) and clouded leopards (Salakij et al. 2008a). Interestingly and contrary to this study's findings, reports on captive leopards in Thailand showed that female leopards had higher lymphocyte counts than males (Salakij et al. 2009).

Eosinophil counts

As reported from other felids, eosinophils constituted the smallest quantifiable proportion of leukocytes. Leopards from the current study had relatively high eosinophil counts when compared to that reported from other felids. Among the big cats, this study's leopards had more circulating eosinophils than captive leopards (Thailand) (Salakij et al. 2009), wild and captive jaguars (Widmer et al. 2012, Perez & Paredes 2013) and captive Bengal tigers (Proverbio et al. 2021). Counts also exceeded what were reported for captive clouded leopards (Salakij et al. 2008a). Differences reported between eosinophil counts of big cat males and females, where males had more circulating eosinophils, include reports from leopards (Thailand) (Salakij et al. 2009) and jaguars (Brazil) (Widmer et al. 2012). Contrarily, in smaller cats, male clouded leopards had lower eosinophil counts than females (Salakij et al. 2008a).

According to Maceda-Veiga et al. (2015) increased numbers of eosinophils could indicate the presence of parasitic infections, which may possibly explain the high counts in the leopards from this study as some were infected with a species of *Hepatozoon*.

Neutrophil-to-lymphocyte ratio's

Several studies have shown NLR values to be good diagnostic indicators of stress or innate immune response, even though NLRs can be quite variable among feline species (Davis et al. 2008, Pavlova et al. 2015, Gori et al. 2021, Petrucci et al. 2021). In cats, neutrophils form an important part of inflammatory response and usually serve as a first line of defence against infections (Valenciano et al. 2014). Increased NLR could indicate increased stress, infection or inflammation (Maceda-Veiga et al. 2015, Gori et al. 2021, Petrucci et al. 2021). The numbers of neutrophils and lymphocytes are disparately affected by stress and therefore NLR values, or the proportion of neutrophils to lymphocytes within an individual, is often considered a relatively accurate measure of stress-levels (Pavlova et al. 2015). NLRs are also indicative of disease and infections (Davis et al. 2008), and there is often a positive correlation between the NLR value and how much stress an individual was under, or the severity of infection, at the time of sampling (Pavlova et al. 2015).

Neutrophils were the most numerous circulating leukocyte, followed by lymphocytes and eosinophils. This trend is similar to reports from both small cats (domestic cats (Latimer et al. 2003, Lin et al. 2019), flat-headed cats infected with a *Hepatozoon* sp. (Salakij et al. 2008b), clouded leopards (Salakij et al. 2008a), jungle cats (Salakij et al. 2011)) and big cats (captive leopards in Thailand (Salakij et al. 2009), captive Far eastern leopards (Pavlova et al. 2015), Indian leopards (Sabapara et al. 2008), Bengal tigers (Shrivastav & Singh 2012) and Siberian tigers (Larsson et al. 2015, Pavlova et al. 2015)). The only dissimilar trend, where lymphocytes were the most prevalent leukocyte, was reported from captive lions, leopard cats and Pallas cats (Salakij et al. 2010, Larsson et al. 2015, Pavlova et al. 2015). DLCs of captive lions showed they have higher counts of lymphocytes than eosinophils, but the same study reported Siberian tigers to have more eosinophils than lymphocytes (Larsson et al. 2015). Khoshnegah et al. (2012) also found that the number of eosinophils exceed that of the lymphocytes in a wild, *Hepatozoon*-infected Persian leopard, but this was not observed in the leopards from this study.

A study done by Pavlova et al. (2015) on wild Pallas cats, Siberian tigers and Far eastern leopards, found that the leopards had the largest NLR of these three cat species, meaning these leopards had relatively more neutrophils circulating than lymphocytes. Interestingly, Pallas cats had a much smaller NLR than their big cat counterparts, with their lymphocytes exceeding their neutrophils in count. Pavlova et al. (2015) also reported a positive correlation between NLR and pathogenic seropositive results, relaying that a higher ratio may be indicative of a more severe pathogenic infection. Ultimately, Pavlova et al. (2015) concluded that higher NLRs could be the result of both disease-induced immunosuppression and stress, either from capture or other

sources. Both Gori et al. (2021) and Petrucci et al. (2021) came to the conclusions that NLR in domestic cats could serve as a good early diagnostic tool to detect degree of sicknesses such as systemic inflammatory responses. Their studies also concluded that the higher the NLR in domestic cats, the higher the likelihood of severe systemic inflammatory response in sick cats. Pavlova et al. (2015)'s findings suggested that cats with the lowest NLR are in the best physiological condition. If this same premise is applied to the current study's leopards, it could be concluded that wild leopards, female leopards and regular leopards (as analysed in their respective categories against captive, male and black leopards) are physiologically the healthier groups. It also indicates that, although all leopards from this study were deemed clinically healthy, very few were in pristine health.

Differential platelet counts

It is not unusual to find interspecific differences in feline platelet counts (Hawkey & Hart 1986, Du Plessis 2009). Even though platelet counts usually remain fairly constant within a species, intraspecific variation in platelet counts have been reported from two groups of wild lions in South Africa, at geographically disjunct locations (Du Plessis 2009). It may be that a lower platelet count indicates some underlying physiological issue in felids, such as the presence of infection (Du Plessis et al. 2004, Du Plessis 2009).

The current study's leopards' platelet counts were within the range of those reported for other members of the genus *Panthera*. Platelet counts were similar to those reported from cheetahs (South Africa) (Du Plessis et al. 2004), African lions (South Africa) (Hawkey & Hart 1986, Du Plessis 2009) and snow leopards (Pakistan) (Hussain et al. 2016), but much higher than that of Amur tigers (China) (Liu et al. 2021). Wild leopards from the current study had higher platelet counts than our captive population. Observations from wild and captive lions have, however, shown a different trend. Wild lion platelet counts were reported to be within the same range (Du Plessis 2009) as that of captive lions (Hawkey & Hart 1986). The current study's wild leopards had fewer circulating platelets than wild Persian leopards (Khoshnegah et al. 2012), more platelets than wild jaguars (Widmer et al. 2012) and a similar count to that of wild lions (South Africa) (Du Plessis 2009). Captive leopards had higher platelet counts than captive Bengal tigers (Proverbio et al. 2021), jaguars (Hawkey & Hart 1986, Deem 2004) and Amur tigers (Liu et al. 2021), but fewer circulating platelets than captive snow leopards (Pakistan) (Hussain et al. 2016), African lions (Hawkey & Hart 1986), leopards (Thailand) (Salakij et al. 2009) and cheetahs (Du Plessis et al. 2004). Again this study confirms that there is a variation on platelet counts among members of the genus *Panthera*.

Female leopards of the current study had more circulating platelets at the time of sampling than their male counterparts. A similar sex-based trend was reported in other captive leopards from Thailand (Salakij et al. 2009), but in jaguars the roles were reversed (Widmer et al. 2012). Male leopard platelet counts were most similar to that of wild male jaguars (Widmer et al. 2012) and lower than that reported from captive male leopards (Thailand) (Salakij et al. 2009),

and females had much lower platelet counts than that reported for captive leopard females (Thailand) (Salakij et al. 2009). However, this study's females had more circulating platelets than that reported for wild female snow leopards (Hussain et al. 2016) and wild female jaguars (Widmer et al. 2012). Captive black leopards from the current study, with a similar platelet count to that of captive male leopards in Thailand (Salakij et al. 2009), had much higher platelet counts than their regular counterparts. Regular captive leopards from this study had similar counts to that of captive Amur tigers (China) (Liu et al. 2021).

The presence of all the forms of blood cells, and subsequent differential counts, reported in this chapter may indicate that seemingly healthy leopards may not be in pristine health. This study confirms great variation in blood cell counts and morphologies found in other members of the family Felidae.

3.5 Conclusions

The transporting quality of blood for both resources needed and wastes produced by the body makes it an excellent resource for health screening in larger vertebrates. Although wildlife haematology is still considered an emerging science, peripheral blood is relatively easy to collect and can serve as an early indicator for health-related issues. Possibilities for resampling individuals are also possible and comparisons should be relatively straight forward to carry out. Results presented in this chapter highlight the haematological differences among big cats, stressing that so-called 'normal' haematological values for one species should not be applied to assess that of another species, even within the same genus. Extensive information on the haematological parameters of both wild and captive leopards will prove helpful in health assessment and monitoring, and this study adds detailed knowledge on the subject. The results presented in this chapter adds to current knowledge on blood cell morphology, staining properties and provides ranges of differential counts for different leukocytes and platelets. This chapter also provides a guide for haematological characteristics in captive and wild African leopards, which may be useful for health assessment and monitoring of this species.

3.6 References

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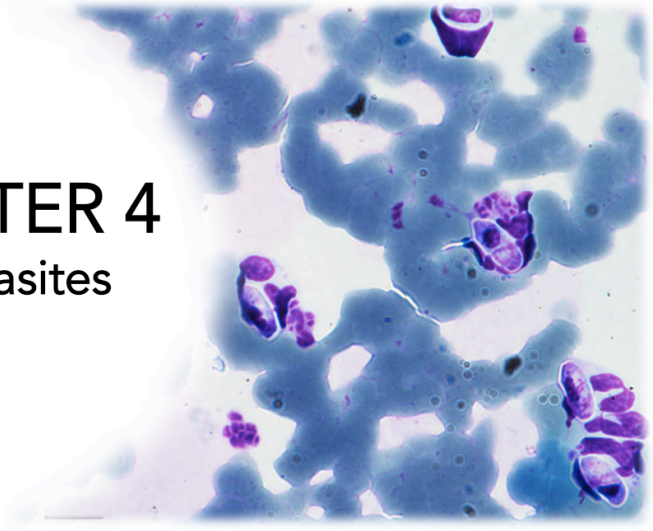
CHAPTER 4
Intraleukocytic haemoparasites of leopards



Sections of this chapter has been published in the following peer-reviewed publication:
VAN AS, M., NETHERLANDS, E. C. & SMIT, N. J. 2020. Molecular characterisation and morphological description of two new species of *Hepatozoon* Miller, 1908 (Apicomplexa: Adeleorina: Hepatozoidae) infecting leukocytes of African leopards *Panthera pardus pardus* (L.). *Parasites & Vectors* 13:1–16. BioMed Central.

4.1 Introduction

It is only recently that researchers have started to focus on the potential role of infectious disease in wildlife conservation, even though it is commonly acknowledged that it can be a determinant of the abundance and distribution of many animal species (e.g. Dobson & May 1986, Williams & Thorne 1996, Murray et al. 1999). The effects of even minor diseases may also be intensified when it interacts with stress-related factors in populations of endangered species (O'Brien et al. 1985, Ullrey 1993, Lloyd 1995), and therefore the threat of disease epidemics may be serious in the case of large carnivores (Murray et al. 1999), many of which are considered to be endangered. Within all mammalian species, the threat of pathogens is greatest to members of the Canidae and Felidae (Pedersen et al. 2007) and according to Mussart et al. (2009), biochemical and haematological studies are needed to enhance the accuracy of health assessment of big cats. From a parasitological perspective, the leopard is one of the least studied big cat hosts. According to Bothma & Walker (1999) and Murray et al. (1999), there are records indicating that the health of leopards can be threatened by rabies, canine distemper, feline leukaemia, feline immunodeficiency virus, anthrax, *Toxoplasma* spp. Nicolle & Manceaux, 1909, *Sarcocystis* spp. Lankester, 1882, *Hepatozoon* spp. Miller 1908, *Giardia* spp. Künstler, 1882, and *Isosopora* spp. Schneider, 1881.

4.1.1 Hepatozoans of African wildlife

Members of the genus *Hepatozoon* Miller, 1908 are intracellular apicomplexan haemogregarines (Apicomplexa Levine, 1970: Adeleorina Léger, 1911: Hepatozoidae Wenyon,

1926) widely reported from amphibians, reptiles, birds and mammals, specifically including carnivores such as wild felids (Smith 1996). Various wildlife species can be infected by *Hepatozoon* species (McCully et al. 1975, Mercer et al. 1988, Averbeck et al. 1990, Kocan et al. 2000, East et al. 2008, Metzger et al. 2008) (see Table 4.1). Mammalian records include members of the orders Rodentia, Lagomorpha (refer to Craig 2001), Artiodactyla and Carnivora (Table 4.1). Various wild canids are infected by these haemogregarines. There is numerous unspecific records of *Hepatozoon* parasites in African wild dog *Lycaon pictus* (Van Heerden et al. 1995, Williams et al. 2014), black-backed jackal *Canis mesomelas* (Schreber, 1775) (Basson et al. 1971, McCully et al. 1975), red fox *Vulpes vulpes* (Linnaues, 1758) (Criado-Fornelio et al. 2003, Maia et al. 2014a), golden jackal *Canis aureus* Linnaeus, 1758 (Duscher et al. 2013, Maia et al. 2014a), Fennec fox *Vulpes zerda* (Zimmermann, 1780), side-striped jackal *Canis adusta* (Sundevall, 1847) (Maia et al. 2014a) and others (see Table 4.1). Kocan et al. (2000) reported *Hepatozoon americanum* Vincent-Johnson, MacIntire, Lindsay, Lenz, Baneth, Shkap & Blagburn, 1997 from North American coyotes *Canis latrans* Say, 1823, (Krampitz et al. 1968) and Keymer (1971) reported *Hepatozoon chattoni* Ledger, 1912 from golden jackals and *Hepatozoon canis* (James 1905) have been reported from various other wild canid hosts (see Table 4.1).

There have also been reports from various Bovids (e.g. Basson et al. 1967, 1971, McCully et al. 1975), Hyaenids (e.g. McCully et al. 1975, East et al. 2008), Mustelids (e.g. Geisel et al. 1979, Criado-Fornelio et al. 2009), Procyonids (e.g. Richards 1961, Criado-Fornelio et al. 2009) and Viverrids (e.g. Laird 1959, Keymer 1971). Hepatozoans have been quite extensively reported from African wildlife (see Table 4.1). South African records on hepatozoonosis in larger wildlife species include records from lions (Basson et al. 1971, McCully et al. 1975, Maddock et al. 1996), African wild dogs (Van Heerden et al. 1995, Matjila et al. 2008b), leopards (McCully et al. 1975), cheetahs (Keep 1970, Basson et al. 1971, McCully et al. 1975), spotted hyaenas *Crocuta crocuta* (Erxleben, 1777) (Keep 1970, Basson et al. 1971, McCully et al. 1975), black-backed jackals (Basson et al. 1971, McCully et al. 1975), impalas *Aepceros melampus* (Lichtenstein, 1812) (Basson et al. 1967, 1971), bushbuck *Tragelaphus sylvaticus* (Sparrman, 1780) (Basson et al. 1971, McCully et al. 1975), nyalas *Tragelaphus angasii* (Angas, 1849) (Basson et al. 1971, McCully et al. 1975), giraffe *Giraffa camelopardalis* Linnaeus, 1758 (Fantham 1920, McCully et al. 1975) and reedbuck *Redunca fulvorufula* (Afzelius, 1815) (Fantham 1920, McCully et al. 1975).

4.1.2 Feline hepatozoonosis

Feline hepatozoonosis was first reported in the early 1900s by Patton (Patton 1908), who described an intraleukocytic parasite *Leucocytozoon felis domestici* Patton, 1908 from a domestic cat in India. Eighteen years later, Wenyon (1926) distinguished a capsule encasing this parasite that was overlooked by Patton (1908), and then reclassified this parasite as a species of *Hepatozoon*, apparently morphologically identical to those found in hyaenas, dogs and jackals (Patton 1908, Wenyon 1926). In Africa, domestic dogs *Canis familiaris* Linnaeus, 1758 and wild carnivores have been reported to have cases of asymptomatic hepatozoonosis, caused

mostly by *Hepatozoon canis* (James 1905) and *Hepatozoon felis* (Brocklesby 1971, McCully et al. 1975, Averbeck et al. 1990, Dubey & Bwangamoi 1994, Peirce et al. 1995, Van Heerden et al. 1995, East et al. 2008). African members of the genus *Genetta* Cuvier, 1816 have also been reported to have these infections (Brocklesby & Vidler 1963, 1965, Keymer 1971). However, *H. canis* and *H. felis* are not respectively specific to either canids or felids but have been reported to infect both carnivore families (Rubini et al. 2006, Jittapalapong et al. 2006, Baneth et al. 2013, Williams et al. 2014). In the late 1990s, Beaufils et al. (1998) proposed that canid and felid *Hepatozoon* are separate species due to their morphological differences. The majority of species of *Hepatozoon* detected in African carnivores were previously identified as *Hepatozoon canis*, until Levine (Levine 1988) suggested that host species should be taken into account when determining the identity of these parasites. Peirce et al. (1995) supported this by suggesting that researchers are wrong to assume that all species of *Hepatozoon* from African carnivore are synonyms of *H. canis*.

Feline hepatozoonosis in African wild felids has been reported from lions from Kenya (Brocklesby & Vidler 1963, 1965), Tanzania (Krampitz et al. 1968) and Zambia (Williams et al. 2014), leopards from Kenya (Brocklesby & Vidler 1963, 1965) and Zimbabwe (Keymer 1971), cheetahs from Tanzania (Averbeck et al. 1990) and caracals from South Africa (Viljoen et al. 2020). Brocklesby & Vidler (1963) were the first to report *Hepatozoon*-like organisms from a free-ranging African leopard in Kenya. In the early 1970s, Keymer (1971) reported *Hepatozoon canis*-like schizonts from the cardiac muscle of a leopard in Central Africa and four years later McCully et al. (1975) reported *Hepatozoon*-like parasites from leopards in South Africa's KwaZulu-Natal Province. More recently, Pawar et al. (2012) and Khoshnegah et al. (2012) detected what they thought to be *Hepatozoon felis* from two free-ranging Indian leopards *Panthera pardus fusca* as well as an unnamed species of *Hepatozoon* from a single free-ranging Caucasian leopard *Panthera pardus ciscausica* (Satunin, 1914).

4.1.3 Asymptomatic infections

Wildlife hepatozoonosis is normally subclinical or asymptomatic (McCully et al. 1975, Mercer et al. 1988, Averbeck et al. 1990, Kocan et al. 2000, East et al. 2008, Metzger et al. 2008, Santos et al. 2013). African carnivores, both wild and domestic, reportedly usually have asymptomatic hepatozoonosis, caused mostly by *Hepatozoon canis* and *Hepatozoon felis* (Brocklesby 1971, McCully et al. 1975, Averbeck et al. 1990, Dubey & Bwangamoi 1994, Peirce et al. 1995, Van Heerden et al. 1995, East et al. 2008). *Hepatozoon* spp. reported from leopards (Keymer 1971), lions (Brocklesby 1971, Dubey & Bwangamoi 1994), cheetahs and spotted hyaenas (Keep 1970) also seem to occur without an apparent clinical response from the host. However, young wild carnivores may occasionally show clinical responses to these infections, as was observed in young coyotes (Kocan et al. 2000, Garret et al. 2005) and spotted hyaenas (East et al. 2008), in which the immune system is still developing. It has been shown that immunocompromised animals may be prone to higher parasitaemia of *Hepatozoon* spp. (Hervas et al. 1995, Baneth et al. 1997, 2001), which may in turn further affect immunological responses to other pathogens. It is therefore reasonable to suggest that the effect of hepatozoonosis on wild carnivore

populations might be quite underestimated, especially when associated with other pathogenic zoonotics.

Prior to the 21st century, classification of species of *Hepatozoon* was based on their life history, host identity and morphological characteristics. However, with recent advances in molecular techniques, phylogenetic analyses on the relationships between species have become possible (Davies & Johnston 2000, Perkins & Keller 2001, Netherlands et al. 2015, 2017). Morphological characteristics used to distinguish between species of *Hepatozoon* include gamont and nucleus dimensions; position of the nucleus within the gamont; number and arrangement of vacuoles and staining properties (Van As et al. 2013, Borges-Nojosa et al. 2017), as well as characteristics of other developmental stages (Baneth et al. 2013, Hodžić et al. 2017). In Africa, domestic dogs and wild carnivores have been reported to have cases of asymptomatic hepatozoonosis, caused mostly by *H. canis* and *H. felis* (Brocklesby 1971, McCully et al. 1975, Averbeck et al. 1990, Dubey & Bwangamoi 1994, Peirce et al. 1995, Van Heerden et al. 1995, East et al. 2008). Although, *H. canis* and *H. felis* are not specific to either canids or felids, they have been reported infecting both carnivore families (Rubini et al. 2006, Jittapalapong et al. 2006, Williams et al. 2014).

Due to constant improvement of molecular techniques, the number of studies on haemogregarines has systematically increased, with several studies relying solely on these methods to detect species in their hosts (Matjila et al. 2008a, André et al. 2010, Pawar et al. 2012, Farkas et al. 2014, Williams et al. 2014, Penzhorn et al. 2018). Recent research has shown that a difference in p-distance of between 1 – 2% of the 18S rRNA gene is sufficient to distinguish between species of haemogregarines if supported by morphological data (Barta et al. 2012, Netherlands et al. 2015, 2017, Borges-Nojosa et al. 2017). Other studies, such as Metzger et al. (2008), incorporate both molecular and some degree of morphological investigations, showing the importance of utilizing a more holistic approach when distinguishing among species of *Hepatozoon*.

The objectives of this part of the study were to investigate whether captive and wild leopards in South Africa are infected with species of *Hepatozoon*, and if these infections would cause clinical symptoms. This study also aimed to specifically identify the hepatozoans infecting the leopards from this study. This was achieved by screening for and identifying hepatozoan infections by means of gamont morphology observed in peripheral blood smears and molecular analysis of a fragment of the 18S rRNA gene. All potential clinical symptoms of infected leopards were also noted. This study is the first report on the molecular and morphological characteristics of two new *Hepatozoon* species infecting captive and wild leopards in South Africa.

Table 4.1 Records of *Hepatozoon* spp. recorded from large mammalian wildlife (excluding members of the order Rodentia).

***Hepatozoon* species**	**Host (wild unless otherwise stated)**	**Species**	**Order**	**Family**	**Country/Region**	**Reference**
H. americanum	Coyote	*Canis latrans*	Carnivora	Canidae	USA	(Kocan et al. 2000)
H. canis	Cheetah	*Acinonyx jubatus*	Carnivora	Felidae	South Africa	(Keep 1970)
	Side-striped jackal	*Canis adustus*	Carnivora	Canidae	East Africa	(Wenyon 1926)
	Golden jackal	*Canis aureus*	Carnivora	Canidae	Hungary	(Farkas et al. 2014)
	Crab-eating fox	*Cerdocyon thous*	Carnivora	Canidae	Brazil	(Alencar et al. 1997)
					Brazil	(Waner et al. 1994)
	spotted hyaena	*Crocuta crocuta*	Carnivora	Hyenidae	South Africa	(Keep 1970)
	African lion	*Panthera leo*	Carnivora	Felidae	Kenya	(Dubey & Bwangamoi 1994)
	African lion✚	*Panthera leo*	Carnivora	Felidae	Zimbabwe	(Kelly et al. 2014)
	Jaguar	*Panthera onca*	Carnivora	Felidae	Venezuela	(Criado-Fornelio et al. 2009)
	African leopard	*Panthera pardus pardus*	Carnivora	Felidae	Zimbabwe	(Keymer 1971)
	Crab-eating raccoon	*Procyon cancrivorus*	Carnivora	Procyonidae	Venezuela	(Criado-Fornelio et al. 2009)
	Red fox	*Vulpes vulpes*	Carnivora	Canidae	Croatia	(Dezdek et al. 2010)
					France	(Rioux et al. 1964)
					Hungary	(Farkas et al. 2014)
					Israel	(Fishman et al. 2004)
					Italy	(Gabrielli et al. 2010)
					Portugal	(Cardoso et al. 2014)
					Portugal	(Cardoso et al. 2014)
					Spain	(Criado-Fornelio et al. 2003)
H. chattoni	Golden jackal	*Canis aureus*	Carnivora	Canidae	Tanzania	(Krampitz et al. 1968)
					Zimbabwe	(Keymer 1971)
	spotted hyaena	*Crocuta crocuta*	Carnivora	Hyenidae	Senegal	(Nuttall 1910)
					Senegal	(Patton 1910)
					Senegal	(Wenyon 1926)

Table 4.1 Records of *Hepatozoon* spp. recorded from large mammalian wildlife (excluding members of the order Rodentia).

***Hepatozoon* species**	**Host (wild unless otherwise stated)**	**Species**	**Order**	**Family**	**Country/Region**	**Reference**
H. felis	European wild cat	*Felis silvestris*	Carnivora	Felidae	Bosnia and Herzegovina	(Hodžić et al. 2017)
	African lion✚	*Panthera leo*	Carnivora	Felidae	Zimbabwe	(Kelly et al. 2014)
	Asiatic lion✚	*Panthera leo persica*	Carnivora	Felidae	India	(Pawar et al. 2012)
	Indian leopard✚	*Panthera pardus fusca*	Carnivora	Felidae	India	(Pawar et al. 2012)
	Indian tiger✚	*Panthera tigris tigris*	Carnivora	Felidae	India	(Pawar et al. 2012)
	Tsushima leopard cat	*Prionailurus bengalensis*	Carnivora	Felidae	Japan	(Tateno et al. 2013, 2015)
	Iriomote cat	*Prionailurus bengalensis iriomotensis*	Carnivora	Felidae	Japan	(Sakuma et al. 2011)
					Japan	(Tateno et al. 2013, 2015)
					Korea	(Kubo et al. 2010)
H. rotundatum	Golden jackal	*Canis aureus*	Carnivora	Canidae	India	(Wenyon 1926)
H. silvestris	European wild cat	*Felis silvestris*	Carnivora	Felidae	Bosnia and Herzegovina	(Hodžić et al. 2017)
Hepatozoon sp.	Cheetah	*Acinonyx jubatus*	Carnivora	Felidae	Tanzania	(Averbeck et al. 1990)
					Tanzania	(Averbeck et al. 1990)
					South Africa	(McCully et al. 1975)
					Tanzania	(Averbeck et al. 1990)
					Tanzania	(Peirce et al. 1995)
					South Africa	(Basson et al. 1971)
					South Africa	(Keep 1970)
	Impala	*Aepyceros melampus*	Artiodactyla	Bovidae	South Africa	(Basson et al. 1971)
					South Africa	(Basson et al. 1967)
	Side-striped jackal	*Canis adustus*	Carnivora	Canidae	Africa	(Nuttall 1910)
					Ethiopia	(Maia et al. 2014a)
	Golden jackal	*Canis aureus*	Carnivora	Canidae	Africa	(Duscher et al. 2013)
					Algeria	(Maia et al. 2014a)
					Mauritania	(Maia et al. 2014a)

Table 4.1 Records of *Hepatozoon* spp. recorded from large mammalian wildlife (excluding members of the order Rodentia).

***Hepatozoon* species**	**Host (wild unless otherwise stated)**	**Species**	**Order**	**Family**	**Country/Region**	**Reference**
Hepatozoon sp.	Golden jackal	*Canis aureus*	Carnivora	Canidae		(Patton 1910)
	Coyote	*Canis latrans*	Carnivora	Canidae	USA	(Davis et al. 1978)
	black-backed jackal	*Canis mesomelas*	Carnivora	Canidae	Kenya	(Brocklesby & Vidler 1963, 1965)
					South Africa	(Basson et al. 1971)
					South Africa	(McCully et al. 1975)
	Maned wolf✚	*Cerdocyon brachyurus*	Carnivora	Canidae	Brazil	(André et al. 2010)
	Crab-eating fox✚	*Cerdocyon thous*	Carnivora	Canidae	Brazil	(André et al. 2010)
	spotted hyaena	*Crocuta crocuta*	Carnivora	Hyenidae	Kenya	(Brocklesby & Vidler 1963, 1965)
					South Africa	(McCully et al. 1975)
					South Africa	(Basson et al. 1971)
					Tanzania	(East et al. 2008)
					Tanzania	(Krampitz et al. 1968)
					West Africa	(Leger 1912)
					Zambia	(Williams et al. 2014)
	Pallas cat	*Felis manul*	Carnivora	Felidae	Russia	(Barr et al. 1993)
	Common genet	*Genetta genetta*	Carnivora	Viverridae	Kenya	(Brocklesby & Vidler 1963, 1965)
	Large-spotted Genet	*Genetta tigrina*	Carnivora	Viverridae	Zimbabwe	(Keymer 1971)
	Giraffe	*Giraffa camelopordalis*	Artiodactyla	Bovidae	South Africa	(McCully et al. 1975)
					South Africa	(Fantham 1920)
	Ocelot	*Leopardus pardalis*	Carnivora	Felidae	Brazil	(Metzger et al. 2008)
					USA	(Mercer et al. 1988)
	Little spotted cat	*Leopardus tigrinus*	Carnivora	Felidae	Brazil	(Metzger et al. 2008)
	Little spotted cat✚	*Leopardus tigrinus*	Carnivora	Felidae	Brazil	(André et al. 2010)
	Hoary fox	*Lycaloplex vetulus*	Carnivora	Canidae	Brazil	(Santos et al. 2013)

Table 4.1 Records of *Hepatozoon* spp. recorded from large mammalian wildlife (excluding members of the order Rodentia).

***Hepatozoon* species**	**Host (wild unless otherwise stated)**	**Species**	**Order**	**Family**	**Country/Region**	**Reference**
Hepatozoon sp.	African wild dog	*Lycaon pictus*	Carnivora	Canidae	South Africa	(Matjila et al. 2008a)
					Tanzania	(Peirce et al. 1995)
					Zambia	(Williams et al. 2014)
					South Africa	(Van Heerden et al. 1995)
	Bobcat	*Lynx rufus*	Carnivora	Felidae	USA	(Lane & Kocan 1983)
					USA	(Mercer et al. 1988)
	Stone marten	*Martes foiva*	Carnivora	Mustelidae	Germany	(Geisel et al. 1979)
	Pine marten	*Martes martes*	Carnivora	Mustelidae	Germany	(Geisel et al. 1979)
					Spain	(Criado-Fornelio et al. 2009)
	Japanese marten	*Martes melampus*	Carnivora	Mustelidae	Japan	(Yanai et al. 1995)
	Weasel	*Mustela erminea*	Carnivora	Mustelidae	Germany	(Geisel et al. 1979)
	Siberian polecat	*Mustela eversmanni*	Carnivora	Mustelidae	Siberia	(Novilla et al. 1978)
	Mink	*Mustela vison*	Carnivora	Mustelidae	Canada	(Presidente & Karstad 1975)
	White-tailed deer	*Odocoileus virginianus*	Artiodactyla	Cervidae	USA	(Clark et al. 1973)
	African lion	*Panthera leo*	Carnivora	Felidae	South Africa	(Maddock et al. 1996)
					Kenya	(Brocklesby & Vidler 1963, 1965)
					Tanzania	(Averbeck et al. 1990)
					Tanzania	(Averbeck et al. 1990)
					South Africa	(Basson et al. 1971)
					South Africa	(McCully et al. 1975)
					Tanzania	(Krampitz et al. 1968)
					Zambia	(Williams et al. 2014)
	Persian leopard	*Panthera pardus ciscaucasica*	Carnivora	Felidae	Iran	(Khoshnegah et al. 2012)
	African leopard	*Panthera pardus pardus*	Carnivora	Felidae	Kenya	(Brocklesby & Vidler 1963, 1965)
					South Africa	(McCully et al. 1975)
	Palm civet	*Paradoxurus hermaphroditus*	Carnivora	Viverridae	Malaysia	(Laird 1959)

Table 4.1 Records of *Hepatozoon* spp. recorded from large mammalian wildlife (excluding members of the order Rodentia).

Hepatozoon species	Host (wild unless otherwise stated)	Species	Order	Family	Country/Region	Reference
Hepatozoon sp.	Tsushima leopard cat	*Prionailurus bengalensis*	Carnivora	Felidae	Japan	(Kubo et al. 2006)
	Leopard cat	*Prionailurus bengalensis*	Carnivora	Felidae	Thailand	(Salakij et al. 2010)
	Iriomote cat	*Prionailurus bengalensis iriomotensis*	Carnivora	Felidae	Japan	(Kubo et al. 2006)
	Flat-headed cat	*Prionailurus planiceps*	Carnivora	Felidae	Thailand	(Salakij et al. 2008b)
	Crab-eating raccoon	*Procyon cancrivorus*	Carnivora	Procyonidae	Panama	(Schneider 1968)
	Raccoon	*Procyon lotor*	Carnivora	Procyonidae	USA	(Richards 1961)
	Hoary fox✚	*Pseudalopex vetulus*	Carnivora	Canidae	Brazil	(André et al. 2010)
	Puma✚	*Puma concolor*	Carnivora	Felidae	Brazil	(André et al. 2010)
	Jaguarondi✚	*Puma yagouaroundi*	Carnivora	Felidae	Brazil	(André et al. 2010)
	Reed buck	*Redunca arundinum*	Artiodactyla	Bovidae	South Africa	(McCully et al. 1975)
					South Africa	(Fantham 1920)
	Bush dog✚	*Speothos venaticus*	Carnivora	Canidae	Brazil	(André et al. 2010)
	Nyala	*Tragelaphus angasi*	Artiodactyla	Bovidae	South Africa	(McCully et al. 1975)
					South Africa	(Basson et al. 1971)
	Bushbuck	*Tragelaphus scriptus*	Artiodactyla	Bovidae	South Africa	(McCully et al. 1975)
					South Africa	(Basson et al. 1971)
	Pale fox	*Vulpes pallida*	Carnivora	Canidae	Mauritania	(Maia et al. 2014a)
					Niger	(Maia et al. 2014a)
					Senegal	(Maia et al. 2014a)
	Rüppell's fox	*Vulpes rueppellii*	Carnivora	Canidae	Mauritania	(Maia et al. 2014a)
					Morocco	(Maia et al. 2014a)
	Red fox	*Vulpes vulpes*	Carnivora	Canidae	Africa	(Gabrielli et al. 2010)
					Japan	(Maede et al. 1982)
					Japan	(Maede et al. 1982)
					Morocco	(Maia et al. 2014a)
					Tunisia	(Maia et al. 2014a)
					Portugal	(Conceição-Silva et al. 1988)

Table 4.1 Records of *Hepatozoon* spp. recorded from large mammalian wildlife (excluding members of the order Rodentia).

***Hepatozoon* species**	**Host (wild unless otherwise stated)**	**Species**	**Order**	**Family**	**Country/Region**	**Reference**
Hepatozoon sp.	Red fox	*Vulpes vulpes*	Carnivora	Canidae	Spain	(Criado-Fornelio et al. 2006)
					Spain	(Gimenez et al. 2009)
					Morocco	(Maia et al. 2014a)
					West Sahara	(Maia et al. 2014a)

* presumed *H. canis*

✚ captive animal

4.2 Materials and methods

Description of study area, characterisation of wild and captive leopards and blood collection was presented in Chapter 2 of this thesis.

4.2.1 Thin blood smears

Thin blood smear preparation

Small blood droplets (enough to provide three to four duplicate blood smears) were placed onto clean, pre-labelled microscope slides to make thin blood smears. Blood smears were air-dried and subsequently fixed with absolute methanol for one minute. Once dry, blood smears were stored in slide boxes for further processing in the lab. A modified Giemsa (Fluka, Sigma-Aldrich, Steinheim, Germany) stain solution was prepared with distilled water (ratio of 9:1) in a 50 ml staining container. Air-dried blood smears were stained in the Giemsa solution for 20 min, rinsed with a slow stream of distilled water and again left to air dry.

Screening of blood smears

Stained smears were examined under the 100× oil immersion objective of a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, The Netherlands) and digital images of any infections detected were captured with an attached Nikon DS-Fi1 digital camera and accompanying software. Haemoparasites were identified through comparison of morphometric data to previous studies on species of *Hepatozoon* from carnivores (Khoshnegah et al. 2012, Baneth et al. 2013). Parasitaemia was calculated per 100 host cells, with ~ 500 host cells (ten fields of 50 host cells) examined per blood smear. Photomicrographs of blood smears were calibrated according to the guidelines stipulated by the ImageJ Image Processing and Analysis software (Rasband 2014). Measurements of parasites and leopard blood cells were taken with the ImageJ version 1.47 software program (Wayne Rasband National Industries of Health, USA) (http://imagej.nih.gov/ij). All measurements are in micrometres (μm) and are given as the range followed by the mean ± standard deviation (SD) in parentheses.

4.2.2 Whole blood

Molecular Analyses

Blood samples collected directly into Vacutainer® EDTA tubes were thawed and used for molecular protocols. DNA was extracted with the KAPA Blood PCR Kit B (Kapa Biosystems, Cape Town, South Africa) according to the protocol provided by the manufacturer. DNA for the dried blood collected from the erythristic leopard was extracted by using the Kapa Express DNA extraction kit (Kapa Biosystems, Cape Town, South Africa), following the manufacturer's protocols. Haemogregarine-specific primers 4558F (5'-GCT AAT ACA TGA GCA AAA TCT CAA-3') and 2733R (5'-CGG AAT TAA CCA GAC AAA T-3') (Mathew et al. 2000) were used for the detection of *Hepatozoon* species through PCR (polymerase chain reaction) amplification of the 18S rRNA gene. Fragments of between 995 and 1002 nucleotide (nt) were amplified using the primer set as mentioned above. PCRs were performed with 1.25 μl (10 μM) of each primer, 12.5 μl Kapa Blood Mix B, 7.5 μl molecular grade nuclease-free water (Thermo Fisher Scientific, Vilnius, Lithuania) and 2.5 μl whole blood to make up a final volume of 25 μl per sample. The PCR was undertaken in a Bio-Rad C1000 TouchTM Thermal Cycler PCR machine (Bio-Rad, Hemel Hempstead, UK), under the following conditions: Initial denaturation step of 5 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 50 °C and extension for 1 min at 72 °C. This was followed by a final extension of 7 min at 72 °C, and products were held at 4 °C. Resulting amplicons were visualised on a 1% agarose gel stained with gel red and using a Bio-Rad Gel-DocTM XR+ imaging system (Bio-Rad, Hemel Hempstead, UK) under ultraviolet light.

All positive, purified PCR products were sent for sequencing to Inqaba Biotechnical Industries (Pty) Ltd. (IBSA) (Pretoria, South Africa), a commercial sequencing company, for sequencing in both directions. Resultant sequences species identity was verified against previously published sequences using the Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990). Haemogregarine species identity was determined by establishing the closest BLAST match (97 – 100% to existing sequences available on the GenBank database). All sequences matching *Hepatozoon* spp. were considered positive and, since they were identical within each new species, only one representative sequence of each was included in further analysis.

The software package Geneious R11 (http://www.geneious.com (Kearse et al. 2012)) was used to assemble and edit resultant sequence fragments. Sequences were aligned using the Clustal W 2.1 alignment tool (Larkin et al. 2007) implemented within Geneious R11. A model test was performed using jModelTest 2·1·7 (Darriba et al. 2012), to determine the most suitable nucleotide substitution model, according to the Bayesian information criterion (BIC). The model with the best BIC score was the General Time Reversible (Tavaré 1986) model with estimates of invariable sites and a discrete Gamma distribution (GTR+I+G). 18S rDNA sequences for species of *Hemolivia* Petit, Landau, Baccam & Lainson, 1990, *Hepatozoon* Miller, 1908, *Karyolysus* Labbé, 1894, *Haemogregarina* Danilewsky, 1885 and *Dactylosoma* Labbé, 1894 (parasitising amphibian, reptilian and mammalian hosts) were downloaded from GenBank and aligned with

the sequences generated in this study (Table 4.2). *Adelina dimidiate* Schneider, 1875, *Adelina grylli* Butaeva, 1996 (GenBank: DQ096835-DQ096836) and *Klossiella equi* Smith & Johnson, 1902 (GenBank: MH211602), from the suborder Adeleiorina Léger, 1911 were selected as outgroup. Although eight sequences were obtained from infected leopards, only a single representative of each species of *Hepatozoon* amplified in the present study was used for phylogenetic analyses. Phylogenetic analyses consisted of two datasets, the first alignment a large dataset (n=297) including all representative *H. felis* sequences from GenBank, and the second alignment based on the results of the first included 57 representative sequences (Table 4.2). Bayesian inference (BI) was used to infer phylogenetic relationships. The BI analysis was performed using MrBayes 3.2.2 (Huelsenbeck & Ronquist 2001) implemented from within Geneious R11. To assess posterior probability support the Markov Chain Monte Carlo (MCMC) algorithm was run for one million generation for the first larger dataset (297 sequences), and 10 million generations for the second the smaller dataset (57 sequences), sampling every 100 generations and using the default parameters. The first 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained. Results were visualised in Trace, to assess convergence and the 'burn-in' period. Furthermore, uncorrected p-distances for the sequences used were also calculated in PAUP (Phylogenetic Analysis Using Parsimony) version 4.0a152.

4.3 Results

Refer to Chapter 2 Tables 2.3 and 2.4 to see which samples were obtained for which captive and wild leopards in this study.

4.3.1 Taxonomy

Phylum Apicomplexa Levine, 1970
Class Conoidasida Levine, 1988
Order Eucoccidiorida Léger & Dubosq, 1910
Suborder Adeleorina Léger, 1911
Family Hepatozoidae Wenyon, 1926
Genus *Hepatozoon* Miller, 1908

Hepatozoon luiperdjie Van As et al. 2020

Type-host: *Panthera pardus pardus* (Linnaeus, 1758) (Carnivora: Felidae).
Type-locality: In-situ Site1 (23°02'17.1"S, 29°26'26.5"E), Limpopo Province, South Africa.
Other localities: In-situ Site2 (25°9'51.31"S, 30°26'55.46"E); In-situ Site3 (24°34'43.50"S, 31°25'46.48"E), Mpumalanga Province Mpumalanga Province, South Africa.
Type-material: Hapantotype, 1 peripheral blood smear from the type-host *P. p. pardus* and type-locality (23°02'17.1"S, 29°26'26.5"E), deposited under the accession number NMBP392 in the protozoan collection of the National Museum, Bloemfontein, South Africa.

Vector: Unknown.

Representative DNA sequences: Two sequences, of a 995 nt fragment of the 18S rRNA gene of *Hepatozoon luiperdjie* Van As et al. 2020, isolated from the type-host *P. p. pardus*, deposited under the accession numbers MN793002 and MN793003 in the GenBank database.

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN 2012), details of the new species have been sub- mitted to ZooBank. The Life Science Identifier (LSID) of the article is urn:lsid:zoobank.org:pub:9E65A924-729F-43A5-AE1F-8001204B6A6A. The LSID for the new name *Hepatozoon luiperdjie* Van As et al. 2020 is urn:lsid:zoobank.org:act:2293B0B3-3B91-4BDC-8246-50215B80F8D5.

Etymology: The species epithet is derived from the Afrikaans language diminutive name for the host *P. p. pardus*, which in Afrikaans is referred to as “luiperdjie”.

Description

Gamonts: Most abundant stage in peripheral blood smears (Fig. 4.1 a – f). Extracellular forms (Fig. 4.1 c) and immature gamonts (Fig. 4.1 d) rarely observed and no division stages detected. Mature gamonts measure 9.9–12.6 × 4.1–5.0 (11.0 ± 0.9 × 4.7 ± 0.4) μm (n=53), area of 39.5–46.2 (42.0 ± 2.9) μm^2 (n=53). Mature gamonts mostly conspicuous within neutrophil cytoplasm (Fig. 4.1 a – c), sometimes hardly visible and concealed by host cell nucleus; elongate, with bluntly rounded extremities, thin visible capsule (Fig. 4.1 a); cytoplasm stained pale purple with some gamonts containing bright magenta and basophilic staining granules (Fig. 4.1 a, f). Some gamonts with 2 to 3 small, slightly noticeable posteriorly situated vacuoles (Fig. 4.1 e, thick arrow). Gamont nuclei measure 3.0–3.6 × 3.1–3.8 (3.5 ± 0.3 × 3.4 ± 0.3) μm (n=53), area of 8.8–9.6 (9.2 ± 0.3) μm^2 (n=53); rounded and acentric, usually as wide as gamont at widest point, mostly located closer to anterior than posterior of gamont and stained dark purple, with densely stranded chromatin. Capsules 0.3–0.7 (0.5 ± 0.2) μm thick (n=53) and observable in most gamonts (Fig. 4.1 e, thin arrow).

Prevalence and parasitaemia: *Hepatozoon luiperdjie* occurred in peripheral blood of 8/16 (prevalence 50%) individual *P. p. pardus* sampled. This haemogregarine formed co-infections with *Hepatozoon ingwe* Van As et al. 2020 (see below) in 6 out of 16 leopards (prevalence of 38%), and was the sole species of *Hepatozoon* detected in 2 out of 16 individuals (prevalence of 13%). Prevalence of 63% (5/8) in males and 38% (3/8) in females infected. No captive individuals infected by this haemogregarine.

Table 4.2 List of taxa used in the phylogenetic analyses of this study, with associated GenBank accession numbers, host, host family, host common name, country and references.

Accession number	Haemoparasite	Host species	Class	Family	Common name	Country	Reference
AF176836	*Hepatozoon americanum*	*Canis familiaris*	Mammalia	Canidae	Domestic dog	USA	(Mathew et al. 2000)
AY461376	*Hepatozoon canis*	*Pseudalopex gymnocercus*	Mammalia	Canidae	Pampas fox	Brazil	(Kubo et al. 2006)
AY461377	*Hepatozoon* sp.	*Cerdocyon thous*	Mammalia	Canidae	Crab-eating fox	Brazil	(Kubo et al. 2006)
AY620232	*Hepatozoon felis*	*Felis catus*	Mammalia	Felidae	Domestic cat	Spain	(Kubo et al. 2006)
AY628681	*Hepatozoon felis*	*Felis catus*	Mammalia	Felidae	Domestic cat	Spain	(Kubo et al. 2006)
DQ096835	*Adelina dimidiata*	*Scolopendra cingulata*	Scolopendromorpha	Scolopendridae	Megarian banded centipede	Bulgaria	(Criado-Fornelio et al. 2009)
DQ096836	*Adelina grylli*	*Gryllus bimaculatus*	Insecta	Gryllidae	African field cricket	Bulgaria	(Criado-Fornelio et al. 2009)
DQ439540	*Hepatozoon canis*	*Canis lupus familiaris*	Mammalia	Canidae	Domestic dog	Venezuela	(Kopečná et al. 2006)
EF157822	*Hepatozoon ayorgbor*	*Python regius*	Reptilia	Pythonidae	Ball python	Ghana	(Criado-Fornelio et al. 2007)
EF222257	*Hepatozoon* sp.	*Martes martes*	Mammalia	Mustelidae	European pine marten	Spain	(Ortuño et al. 2007)
EF222259	*Hepatozoon* sp.	*Sciurus vulgaris*	Mammalia	Sciuridae	Eurasian red squirrel	Spain	(Ortuño et al. 2007)
EU041717	*Hepatozoon ursi*	*Ursus thibetanus japonicus*	Mammalia	Ursidae	Asian black bear	Japan	(Sloboda et al. 2007)
EU041718	*Hepatozoon ursi*	*Ursus thibetanus japonicus*	Mammalia	Ursidae	Asian black bear	Japan	(Sloboda et al. 2007)
HQ224957	*Dactylosoma ranarum*	*Pelophylax* kl. *esculentus*	Amphibia	Ranidae	Edible frog	Canada	(Barta et al. 2012)
HQ224959	*Haemogregarina balli*	*Chelydra serpentina*	Reptilia	Chelydridae	Common snapping turtle	Canada	(Barta et al. 2012)
HQ734791	*Hepatozoon* sp.	*Scelarcis perspicillata*	Reptilia	Lacertidae	Moroccan rock lizard	Morocco	(Kubo et al. 2008)
HQ734792	*Hepatozoon* sp.	*Podarcis vaucheri*	Reptilia	Lacertidae	Andalusian wall lizard	Morocco	(Kubo et al. 2008)
HQ829437	*Hepatozoon ursi*	*Melursus ursinus*	Mammalia	Ursidae	Sloth bear	India	(Maia et al. 2011)
HQ829440	*Hepatozoon felis*	*Panthera leo persica*	Mammalia	Felidae	Asiatic lion	India	(Pawar et al. 2012)
HQ829444	*Hepatozoon felis*	*Panthera pardus fusca*	Mammalia	Felidae	Indian leopard	India	(Pawar et al. 2012)
HQ829445	*Hepatozoon felis*	*Panthera tigris tigris*	Mammalia	Felidae	Bengal tiger	India	(Pawar et al. 2012)
JN123435	*Hepatozoon felis*	*Felis catus*	Mammalia	Felidae	Domestic cat	Brazil	(Pawar et al. 2012)
JN181157	*Hepatozoon sipedon*	*Nerodia sipedon sipedon*	Reptilia	Colubridae	Northern water snake	Canada	(Barta et al. 2012)
KC138535	*Hepatozoon canis*	*Canis lupus familiaris*	Mammalia	Canidae	Domestic dog	Israel	(East et al. 2008)
KC696565	*Hepatozoon* sp.	*Psammophis schokari*	Reptilia	Lamprophiidae	Schokari sand racer	Algeria	(de Bortoli et al. 2011)
KF257925	*Haemogregarina* sp.	*Pelusios subniger*	Reptilia	Pelomedusidae	East African black mud turtle	Mozambique	(Tomé et al. 2013)
KF257926	*Haemogregarina stepanowi*	*Mauremys caspica*	Reptilia	Geoemydidae	Striped-neck terrapin	Iran	(Tomé et al. 2013)

Table 4.2 List of taxa used in the phylogenetic analyses of this study, with associated GenBank accession numbers, host, host family, host common name, country and references.

Accession number	Haemoparasite	Host species	Class	Family	Common name	Country	Reference
KF992699	*Hemolivia mauritanica*	*Testudo marginata*	Reptilia	Testudinidae	Marginated tortoise	Greece	(Dvořáková et al. 2013)
KF992711	*Hemolivia mariae*	*Egernia stokesii*	Reptilia	Scincidae	Gidgee skink	Australia	(Dvořáková et al. 2013)
KF992713	*Hemolivia* sp.	*Rhinoclemmys pulcherrima manni*	Reptilia	Geoemydidae	Central American painted wood turtle	Nicaragua	(Dvořáková et al. 2013)
KJ461939	*Karyolysus latus*	*Podarcis muralis*	Reptilia	Lacertidae	European wall lizard	Slovakia	(Ortuño et al. 2007)
KJ461943	*Karyolysus lacazei*	*Lacerta viridis*	Reptilia	Lacertidae	European green lizard	Slovakia	(Ortuño et al. 2007)
KJ461945	*Karyolysus* sp.	*Zootoca vivipara*	Reptilia	Lacertidae	Viviparous lizard	Poland	(Kvičerová et al. 2014)
KM234646	*Hepatozoon domerguei*	*Madagascarophis colubrinus*	Reptilia	Lamprophiidae	Madagascar cat-eyed snake	Madagascar	(Haklová-Kočíková et al. 2014)
KM887508	*Haemogregarina pellegrini*	*Malayemys subtrijuga*	Reptilia	Geoemydidae	Mekong snail-eating turtle	Vietnam	(Maia et al. 2014b)
KP881349	*Hemolivia stellata*	*Rhinella marina*	Amphibia	Bufonidae	Cane toad	Brazil	(Dvořáková et al. 2015)
KR069082	*Hemolivia parvula*	*Kinixys zombensis*	Reptilia	Testudinidae	Eastern hinged back tortoise	South Africa	(Karadjian et al. 2015)
KR069084	*Hepatozoon fitzsimonsi*	*Kinixys zombensis*	Reptilia	Testudinidae	Eastern hinged back tortoise	South Africa	(Karadjian et al. 2015)
KU198330	*Hepatozoon* sp.	*Meles meles*	Mammalia	Mustelidae	European badger	Spain	(Barandika et al. 2016)
KX011040	*Karyolysus paradoxa*	*Varanus albigularis*	Reptilia	Varanidae	Rock monitor	South Africa	(Cook et al. 2016)
KX757032	*Hepatozoon silvestris*	*Felis silvestris silvestris*	Mammalia	Felidae	European wild cat	Bosnia and Herzegovina	(Hodžić et al. 2017)
KY056823	*Hepatozoon felis*	*Panthera leo persica*	Mammalia	Felidae	Asiatic lion	Thailand	(Bhusri et al. 2017)
LC314791	*Hepatozoon apri*	*Sus scrofa leucomystax*	Mammalia	Suidae	Japanese boar	Japan	(Yamamoto et al. 2017)
MG041594	*Hepatozoon involucrum*	*Hyperolius marmoratus*	Amphibia	Hyperoliidae	Painted reed frog	South Africa	(Netherlands et al. 2017)
MG041596	*Hepatozoon tenuis*	*Afrixalus fornasini*	Amphibia	Hyperoliidae	Greater leaf-folding frog	South Africa	(Netherlands et al. 2017)
MG041602	*Hepatozoon thori*	*Hyperolius marmoratus*	Amphibia	Hyperoliidae	Marbled reed frog	South Africa	(Netherlands et al. 2017)
MG041604	*Hepatozoon ixoxo*	*Sclerophrys pusilla*	Amphibia	Bufonidae	Merten's Striped Toad	South Africa	(Netherlands et al. 2017)

Table 4.2 List of taxa used in the phylogenetic analyses of this study, with associated GenBank accession numbers, host, host family, host common name, country and references.

Accession number	Haemoparasite	Host species	Class	Family	Common name	Country	Reference
MG041605	*Hepatozoon theileri*	*Amietia delalandii*	Amphibia	Pyxicephalidae	Delalande's river frog	South Africa	(Netherlands et al. 2017)
MG136687	*Hepatozoon martis*	*Martes martes*	Mammalia	Mustelidae	European pine marten	Bosnia and Herzegovina	(Hodžić et al. 2018)
MG519502	*Hepatozoon angeladaviesae*	*Philothamnus semivariegatus*	Reptilia	Colubridae	Spotted bush snake	South Africa	(Cook et al. 2018)
MG519504	*Hepatozoon cecilhoarei*	*Philothamnus natalensis natalensis*	Reptilia	Colubridae	Natal green snake	South Africa	(Cook et al. 2018)
MG919977	*Hepatozoon* sp.	*Canis mesomelas*	Mammalia	Canidae	black-backed jackal	South Africa	(Matjila et al. 2008a)
MG919980	*Hepatozoon* sp.	*Canis mesomelas*	Mammalia	Canidae	black-backed jackal	South Africa	(Matjila et al. 2008a)
MH211602	*Klossiella equi*	*Equus ferus caballus*	Mammalia	Equidae	Horse	Canada	(Léveillé et al. 2019b)
MH615006	*Hepatozoon canis*	*Canis lupus familiaris*	Mammalia	Canidae	Domestic dog	Israel	(Léveillé et al. 2019a)
MK621310	*Hepatozoon felis*	*Caracal caracal*	Mammalia	Felidae	Caracal	South Africa	(Viljoen et al. 2020)
MK621318	*Hepatozoon felis*	*Caracal caracal*	Mammalia	Felidae	Caracal	South Africa	(Viljoen et al. 2020)
MK621319	*Hepatozoon felis*	*Caracal caracal*	Mammalia	Felidae	Caracal	South Africa	(Viljoen et al. 2020)

Parasitaemia varied between individuals and could only be determined in WM1 (11.3%), WF1 (21.5%), WM5 (1.9%), WF3 (15.4%) and WM4 (4.8%). Average parasitaemia in all five leopards was 11.0%.

Effect on host cells: Gamonts sometimes compressed the lobulated nucleus of neutrophils, either towards the periphery of the host cell (Fig. 4.1 a), or towards one side (Fig. 4.1 b). Parasitized neutrophils measured 12.7–14.3 × 12.1–14.1 (13.6 ± 0.8 × 13.1 ± 0.7) μm (n=53), area of 130.2–186.0 (146.3 ± 22.9) μm^2 (n=53). Healthy, uninfected neutrophils measured 7.6–14.4 × 7.0–13.0 (11.1 ± 1.5 × 10.3 ± 1.3) μm (n=450), area of 46.3–127.2 (89.1 ± 18.3) μm^2 (n=450). Nuclei of parasitized neutrophils measured 12.8–25.3 × 3.3–13.3 (19.8 ± 5.2 ×25.7 ± 4.2) μm (n=53), area of 53.3–116.9 (68.8 ± 27.0) μm^2 (n=53). Dimensions of healthy neutrophil nuclei were 10.3–29.3 × 1.7–4.5 (20.5 ± 3.9 ×22.7 ± 0.6) μm (n=450), area of 25.1–58.1 (41.7 ± 7.4) μm^2 (n=450). Thus, infected neutrophils were slightly longer and wider, with larger surface area. Nuclei of infected neutrophils were slightly longer and narrower, with greater surface area.

Remarks

Prior to this study, *Hepatozoon* spp. reported from wild carnivores were only *H. felis*, *H. canis* and mostly as an unidentified species of *Hepatozoon* (Brocklesby & Vidler 1965, McCully et al. 1975, Davis et al. 1978, Lane & Kocan 1983, Mercer et al. 1988, Averbeck et al. 1990, Waner et al. 1994, Peirce et al. 1995, Van Heerden et al. 1995, Alencar et al. 1997, Kubo et al. 2010, Criado-Fornelio et al. 2006, Kubo et al. 2006, Salakij et al. 2008a, 2010, East et al. 2008, André et al. 2010, Sakuma et al. 2011, Khoshnegah et al. 2012, Pawar et al. 2012, Okubanjo et al. 2013, Tateno et al. 2013, Kelly et al. 2014), except by Hodžić et al. (2017) who recently described *Hepatozoon silvestris* Hodžić, Alić, Prašović, Otranto, Baneth & Duscher, 2017 in an European wildcat *Felis silvestris* Schreber, 1777 from eastern Europe. Infection with a morphologically and genetically distinct *Hepatozoon* sp. was confirmed by the present study in wild African leopards in various areas throughout South Africa, both in male and female hosts. The haemogregarine described here appears to develop only gamont stages in the peripheral blood of *P. p. pardus*. Therefore, with no division stages detected, it was placed within the genus *Hepatozoon*.

Hepatozoon luiperdjie was on average longer than *H. canis* from Crab-eating fox *Cerdocyon thous* (Linnaeus, 1766) in Brazil (Waner et al. 1994), and longer, but with a similar average width to *H. felis* from *Felis catus* in Israel (Baneth et al. 2013), therefore within the relative morphometrical range of both species (Table 4.3). This haemogregarine was morphometrically most similar to an unnamed species of *Hepatozoon* detected in *Lynx rufus* from the USA (Lane & Kocan 1983). It also differed from the other species of *Hepatozoon* detected in the monocytes of leopards during this study. The most striking feature of *H. luiperdjie* is the densely chromatisized, acentric nucleus, relatively smaller than that of *H. felis* (Baneth et al. 2013, Hodžić et al. 2017). *Hepatozoon luiperdjie* also seemed to exclusively infect the neutrophils of

the host. The lifecycle of this parasite remains to be determined, but possible developmental stages in an associated vector will be described in Chapter 5.

Hepatozoon ingwe Van As et al. 2020

Type-host: *Panthera pardus pardus* (Linnaeus, 1758) (Carnivora: Felidae).
Type-locality: In-situ Site1 (23°02'17.1″S, 29°26'26.5″E), Limpopo Province, South Africa.
Other localities: In-situ Site2 (25°9'51.31″S, 30°26'55.46″E), Mpumalanga Province; In-situ Site3 (24°34'43.50″S, 31°25'46.48″E), Mpumalanga Province; Ex-situ Site3 (24°30'52.44″S, 30°54'8.82″E), Mpumalanga Province, South Africa.
Type-material: Hapantotype, 1 peripheral blood smear from the type-host *P. p. pardus* and type-locality (23°02'17.1″S, 29°26'26.5″E), deposited under accession number NMBP393 in the protozoan collection of the National Museum, Bloemfontein, South Africa.
Vector: Unknown.
Representative DNA sequences: Two sequences, representing a 995 nt fragment of the 18S rRNA gene of *Hepatozoon ingwe* Van As et al. 2020, isolated from the type-host *P. p. pardus*, deposited under the accession numbers MN793000 and MN793001 in the GenBank database.
ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the ICZN (ICZN 2012), details of the new species have been submitted to ZooBank. The LSID of the article is urn:lsid:zoobank.org:pub:9E65A924-729F-43A5-AE1F-8001204B6A6A. The LSID for the new name *Hepatozoon ingwe* Van As et al. 2020 is urn:lsid:zoobank.org:act:65A2DC3D-BABB-443A-82A7-DBCFFCAE3636.
Etymology: The species epithet is derived from that of the Zulu language name for the host *P. p. pardus*, which in Zulu is referred to as "ingwe". Noun in apposition.

Description

Gamonts: Most abundant stage in peripheral blood smears (Fig. 4.1 g – k). Extracellular forms (Fig. 4.1 i) and immature gamonts (Fig. 4.1 j) rarely observed, no division stages detected. Mature gamonts measure 9.8–12.6 × 4.5–5.0 (11.4 ± 1.2 × 4.8 ± 0.2) μm (n=87), surface area of 38.7–48.9 (44.2 ± 4.4) μm^2 (n=87); mostly visible within leukocyte cytoplasm (Fig. 4.1 g, h, k), but in some cases gamonts were concealed by leukocyte nucleus; elongate with round extremities, cytoplasm stained pale blue, slight granulation, minimal basophilic stippling anteriorly; cytoplasm contained bright pink staining granules (Fig. 4.1 g, h). Gamonts with thin visible capsules (Fig. 4.1 g, thick arrow) and 2 to 4 prominent vacuoles posteriorly situated (Fig. 4.1 h, thin arrow); gamont nuclei measure 4.4–5.7 × 2.5–3.7 (5.1 ± 0.6 × 3.0 ± 0.6) μm (n=87), area of 8.7–16.5 (12.2 ± 3.3) μm^2 (n=87). Nuclei stained dark purple with loosely stranded chromatin, through which parts of cytoplasm were often visible (Fig. 4.1 g, h). Nuclei elongate, usually narrower than gamont at

Table 4.3 Details and measurements of *Hepatozoon luiperdjie* and *Hepatozoon ingwe* and closely related *Hepatozoon* species in wild and domestic carnivores.

Host species	Country	*Hepatozoon* species	Host cells infected	GenBank Accession number	Intracellular gamont dimensions (L x W in $\mu\mu$m); [LW in $\mu\mu m^2$]	Gamont nuclei dimensions (L x W in $\mu\mu$m); [LW in $\mu\mu m^2$]	Number measured	Author
Canis familiaris	India	*H. canis*	Neutrophils	-	(9.50 - 11.80 x 9 5.10 – 6.00)	-	-	(Eljadar et al. 2013)
Cerdocyon thous	Brazil	*H. canis*	Leukocytes	-	(11.42 x 5.39) [45.88]	-	-	(Waner et al. 1994)
Cerdocyon thous	Brazil	*H. canis*	Neutrophils	-	(9.1 ± 0.54 x 5.3 ± 0.46)	-	-	(Alencar et al. 1997)
Felis catus	Brazil	*Hepatozoon* sp.	Neutrophils	-	(9.88 ± 0.39 x 5.3 ± 0.19) [45.85 ± 4.9]	-	-	(Perez et al. 2004)
Felis catus	Israel	*H. felis*	Neutrophils.	KC138534	(10.5 ± 0.6 x 4.7 ± 0.8)	(4.0 ± 0.3 x 3.2± 0.5)	13	(Baneth et al. 2013)
Felis silvestris	Bosnia and Herzegovina	*H. felis*	Extracellular	KX757033	(10.5 ± 0.4 x 4.4 ± 0.4)	(4.7 ± 0.3 x 4.4 ± 0.3)	-	(Hodžić et al. 2017)
Felis silvestris	Bosnia and Herzegovina	*H. silvestris*	Extracellular	KX757032	(11.7 ± 0.5 x 5.2 ± 0.7)	(6.3 ± 1.3 x 3.0 ± 0.8)	11	(Hodžić et al. 2017)
Leopardus pardalis	Brazil	*Hepatozoon* sp.	Neutrophils	EU028344	(7.43 x 4.23) [26.95]	-	1	(Metzger et al. 2008)
Lynx rufus	United States of America	*Hepatozoon* sp.	Leukocytes	-	(11.0 x 2.5)	-	-	(Lane & Kocan 1983)
Panthera pardus ciscaucasica	Iran	*Hepatozoon* sp.	Neutrophils	-	(11.37 ± 0.27 x 5.2 ± 0.17) [39.52 ± 3.2]	-	-	(Khoshnegah et al. 2012)
Panthera pardus pardus	South Africa	*H. luiperdjie* n. sp.	Neutrophils	MN793002; MN793003	(10.96 ± 0.87 x 4.69 ± 0.37) [41.98 ± 2.90]	(3.45 ± 0.25 x 3.38 ± 0.28) [9.21 ± 0.31]	53	This study
Panthera pardus pardus	South Africa	*H. ingwe* n. sp.	Lymphocytes	MN793000; MN793001	(11.36 ± 1.20 x 4.84 ± 0.22) [44.23 ± 4.44]	(5.14 ± 0.64 x 3.01 ± 0.59) [12.16 ± 3.26]	87	This study
Prionailurus bengalensis	Thailand	*Hepatozoon* sp.	Neutrophils	GQ926902	(9.8 ± 0.4 x 5.2 ± 0.4)	-	10	(Salakij et al. 2010)

widest point, mostly anteriorly located. Capsule measured 0.4–0.7 (0.5 ± 0.1) μm thick (n=87) and observable in most gamonts.

Prevalence and parasitaemia: Detected in the peripheral blood of 7 out of 16 individual *P. p. pardus* sampled (prevalence of 44%). Of these 6 out of 16 leopards had co-infections with *H. luiperdjie* n. sp. (prevalence of 38%) and 1 out of 16 individuals was solely infected with this species of *Hepatozoon* (prevalence 6%). More males than females were infected, prevalence 50% (4/8) of males and 38% (3/8) of females. One captive female was infected (prevalence in captivity 13% or 1/8). Parasitaemia varied between individuals and could only be determined in WM1 (32%), WF1 (57%), WF3 (13%), WM4 (21%) and WM5 (7%). Average parasitaemia was 30.8%. Gamonts not observed in smears of CF5; however, prevalence confirmed by PCR amplification.

Effect on host cells: Gamonts usually compacted lymphocyte nuclei towards one side and completely usurped lymphocyte cytoplasm (Fig. 4.1 g, h). Parasitized lymphocytes measured 10.9–11.6 × 8.4–9.7 (11.3 ± 0.3 × 9.1 ± 0.6) μm (n=87), area of 73.6–83.4 (79.2 ± 4.3) μm^2 (n=87). Healthy, uninfected lymphocytes measured 6.8–15.7 × 6.1– 13.9 (10.9 ± 2.1 × 9.7 ± 1.9) μm (n=261), area of 36.6–156.8 (81.7 ± 29.4) μm^2 (n=261). Nuclei of parasitized lymphocytes measured 8.4–11.9 × 3.8–5.7 (10.0 ± 1.5 × 4.78 ± 0.8) μm (n=87), area of 30.2–36.9 (33.5 ± 3.0) μm^2 (n=87). Dimensions of healthy lymphocyte nuclei were 6.1–16.1 × 4.2– 10.7 (9.7 ± 2.2 × 7.3 ± 1.4) μm (n=261), area of 26.2–92.1 (56.2 ± 15.4) μm^2 (n=261). Thus, infected lymphocytes were slightly longer and narrower, with smaller surface area. Infected lymphocyte nuclei measured slightly longer and narrower, with smaller surface area.

Remarks

This haemogregarine appears to develop only gamont stages in the peripheral blood of *P. p. pardus*, and with no division stages detected it was placed within *Hepatozoon*. *Hepatozoon ingwe* measured within the same range size as *H. luiperdjie* described above. However, *H. ingwe* seemed to exclusively infect the lymphocytes of the host, unlike the gamonts of *H. luiperdjie*. *Hepatozoon ingwe* was morphometrically similar to an unnamed *Hepatozoon* detected in the neutrophils of *P. p. ciscaucasica* from Iran (Khoshnegah et al. 2012) (Table 4.3). This haemogregarine also measured longer and wider than *H. felis* from domestic cats in Israel (Baneth et al. 2013), widely considered a redescription of *H. felis* (Table 4.3). *Hepatozoon ingwe* was significantly longer (P = 0.0064) and somewhat wider (P = 0.3023), with a comparatively larger surface area than that of *H. luiperdjie* (P = 0.0593) (Table 4.3). Characteristic features of *H. ingwe* include the pale blue staining cytoplasm containing bright pink staining granules, prominent vacuoles at the posterior, and elongated nuclei similar to that of *H. canis*. The life cycle of this parasite remains to be determined.

4.3.2 Differential diagnoses

Each species of haemogregarine described here infected a particular type of leukocyte: *Hepatozoon luiperdjie* were exclusively found in neutrophils and *H. inwe* in lymphocytes. The parasitaemia of *H. ingwe* (30.8%), and *H. luiperdjie* (11.0%), was higher than the range of 0.1–4.0% reported by other studies on domestic cats in Israel (Baneth et al. 1998), an ocelot *Leopardus pardalis* (Linnaeus, 1758) in Brazil (Metzger et al. 2008), a Tsushima leopard cat *Prionailurus bengalensis* in Thailand (Salakij et al. 2010), Iriomote cats *P. b. iriomotensis* (Imaizumi, 1967) in Japan (Tateno et al. 2013) and a Tsushima leopard cat *P. bengalensis* in Japan (Kubo et al. 2006). The parasitaemia of *H. ingwe* was also higher than that of *H. luiperdjie*, possibly indicating that leopard host immune system may be better at suppressing infections by *H. luiperdjie* in neutrophils, than *H. ingwe* in lymphocytes. Additionally, there seemed to be a clear association between sex and parasitaemia in this study, with average parasitaemia in females (*H. luiperdjie* parasitized 18.2% and *H. ingwe* parasitized 35% of host cells) higher than that in males (*H. luiperdjie* parasitized 5.99% and *H. ingwe* parasitized 20% of host cells). This may be a noticeable health concern, since it is still unclear whether feline hepatozoonosis can be transferred within the uterus. However, this trend in parasitaemia needs further investigation over a larger variety and number of felid hosts.

In addition to infecting different types of host cells, the two new species showed clear morphological distinctions of peripheral blood gamont stages on a morphometric basis and differences in staining properties. The gamonts of *H. luiperdjie* are shorter, thinner and with a smaller surface area than those of *H. ingwe*. Furthermore, the staining properties of their cytoplasm, the marked presence of vacuoles in *H. ingwe* and the morphometric and staining differences in their nuclei morphologically distinguished these two species. In terms of nucleus dimensions, the nucleus of *H. luiperdjie* was significantly shorter ($P < 0.0001$), broader ($P < 0.0001$) and larger than that of *H. ingwe* ($P = 0.0025$). Both species described here had a larger surface area compared to the unnamed species of *Hepatozoon* parasitizing neutrophils of *Leopardus pardalis* from Brazil (Metzger et al. 2008) and an unnamed species of *Hepatozoon* parasitising neutrophils of *P. p. ciscaucasica* from Iran (Khoshnegah et al. 2012) (Table 4.3). Both new species of *Hepatozoon* had a smaller surface area as compared to an unnamed species of *Hepatozoon* from domestic cats (Perez et al. 2004) and *H. canis* from *Cerdocyon thous* from Brazil, respectively (Alencar et al. 1997) (Table 4.3). Data on the morphometrics of large numbers of gamonts are scarce, with some studies measuring only a few gamonts (Baneth et al. 2013, Hodžić et al. 2017), or even only a single gamont (Metzger et al. 2008). The current study focused on screening live hosts, while most other studies analysed necropsied or biopsied samples (Baneth et al. 2013, Harris et al. 2018, Hodžić et al. 2017), or did not focus on morphological descriptions of the gamont stage (McCully et al. 1975, Averbeck et al. 1990, Van Heerden et al. 1995, Maddock et al. 1996, Baneth et al. 1998, Rubini et al. 2006, Ortuño et al. 2007, Salakij et al. 2008a, Matjila et al. 2008a, Criado-Fornelio et al. 2009, Kubo et al. 2010, André et al. 2010, Sakuma et al. 2011, Pawar et al. 2012, Tateno et al. 2013). Several of these

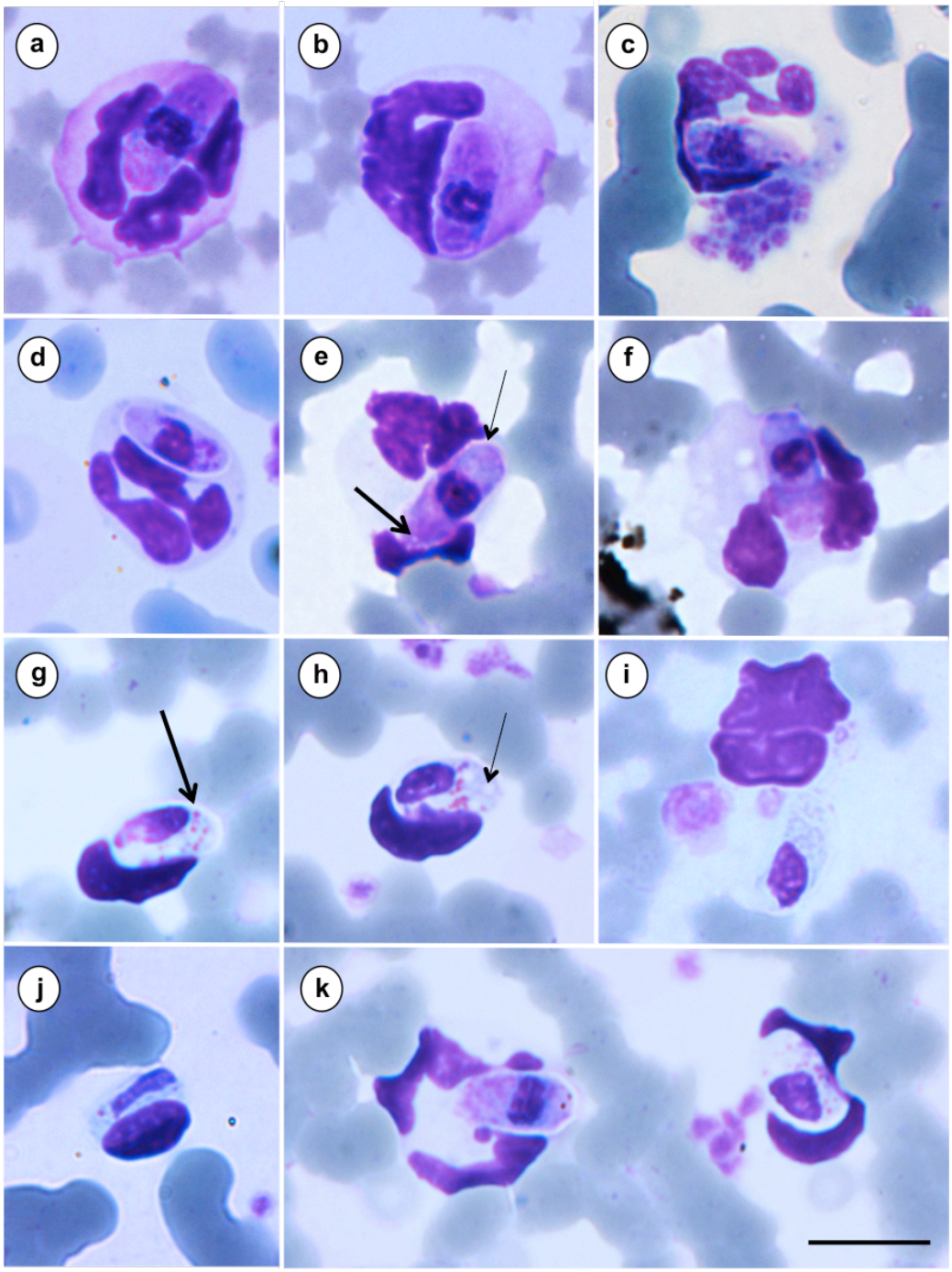


Figure 4.1 a–f Peripheral blood gamont stages of *Hepatozoon luiperdjie* in the African leopard *Panthera pardus pardus* from hapantotype slide (NMBP392). **a**, **b**, **e**, **f** Mature gamonts within neutrophils, where enlargement of host cell and displacement of host cell nucleus is apparent. **c** Extracellular gamont. **d** Immature gamont. **e** Mature gamont in which small posterior vacuoles (thick arrow) and thin capsule (thin arrow) can be seen. **c**, **f** Disintegration of neutrophils by infecting gamonts. **g–k** Peripheral blood gamont stages of *Hepatozoon ingwe* in the African leopard *Panthera pardus pardus* from hapantotype slide (NMBP393). **g**, **h**, **k** Mature gamonts within lymphocytes, where lateral compression of host cell is apparent. **g** Mature gamont in which bright pink granules and thin capsule (thick arrow) can be seen. **h** Mature gamont with prominent posterior vacuoles (thin arrow). **i** Extracellular gamont. **j** Immature gamont. **k** Co-infection of *Hepatozoon luiperdjie* (on the left) and *Hepatozoon ingwe* n. sp. in the same leopard. Scale bar = 10 μm.

studies reported only on molecular detection due to the general low parasitaemia of these haemogregarines (Harris et al. 2018), as confirmed by the absence of gamonts in the peripheral blood smear of CF5 in this study.

4.3.3 Molecular analyses

Amplicons were derived from *H. luiperdjie* and *H. ingwe* from the blood of leopards and the details of all sequences used for analysis are presented in Table 4.2. Although eight sequences were obtained from infected leopards, only a single sequence per species of *Hepatozoon* was used in the phylogenetic analysis (Fig. 4.2), as sequences obtained from samples with single species infections were identical for the respective new species. Sequences obtained from leopards with mixed *Hepatozoon* infections were not included in the phylogenetic analysis, as sequences contained a double chromatogram peak or two separate bases called at the same position (heterozygous positions) from the two new species of *Hepatozoon* amplified. Based on the uncorrected p-distance for the 18S rRNA gene between *H. ingwe* and *H. felis* [amplified from the Asiatic lion *Panthera leo persica* (Linnaeus, 1758) (GenBank: HQ829440) and Indian leopard *Panthera pardus fusca* (GenBank: HQ829444)] interspecific divergence was 1.0%. *Hepatozoon luiperdjie* and *H. felis* amplified from domestic cats, *F. catus* (GenBank: AY620232 and AY628681) and from a Bengal tiger *P. tigris tigris* (GenBank: HQ829445) had an interspecific divergence of 1.0%. The interspecific divergence between *H. luiperdjie* and *H. ingwe* was 1.0%.

For the BI phylogenetic analyses, first a large dataset was used comprising 297 sequences. This analysis included all the available 18S rDNA sequence data of *H. felis* downloaded from GenBank. The genera *Hemolivia*, *Hepatozoon* (parasitising amphibian, reptilian and rodent hosts), *Karyolysus*, *Haemogregarina* and *Dactylosoma* formed separate and well-supported clades at the base of the phylogeny. Species of *Karyolysus* and several species most likely incorrectly identified as species of *Hepatozoon*, formed a sister group to a large clade comprising species of *Hepatozoon* from large mammals. The phylogenetic analysis showed *H. felis* as paraphyletic forming several clusters, along with *Hepatozoon americanum*, *Hepatozoon apri* Yamamoto, Tokiwa, Tobiume, Akamatsu, Matsuo, Moribe & Ike, 2017, *Hepatozoon canis*, *Hepatozoon martis* Hodžić, Alić, Beck, Beck, Huber, Otranto, Baneth & Duscher, 2018, *Hepatozoon silvestris*, *Hepatozoon ursi* Kubo, Uni, Agatsuma, Nagataki, Panciera, Tsubota, Nakamura, Sakai, Masegi & Yanai, 2008 and *H. luiperdjie* and *H. ingwe*. Although several sequences are identified or designated as *H. felis*, the current study considers the sequences isolated from *H. felis* in domestic cats from Spain (GenBank: AY620232; AY628681) as sufficient representatives of *H. felis* based on phylogenetic comparisons to sequences in the formal redescription and molecular characterisation of *H. felis* (Baneth et al. 2013).

The second BI phylogenetic analysis was based on 60 18S rDNA sequences (Fig. 4.2). Species of *Hepatozoon* isolated from large mammal hosts formed a large well- supported clade (Clades A – E, Fig. 4.2). Clades A and B (monophyletic group), and Clades C, D and E, formed a polytomy of four distinct groups. Clade A was a monophyltic clade of *H. canis* and species of *Hepatozoon* isolated from various canid hosts. In Clade B, *H. ingwe* was shown as a sister taxon to a well-

supported monophyletic cluster of *H. felis* in big cats from India, namely the Asiatic lion (GenBank: HQ829440) and Indian leopard (GenBank: HQ829444) (Clade B in Fig. 4.2). In this clade, *H. ingwe* also clustered together with *H. felis* from Caracals (GenBank: MK621318) in South Africa's Namaqualand. Furthermore, *H. silvestris* (GenBank: KX757032), isolated form the European wild cat *Felis silvestris silvestris*, and *H. apri* (GenBank: LC314791) isolated from the Japenese boar, *Sus scrofa leucomystax* Temminck, 1842 formed a polytomy at the base of clade B. In clade C, *H. luiperdjie* clustered with sequences isolated from *H. felis* in domestic cats from Spain (GenBank: AY620232; AY628681), a Bengal tiger, *P. tigris tigris* (GenBank: HQ829445) from India, caracals from South Africa's Central Karoo and Cape Peninsula (GenBank: MK621310; MK621319), and *H. felis* amplified from a tick *Rhipicephalus sanguineus* (Latreille, 1806) that was collected from an Asiatic lion *Panthera leo persica* (GenBank: KY056823). In clade D, *H. martis* isolated from the European badger *Meles meles* (Linnaeus, 1758) and the European pine marten *Martes martes* (Linnaeus, 1758), formed a sister group to *H. ursi* isolated from the Japanese black bear *Ursus thibetanus japonicus* Schlegel, 1857 and the sloth bear *Melursus ursinus* (Shaw, 1791). Clade E comprised *H. americanum*, isolated from domestic dogs, *Canis familiaris* and an unnamed species of *Hepatozoon* in Crab-eating foxes *Dusicyon thous azarae* (Linneus, 1766).

4.4 Discussion

Since the late 1960s, the African leopard has been a favoured research subject of ethologists and ecologists. However, research on haematozoans of large carnivores is sparse and often occurs as non-specific reports, and according to Peirce et al. (1995) researchers often automatically identify species of *Hepatozoon* in African carnivores as *H. canis* or *H. felis*. According to Baneth et al. (2013), most studies on hepatozoonosis have emphasized the detection of the parasite, with little attention given to other aspects such as transmission and epidemiology. It is therefore not unexpected that the scant research on health aspects of African leopards left gaps in knowledge of especially their haemoparasites. The current study addressed these gaps and confirmed co-infection of two morphologically and genetically distinct *Hepatozoon* species in wild and captive African leopards in various areas throughout South Africa, both in male and female hosts.

No leopards sampled during the present study displayed any clinical symptoms associated with hepatozoonosis, confirming similar reports by authors such as Brocklesby & Vidler (1965), Averbeck et al. (1990) and East et al.2008). Prevalence of *Hepatozoon* varies between hosts and regions. The overall prevalence of hepatozoonosis recorded in this study was 56%, which is similar to that reported in Iriomote cats *Prionailurus bengalensis iriomotensis* from Japan (56.7%) (Sakuma et al. 2011), spotted hyenas *Crocuta crocuta* from Zambia (56%) (Williams et al. 2014), and captive Asiatic lions *P. l. persica* from India (55.56%) (Pawar et al. 2012). Prevalence found during the current study was slightly higher than the prevalence of unknown

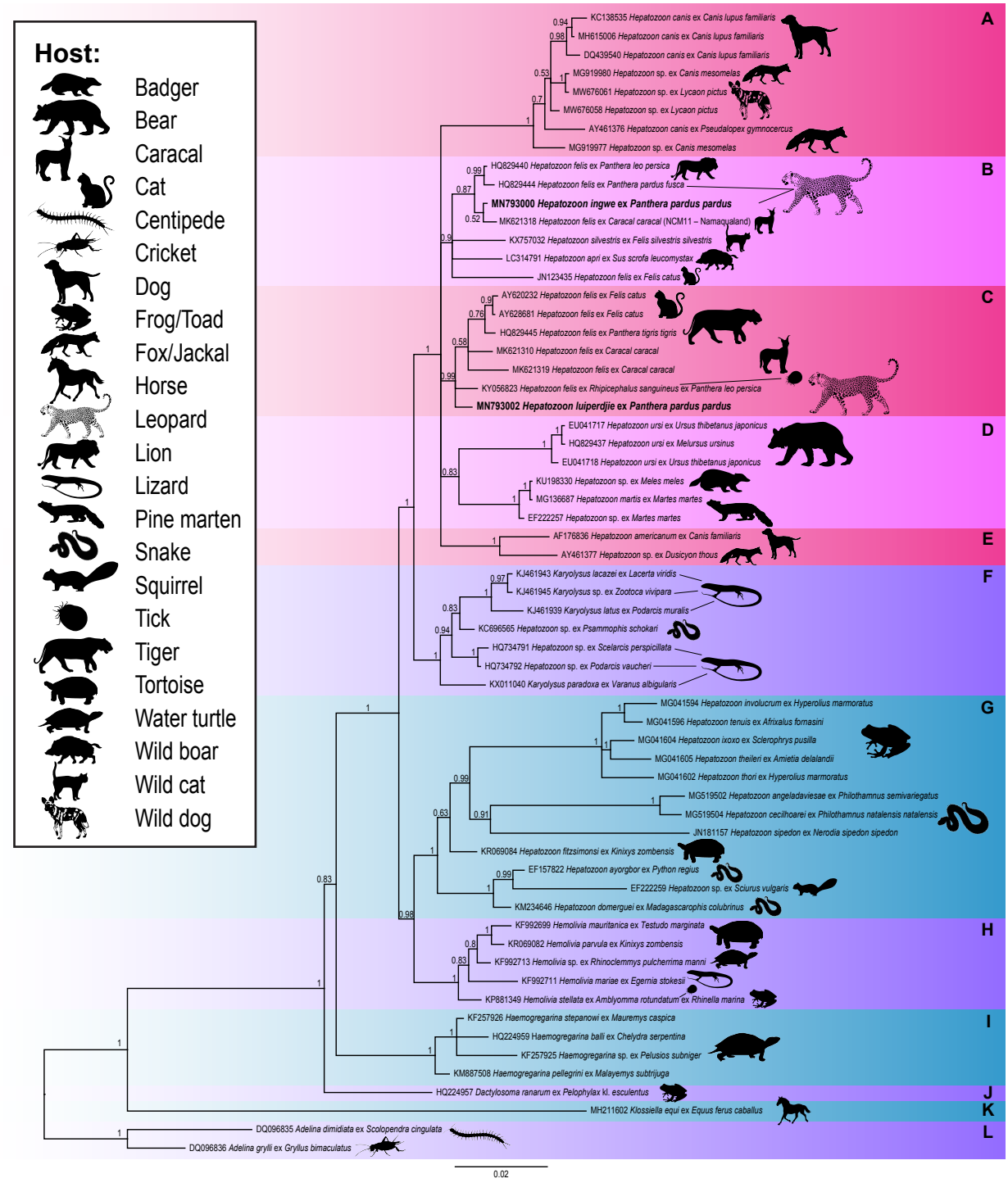


Figure 4.2 Bayesian inference (BI) phylogram based on *18S* rDNA sequences. Phylogram illustrating the phylogenetic relationships between *Hepatozoon luiperdjie* and *Hepatozoon ingwe* (shown in bold) with 55 representative sequences of other species of *Dactylosoma*, *Haemogregarina*, *Hepatozoon*, *Karyolysus* and *Hemolivia* retrieved from GenBank. *Adelina dimidiata*, *Adelina grylli* and *Klossiella equi* were selected as the outgroup. Posterior probability values lower than 0.60 were omitted. The scale-bar represents 0.02 nucleotide substitutions per site. Distinct clades are presented in alternating colors and letters A to L highlight 12 distinct clades.

species of *Hepatozoon* reported from Indian leopards *P. p. fusca* (50%) (Pawar et al. 2012) and much higher than that of African wild dogs *Lycaon pictus* in South Africa (0.7%) (Matjila et al. 2008a). However, hepatozoan prevalence from this study was lower than that of caracals in South Africa's central Karoo (92.6%) and Namaqualand (85.7%) (Viljoen et al. 2020). The prevalence of *H. luiperdjie* (50%) was similar to that of an unknown species of *Hepatozoon* reported from Indian leopards *P. p. fusca* in India (50%) (Pawar et al. 2012), and it was lower than the prevalence of *H. ingwe* (44%). The prevalence of both new species described in this study was higher in males than females, with 63% of males infected with *H. luiperdjie* and 50% of males infected with *H. ingwe*, and 38% of females infected with *H. luiperdjie* and *H. ingwe*, respectively.

The two new haemogregarines had dissimilar effects on their respective host cells. *Hepatozoon luiperdjie* caused enlargement of neutrophil cells and their nuclei and *H. ingwe* reduced the size of lymphocytes and condensed their nuclei. Although Baneth et al. (2013) reported on a species of *Hepatozoon* that infects both neutrophils and lymphocytes, the present study showed that co-infecting species of *Hepatozoon* can inhabit different types of leukocytes, with different effects and morphological characteristics of their gamont stages.

It is evident based on morphological and molecular data that *H. luiperdjie* and *H. ingwe* are distinct species. These species are also distantly related to *H. felis* (GenBank: AY628681) based on 18S rDNA sequence comparisons, isolated by Criado-Fornelio et al. (2006) from domestic cats from Spain. The *H. felis* isolates from Spain (Criado-Fornelio et al. 2006) are widely regarded as the representative *H. felis* isolates to be used for comparison (Perez et al. 2004, East et al. 2008, Sakuma et al. 2011). Thus, based on the phylogenetic relationships and comparisons of *H. felis* and *H. felis*-like species of *Hepatozoon*, the identity of the *Hepatozoon* infecting large carnivores, currently identified as *H. felis* by Pawar et al. (2012) is questioned. Therefore, based on these genetic distinctions, as well as morphological characteristics and effect on host cells, the two haemogregarines described here were deemed to be two different species and new to science. The phylogenetic results from the current study showed *H. luiperdjie* and *H. ingwe* as distinct species as compared to the currently recognised species of *Hepatozoon* infecting large mammal hosts, i.e. *H. americanum*, *H. apri*, *H. canis*, *H. felis*, *H. martis*, *H. silvestris* and *H. ursi*. This study therefore suggests that these haemogregarines may need to be re-classified based on morphological, morphometric and molecular analysis.

Prior to this study, only *H. felis*, *H. canis* and several unknown species of *Hepatozoon* have been reported from African carnivores (Brocklesby & Vidler 1963, McCully et al. 1975, Lane & Kocan 1983, Averbeck et al. 1990, Peirce et al. 1995, Van Heerden et al. 1995, Baneth et al. 1998, Perez et al. 2004, Williams et al. 2014, Penzhorn et al. 2018), but the current study confirmed a mixed population of two genetically distinct haemogregarines from both captive and wild leopards, across males and females and from leopards representative from three core populations as identified by Daly et al. (2005). The topology of the BI tree confirmed the suggestion of Hodžić et al. (2017), that *H. felis* should be viewed as a species complex.

4.5 Conclusions

As shown in this study, morphology and the effect on host cells are important parameters that should be considered when identifying species of *Hepatozoon*. By using different techniques of identification, a better understanding of the parasite and its relation to its host may become possible. Thus, *Hepatozoon* species identified as either *H. felis* or *H. canis* based on the host parasitized should be re-evaluated using both morphological and molecular characteristics, as well as the type of and effect on the host cells. The value of the results from this study, in addition to describing two new haemogregarine species, is that it presents results obtained from live animals in the wild. It is important to identify these parasites to species level in order to better understand potential zoonotic effects on different host species, and to further investigate the possible transfer of haemogregarines from wild to domestic animals. In addition, this paper provides valuable criteria to be considered when describing *Hepatozoon* infections from wild carnivores. Possible future work on these haemogregarines should include in depth investigations on the life cycles and vectors of these species.

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CHAPTER 5

Possible life cycle stages of a *Hepatozoon* species in an *Ixodes* tick

Some sections of this chapter has been submitted for peer review:
VAN AS, M., VAN AS, J., COOK, C.A. & SMIT, N.J. 2022. Morphologies of life cycle stages of a *Hepatozoon* species (Apicomplexa: Adeleorina: Hepatozoidae) in an *Ixodes* tick (Arthropoda: Ixodida: Ixodidae) from an infected African leopard. *International Journal for Parasitology: Parasites and Wildlife*. Under review.

5.1 Introduction

Intracellular haemogregarines from the genus *Hepatozoon* (Phylum Apicomplexa, Suborder Adeleorina, Family Hepatozoidae) infect a wide array of vertebrate hosts (see Smith 1996). Different hepatozoan species have morphologically comparable gamont stages that infect the vertebrate host's (or intermediate host's) blood cells (Levine 1988, Smith 1996). A completely and well-documented life cycle, which includes morphological data from the haematophagous host (or definitive host), is only available for a few *Hepatozoon* species (Smith 1996, Baneth et al. 1998, 2007, Van As et al. 2015).

Hepatozoonosis has been reported from several wild mammalian carnivore species (Order Carnivora) in Africa. Records include infections in side-striped jackal *Canis adusta* (Wenyon 1926), spotted hyaenas *Crocuta crocuta* (Wenyon 1926, Brocklesby & Vidler 1963, 1965, Krampitz et al. 1968, Basson et al. 1971, McCully et al. 1975, East et al. 2008), black-backed jackals *Canis mesomelas* (Brocklesby & Vidler 1963, 1965, Basson et al. 1971, McCully et al. 1975, Penzhorn et al. 2018, Viljoen et al. 2021), African wild dogs *Lycaon pictus* (Matjila et al. 2008, Netherlands et al. 2021), cheetahs *Acinonyx jubatus* (Basson et al. 1971, McCully et al. 1975) and lions *Panthera leo* (Brocklesby & Vidler 1963, 1965, Krampitz et al. 1968, Basson et al. 1971, McCully et al. 1975). Infections have also been reported from ungulates such as nyala *Tragelaphus angasii* (Basson et al. 1971), impala *Aepyceros melampus* (Basson et al. 1967), bushbuck *Tragelaphus sylvaticus* (Basson et al. 1971), reedbuck *Redunca arundinum* and giraffe

Giraffa camelopardalis (Fantham 1920), which all form part of the potential prey basis of African carnivores.

Feline hepatozoonosis was first reported from a domestic cat in India during the early 1900s (Patton 1908), described as an intraleukocytic parasite *Leucocytozoon felis domestici* Patton, 1908. Thereafter it was reassigned to the genus *Hepatozoon* (Wenyon 1926) and accepted as *Hepatozoon felis* (Patton 1908) (more details are presented in Chapter 4). Until recently, it was thought that wild and domestic felines are generally infected with *H. felis* (Baneth et al. 2013), but a more intricate species complex has been shown through descriptions of *Hepatozoon silvestris* in a European wild cat *Felis silvestris silvestris* (Hodžić et al. 2017) and co-infections of *Hepatozoon luiperdjie* and *Hepatozoon ingwe* in African leopards *Panthera pardus pardus* in South Africa (Van As et al. 2020).

Species of *Hepatozoon* typically have a two host life cycle, involving sporogonic development and oocyst formation in the definitive haematophagous invertebrate host, and merogony and gametogony in the intermediate vertebrate host (Siddall 1995, Smith 1996, Telford 2009). Definitive *Hepatozoon* hosts include mites, ticks, tsetse flies, sand flies, mosquitoes, fleas, leeches, lice and reduviid bugs (Smith 1996). Transmission to an intermediate vertebrate host is generally accomplished through ingestion of a mature, oocyst-containing, definitive arthropod host (McCully et al. 1975, Davies & Johnston 2000, Baneth et al. 2007, Van As et al. 2015), but it has also been suggested that infection is possible by predation on other infected intermediate vertebrate hosts (Smith 1996, Ewing et al. 2002, Baneth 2011). Grooming behaviour may also facilitate haemogregarine infections (Ewing et al. 2002, East et al. 2008) and transplacental transmission has been reported in *Hepatozoon canis* infections in mammals (Murata et al. 1993). Baneth et al. (2013) also suggested the possibility of transplacental transmission of *H. felis* in domestic cats.

It has been reported that *H. canis* sporocysts rupture to release sporozoites when coming into contact with bile (Redington & Jachowski 1971, Mathew et al. 1999). It is unknown how *Hepatozoon* sporozoites migrate through a carnivore body following ingestion of an infected haematophagous vector. Baneth et al. (2007) proposed some interesting theories in this regard, all of which remain to be confirmed. Infective sporozoites are released in the intermediate host's gut and penetrate the intestinal wall from where the host's bloodstream carries them to become meronts in the bone marrow, spleen, lungs, liver, lymph nodes and myocardium (McCully et al. 1975, Craig et al. 1978, O'Dwyer 2011). Giannelli et al. (2017) found that sporozoites react to exposure to bile by starting to move in a gliding and flexing manner. It has been shown that the schizogonic cycle of *H. canis* starts in the domestic dog's intestinal wall (Forlano et al. 2005). The *Hepatozoon* gamont stage infects erythrocytes or leukocytes of the vertebrate host, depending on the identity of the host. Reptile erythrocytes and mammalian and avian leukocytes are typically infected by gamont stages (Smith 1996, Telford 2009, Baneth 2011). Small merogonic cysts in vertebrate host tissues, containing various parasitic stages, have been reported for several *Hepatozoon* species (Smith 1996) and these cysts are

considered to be mainly associated with infections incurred by predation (Smith et al. 1994, Vincent-Johnson et al. 1997, Paperna et al. 2002, Baneth et al. 2003, 2007). It also seems that the locality of these cysts may be species-specific to its infecting haemogregarine (Baneth 2011). In mammal hosts, merozoites are released from meronts and invade leukocytes that enter the peripheral bloodstream, where development into gamonts takes place (McCully et al. 1975, O'Dwyer 2011).

Haematophagous ticks ingest *Hepatozoon*-containing leukocytes during a blood meal and the gamonts exit the leukocytes within the gut of the tick. Here, gamonts group into pairs and differentiate into micro- and macrogametes, which fuse to form a zygote. Zygotes then undergo sporogony to develop into mature oocysts, containing many sporocysts with infective sporozoites in the haemocoel of the tick (Baneth et al. 2001, 2003, 2007). The number and morphometrics of intrasporocystic sporozoites could be characteristic of a species of *Hepatozoon* (Telford 2009).

The vectors of many *Hepatozoon* spp. are still unknown and need detailed research (Smith 1996, Forlano et al. 2005, Baneth 2011). Currently, to the authors' best knowledge, all known life cycles of *Hepatozoon* spp. infecting mammalian carnivores have an ixodid tick definitive host (Table 5.1). The development of *H. canis* was first described in *Rhipicephalus sanguineus* by Christophers (1907) and Wenyon (1911). Current mammalian carnivore-associated vector and life cycle stages have only been reported for *H. canis* (Baneth et al. 2007, Rubini et al. 2009, de Miranda et al. 2011, O'Dwyer 2011, Giannelli et al. 2013a, 2017), *Hepatozoon americanum* (Mathew et al. 1998, Ewing et al. 2002), *Hepatozoon ursi* and *Hepatozoon martis* from Japanese martens (Hodžić et al. 2018). Various tick species such as *Haemaphysalis longicornis* Neumann, 1901*, Haemaphysalis hystricis* Supino, 1897*, Haemaphysalis megaspinosa* Saito, 1969*, Haemaphysalis campanulata* Warburton, 1908*, Amblyomma testudinarium* Koch, 1844 and *Ixodes tanuki* Saito, 1964 have been reported to contain *H. felis* DNA (Tateno et al. 2015). The DNA of *H. felis* was also detected in *R. sanguineus* ticks collected from humans in Turkey (Aktas 2014), domestic cats in Portugal (Maia et al. 2014) and captive lions in Thailand (Bhusri et al. 2017).

Several studies only report the presence of oocysts in possible tick vectors from infected carnivores, without studying further possible developmental stages. Further stages such as free-lying sporocysts and extracellular/free-swimming sporozoites are rarely reported, as it is accepted that oocysts are the infective stage for intermediate hosts (Murata et al. 1995, Mathew et al. 1999, Ewing et al. 2002, Forlano et al. 2005, Baneth et al. 2007, Rubini et al. 2009, Baneth 2011, de Miranda et al. 2011, Demoner et al. 2013, Giannelli et al. 2013a). However, studies similar to the report by Giannelli et al. (2013b) on *H. canis* in *R. sanguineus* ticks, have reported further developmental stages. Only two studies described the presence of free-swimming sporozoites; Forlano et al. (2005) and Giannelli et al. (2017) reported free sporozoites of *H. canis* in *Amblyomma ovale* Koch, 1844 and *Rhipicephalus turanicus* Pomerantsev 1936 ticks, respectively.

Various studies only report on the presence of oocysts and not their morphometrics. During a study on hepatozoonosis in spotted hyaenas, lions, jackals, cheetahs and one leopard in South Africa's Kruger National Park, McCully et al. (1975) reported sporogenous development of a *Hepatozoon* sp. in the haemolymph smears of *Rhipicephalus simus* Koch, 1844 females from a Spotted hyaena, with no development in *R. sanguineus* or *Haemaphysalis leachi* (Audouin, 1826) ticks collected from the same hyaena. However, McCully et al. (1975) did find that *R. sanguineus* are successful vectors for a *Hepatozoon* sp. infecting black-backed jackals, but no morphometrics was reported during their study. Other studies reporting on carnivore-related hepatozoan developmental stages within ixodid ticks without noting morphometric data include that of Demoner et al. (2013) (*H. canis*), Bhusri et al. (2017) (*H. felis*), and Hodžić et al. (2018) (*H. martis*).

The clinical properties of feline hepatozoonosis are currently still poorly understood (Kegler et al. 2018) and the potential vectors for feline hepatozoonosis remain relatively unknown (Baneth et al. 2013, Lloret et al. 2015). Little is known about the host-specificity of hepatozoans in African carnivores and it has been suggested by Redington & Jachowski (1971) that more studies should focus on the developmental stages in associated invertebrate hosts. According to Baneth et al. (2013), it can be expected that this haemogregarine is most likely transmitted by haematophagous arthropods, just like other species of the genus *Hepatozoon*. This section of the current study investigated whether ticks collected from an African leopard infected with *H. luiperdjie* can serve as potential vectors for this *Hepatozoon* species. Herewith the author gives the first detailed morphological descriptions of hepatozoan life cycle stages in its wild-collected haematophagous host, an *Ixodes* sp. tick (Arthropoda: Ixodida: Ixodidae). This is the first report of its kind for a leopard-associated *Hepatozoon* species.

5.2 Materials and methods

Description of study area, characterisation of wild and captive leopards and blood collection can be read in Chapter 2 General Materials and Methods. Preparation of thin bloodsmears have been described in Chapter 4 Materials and Methods.

Engorged ticks were collected from a known infected leopard from this study. Ixodid ticks can stay attached to their hosts for several days while feeding (Walker et al. 2003). According to literature, early oocysts of *H. canis* have been observed in ticks within one to four days after removal of an engorged tick from canid hosts (Baneth et al. 2007, Rubini et al. 2009, O'Dwyer 2011). Considering this, the author wanted to see if further development takes place and therefore engorged ticks were dissected only after seven days of being kept in a fasting state. Ticks representing four genera were ventrally incised from posterior to anterior to expose gut contents and haemolymph.

Table 5.1 Morphometrics of mammalian carnivore *Hepatozoon* sp. developmental stages in their haematophagous definitive hosts. NS: not stated; ☐: not relevant.

Vector	*Hepatozoon* species	Measurements (L±SD x W±SD (Min - Max) or diameter (Min – Max) or range (Min - Max)) μm	Number measured/ counted	Next developmental stage/ within previous stage	Number of intracellular developmental stage	Associated intermediate host	Reference
			Immature oocysts				
Rhipicephalus sanguineus	*Hepatozoon canis*	188.3±34.7 × 171.1±40.0	49	☐	☐	*Canis familiaris*	(Giannelli et al. 2013b)
		78.8±12.4 x 78.8±12.4	3	☐	☐	*Canis familiaris*	(Baneth et al. 2007)
Rhipicephalus turanicus	*Hepatozoon canis*	201±72.8 x 138.8±48.6	NS	☐	☐	*Canis familiaris*	(Giannelli et al. 2017)
			Maturing oocysts				
Amblyomma maculatum	*Hepatozoon americanum*	269 (188–323)	40	☐	☐	*Canis familiaris*	(Ewing et al. 2002)
		☐	50	Sporocysts within maturing oocyst	311 (91–458) (n=50 oocysts)	*Canis familiaris*	(Ewing et al. 2002)
Rhipicephalus sanguineus	*Hepatozoon canis*	252.6±68.4 x 247.3±76	2	☐	☐	*Canis familiaris*	(Baneth et al. 2007)
			Mature/sporulated oocysts				
Amblyomma maculatum	*Hepatozoon americanum*	339 (159–438)	50	☐	☐	*Canis familiaris*	(Ewing et al. 2002)
Amblyomma ovale	*Hepatozoon canis*	244.34 x 255.46	4	☐	☐	*Canis familiaris*	(Rubini et al. 2009)
Rhipicephalus microplus	*Hepatozoon canis*	251.3±13.7 x 173.9±5.34	8	☐	☐	*Canis familiaris*	(de Miranda et al. 2011)
Rhipicephalus sanguineus	*Hepatozoon canis*	237.1±27.1 × 226.9±24.0	2	☐	☐	*Canis familiaris*	(Giannelli et al. 2013b)
		309.8±31.8 x 255.8±48.0	5	☐	☐	*Canis familiaris*	(Baneth et al. 2007)
Haemaphysalis flava	*Hepatozoon ursi*	297.5 (263.2–331.8) x 232.85 (231.70–234.0)	2	☐	☐	*Ursus thibethanus japonicus*	(Kubo et al. 2008)
Rhipicephalus turanicus	*Hepatozoon canis*	259.9±36.1 x 246.1±33.9	NS	☐	☐	*Canis familiaris*	(Giannelli et al. 2017)

Table 5.1 Morphometrics of mammalian carnivore *Hepatozoon* sp. developmental stages in their haematophagous definitive hosts. NS: not stated; ❐: not relevant.

Vector	*Hepatozoon* species	Measurements (L±SD x W±SD (Min - Max) or diameter (Min – Max) or range (Min - Max)) μm	Number measured/ counted	Next developmental stage/ within previous stage	Number of intracellular developmental stage	Associated intermediate host	Reference
			Mature/sporulated oocysts				
Amblyomma maculatum	*Hepatozoon americanum*	390.0±59.9 x 35.6±58.9 (310–480 x 260–460)	24	❐	❐	*Canis familiaris*	(Vincent-Johnson et al. 1997)
Rhipicephalus sanguineus	*Hepatozoon canis*	214.8±45.5 x 192.9±36.5 (160–325 x 138–258)	15	❐	❐	*Canis familiaris*	(Vincent-Johnson et al. 1997)
Haemaphysalis longicornis	*Hepatozoon canis*	300 x 150	2	❐	❐	*Canis familiaris*	(Murata et al. 1995)
Amblyomma ovale	*Hepatozoon canis*	214 x 209	2	❐	❐	*Canis familiaris*	(Forlano et al. 2005)
Amblyomma maculatum	*Hepatozoon americanum*	294±43 (175–495)	100	❐	❐	*Canis familiaris*	(Mathew et al. 1999)
		225±37 (145–405)	100	❐	❐	*Canis familiaris*	(Mathew et al. 1999)
		300–1000	20	❐	❐	*Canis familiaris*	(Mathew et al. 1998)
Ixodes sp.	*Hepatozoon* sp.	190.88±16.35 (179.52–209.62) x 157.74±18.78 (136.39–171.76)	3	❐	❐	*Panthera pardus pardus*	**This study**
			Intraoocysctic sporocysts				
Rhipicephalus sanguineus	*Hepatozoon canis*	25.5±2.9 × 20.2±5.8	NS	❐	❐	*Canis familiaris*	(Giannelli et al. 2013b)
		32.3±0 x 13.95±1.5	2	❐	❐	*Canis familiaris*	(Baneth et al. 2007)
Haemaphysalis flava	*Hepatozoon ursi*	31.2±2.5 × 27.0±2.9 (28.0–34.6 × 23.7–32.0)	5	❐	❐	*Ursus thibethanus japonicus*	(Kubo et al. 2008)
		❐	2	Sporocysts within mature oocysts	45 (40–50) (n=2 oocysts)	*Ursus thibethanus japonicus*	(Kubo et al. 2008)
Rhipicephalus turanicus	*Hepatozoon canis*	32.1±4.7 x 20.2±2	NS	❐	❐	*Canis familiaris*	(Giannelli et al. 2017)

Table 5.1 Morphometrics of mammalian carnivore *Hepatozoon* sp. developmental stages in their haematophagous definitive hosts. NS: not stated; ☐: not relevant.

Vector	*Hepatozoon* species	Measurements (L±SD x W±SD (Min - Max) or diameter (Min – Max) or range (Min - Max)) μm	Number measured/ counted	Next developmental stage/ within previous stage	Number of intracellular developmental stage	Associated intermediate host	Reference
			Intraoocysctic sporocysts				
Haemaphysalis longicornis	*Hepatozoon canis*	30 x 30	NS	☐	☐	*Canis familiaris*	(Murata et al. 1995)
		☐	2	Sporocysts within mature oocysts	50 – 70 (n=2 oocysts)	*Canis familiaris*	(Murata et al. 1995)
Amblyomma ovale	*Hepatozoon canis*	3.5 x 21.5	NS	☐	☐	*Canis familiaris*	(Forlano et al. 2005)
		☐	2	Sporocysts within mature oocysts	240 (n=2 oocysts)	*Canis familiaris*	(Forlano et al. 2005)
Amblyomma maculatum	*Hepatozoon americanum*	27.8±4.8 (18–39)	100	☐	☐	*Canis familiaris*	(Mathew et al. 1999)
		☐	20	Sporocysts within mature oocysts	656±176 (260–1040) (n=20 oocysts)	*Canis familiaris*	(Mathew et al. 1999)
			Sporozoites within intraoocystic sporocysts				
Haemaphysalis flava	*Hepatozoon ursi*	12.2±1.4 × 3.5±0.5 (10.0–14.0 × 2.9–4.2)	4	☐	☐	*Ursus thibethanus japonicus*	(Kubo et al. 2008)
		☐	4	Sporozoites within intraoocystic sporocysts	8 – 16 (n=4 intraoocystic sporocysts)	*Ursus thibethanus japonicus*	(Kubo et al. 2008)
Amblyomma ovale	*Hepatozoon canis*	☐	NS	Sporozoites within intraoocystic sporocysts	20 (n= NS)	*Canis familiaris*	(Forlano et al. 2005)
Amblyomma maculatum	*Hepatozoon americanum*	15±1.2 x 5	NS	☐	☐	*Canis familiaris*	(Mathew et al. 1999)
		☐	15	Sporozoites within intraoocystic sporocysts	10 – 26 (n=15 intraoocystic sporocysts)	*Canis familiaris*	(Mathew et al. 1999)

Table 5.1 Morphometrics of mammalian carnivore *Hepatozoon* sp. developmental stages in their haematophagous definitive hosts. NS: not stated; ❐: not relevant.

Vector	*Hepatozoon* species	Measurements (L±SD x W±SD (Min - Max) or diameter (Min – Max) or range (Min - Max)) μm	Number measured/ counted	Next developmental stage/ within previous stage	Number of intracellular developmental stage	Associated intermediate host	Reference
			Free Sporocysts				
Amblyomma maculatum	*Hepatozoon americanum*	25 (20–31)	50	❐	❐	*Canis familiaris*	(Ewing et al. 2002)
		❐	28	Sporozoites within free sporocysts	18 (12–26) (n=28 sporocysts)	*Canis familiaris*	(Ewing et al. 2002)
Rhipicephalus sanguineus	*Hepatozoon canis*	37.0±4.2 × 21.6±1.5	NS	❐	❐	*Canis familiaris*	(Giannelli et al. 2013b)
Amblyomma maculatum	*Hepatozoon americanum*	26.1±2.0 x 24.8±1.8 (20–30 x 20–29)	58	❐	❐	*Canis familiaris*	(Vincent-Johnson et al. 1997)
Rhipicephalus sanguineus	*Hepatozoon canis*	35.6±3.7 x 25.7±2.8 (29–41 x 17–30)	31	❐	❐	*Canis familiaris*	(Vincent-Johnson et al. 1997)
Ixodes sp.	*Hepatozoon* sp.	30.3±2.0 (26.5–34.2) x 29.8±2.3 (24–32.8)	20	❐	❐	*Panthera pardus pardus*	**This study**
Ixodes sp.	*Hepatozoon* sp.	❐	14	Sporozoites within free sporocysts	30±6 (21–41) (n=14 sporocysts)	*Panthera pardus pardus*	**This study**
Haemaphysalis longicornis	*Hepatozoon canis*	❐	NS	Sporozoites within free sporocysts	10 – 16 (n= NS)	*Canis familiaris*	(Murata et al. 1995)
Amblyomma maculatum	*Hepatozoon americanum*	20–30	20	❐	❐	*Canis familiaris*	(Mathew et al. 1998)
		❐	20	Sporozoites within free sporocysts	8 – 16 (n=20 sporocysts)	*Canis familiaris*	(Mathew et al. 1998)
			Intrasporocystic sporozoites in free sporocysts				
Ixodes sp.	*Hepatozoon* sp.	12.86±4.22 (6.56–19.92) x 2.00±0.60 (1.17–2.81)	9	❐	❐	*Panthera pardus pardus*	**This study**
Haemaphysalis longicornis	*Hepatozoon canis*	10 x 3	NS	❐	❐	*Canis familiaris*	(Murata et al. 1995)

Table 5.1 Morphometrics of mammalian carnivore *Hepatozoon* sp. developmental stages in their haematophagous definitive hosts. NS: not stated; ❐: not relevant.

Vector	*Hepatozoon* species	Measurements (L±SD x W±SD (Min - Max) or diameter (Min – Max) or range (Min - Max)) μm	Number measured/ counted	Next developmental stage/ within previous stage	Number of intracellular developmental stage	Associated intermediate host	Reference
			Intrasporocystic sporozoite nuclei				
Ixodes sp.	*Hepatozoon* sp.	1.72±0.56 (0.93–3.4) x 1.65±0.46 (2.00–2.60)	23	❐	❐	*Panthera pardus pardus*	**This study**
			Free swimming mature sporozoites				
Rhipicephalus turanicus	*Hepatozoon canis*	15.5±4.1 x 3±0.6	NS	❐	❐	*Canis familiaris*	(Giannelli et al. 2017)
Amblyomma maculatum	*Hepatozoon americanum*	13–17 x 4–7	20	❐	❐	*Canis familiaris*	(Mathew et al. 1998)
Ixodes sp.	*Hepatozoon* sp.	17.11±1.22 (14.89–20.11) x 3.09±0.47 (2.07–4.19)	22	❐	❐	*Panthera pardus pardus*	**This study**
			Nuclei of free swimming mature sporozoites				
Ixodes sp.	*Hepatozoon* sp.	2.92±0.44 (2.16–3.67) x 2.81±0.57 (1.91–3.79)	22	❐	❐	*Panthera pardus pardus*	**This study**

Gut contents containing haemolymph were then smeared on clean microscope slides, fixed in absolute methanol for one minute, air-dried and stained with 10% Giemsa (Sigma-Aldrich®) solution for 20 minutes according to Van As et al. (2015). Peripheral blood and tick smears were scanned with a Nikon Eclipse E800 compound microscope (Nikon, Amsterdam, The Netherlands) under a 40x objective to find possible *Hepatozoon* life stages. Identified stages were micrographed with an attached Nikon DS-Fi1 digital camera and accompanying software. Identified and clearly visible developmental life cycle stages (and their subsequent characteristics) were digitally measured with the ImageJ version 1.47 software program (Wayne Rasband National Industries of Health, USA) (Rasband 2014) (http://imagej.nih.gov/ij).

5.2.1 Morphological identification of *Hepatozoon* life cycle stages

A compilation of several literature sources was used in the identification of possible life cycle stages of *Hepatozoon* species (Table 5.2). This study followed the suggestion by Giannelli et al. (2013a) that the presence of *Hepatozoon* sp. oocysts in a tick vector collected from a known infected carnivore with the same *Hepatozoon* sp. infection is indicative that the tick may be a potential vector for that *Hepatozoon* species. Ticks were identified using Walker et al. (2003) and identification was confirmed by tick specialists at The Agricultural Research Council-Onderstepoort Veterinary Research Campus (ARC-OVR), South Africa.

5.3. Results

Refer to Chapter 2 Tables 2.3 and 2.4 to see which samples were obtained from which captive and wild leopards in this study.

All *Hepatozoon* sporogonic stages were found within a single hard-bodied tick, identified as an unknown *Ixodes* Latreille, 1795 species (Order Ixodida; Family Ixodidae) (Fig. 5.1) (Table 5.1). Other ticks collected from this leopard included *Haemaphysalis elliptica* (Koch, 1844) (Order Ixodida; Family Ixodidae; Subfamily Haemaphysalinae), *Hs. leachi* (Order Ixodida; Family Ixodidae; Subfamily Haemaphysalinae), *R. simus* (Order Ixodida; Family Ixodidae) and an unknown *Haemaphysalis* Koch, 1844 species. No hepatozoan developmental stages were observed in these ticks.

Peripheral blood smears of this particular leopard had gamont stages of *H. luiperdjie* at various degrees of maturity. This specific infection from this specific leopard was previously reported, both morphologically and molecularly by Van As et al. (2020) (see Chapter 4 Fig. 4.1 a – f). This specific leopard was only infected with *H. luiperdjie* (Van As et al. 2020), therefore the author assumes that the sporogonic stages seen in this dissected *Ixodes* tick, collected from this leopard while fully engorged, represent this hepatozoan species.

For clarity, a detailed, illustrated representation of the possible life cycle of this *Hepatozoon* species has been constructed (Fig. 5.2). Merogonic stage sections of this illustration (Fig. 5.2 l – p) include redrawn portrayals of merogony of *H. felis* in domestic cats (Baneth et al. 2013), and an unnamed *Hepatozoon* in spotted hyaenas (from the same area in South Africa where the leopard from this study was sampled (McCully et al. 1975) (Fig. 5.2 l – p).

5.3.1 Description of sporogonic stages in a wild-collected, engorged *Ixodes* sp. tick

Figure 5.1 a – h.
Figure 5.2 c – j.

Stages found within the haemocoel of an *Ixodes* sp. tick included maturing sporulated oocysts, free sporocysts with growing sporozoites at various stages of development, mature sporocysts with mature sporozoites, ruptured sporocysts with exuding sporozoites and free-swimming sporozoites. No gametogenesis and subsequent fertilization was observed. Development of sporogonic stages, with corresponding micrographs (Fig. 5.1), are described below.

Maturing, sporulated oocysts (Figure 5.1 a and Figure 5.2 f): Suspended free in the haemocoel. Spherical/oval shape. Mature oocysts measured 190.88 ± 16.35 (179.52–209.62) x 157.74 ± 18.78 (136.39–171.76) μm, area 23302.27 ± 3035.21 (19932.52–25821.45) μm^2 (n=4). Contained 23–25 sporocysts each (n=4). Faint, thin, dark blue membrane. Cytoplasm uniform purplish blue, more foamy at apical area. Differentiating nuclei (average 19 per oocyst) equally distributed in cytoplasm, dense and uniform dark indigo, round to oval shape.

Remarks (oocysts): Sporulated oocysts were clearly visible in the tick's haemocoel as free-lying dots on Giemsa-stained microscope slides, similar to sporulated oocysts of *H. americanum* (Mathew et al. 1998, 1999) in *Amblyomma maculatum* Koch, 1844 (Vincent-Johnson et al. 1997), *H. canis* (Murata et al. 1995) and an unknown *Hepatozoon* species (McCully et al. 1975). Maturing hepatozoan oocysts have also been reported in *A. maculatum* (*H. americanum*) (Ewing et al. 2002) and *R. sanguineus* (*H. canis*) (Baneth et al. 2007) and *Rhipicephalus turanicus* (*H. canis*) (Giannelli et al. 2017). Similar to the studies of Kubo et al. (2008) and Murata et al. (1995), no immature or ruptured oocysts releasing sporocysts were observed. The number of intraoocystic sporocysts were closest to that of *H. ursi* in *Haemaphysalis flava* Neumann, 1897 (Kubo et al. 2008) (Table 5.1) and *H. canis* in *Hs. longicornis* (Murata et al. 1995), but far fewer than that of *H. americanum* in *A. maculatum* (Ewing et al. 2002) and *A. ovale* (Forlano et al. 2005) (Table 5.1).

Table 5.2 Morphological characteristics of various *Hepatozoon* life cycle stages (mainly compiled from Smith 1996, Telford 2009).

Life cycle stage	Morphological description	Additional References
Immature oocyst	Near-spherical in shape with a dense central matrix, surrounded by lighter staining basophilic cytoplasm and visibly evident outer membrane. No sporocysts formed.	Baneth et al. (2007) Giannelli et al. (2013b, 2017)
Maturing oocysts	Early sporocyst formation visible as nonhomogenic cytoplasm. Thick visible membrane.	Ewing et al. (2002) Baneth et al. (2007) Van As et al. (2015)
Mature oocysts	Appear as a thin structure encompassing sporocysts. Sporulated with defined sporocysts containing developing sporozoites. Thin membrane. Considered the infective stage to intermediate vertebrate host.	Vincent-Johnson et al. (1997) Ewing et al. (2002) Baneth et al. (2007) de Miranda et al. (2011) Giannelli et al. (2013b)
Sporocysts	Round to ellipsoidal within maturing or mature oocysts; sometimes free in tick haemocoel. Membrane wall could be thick or thin. Internal cytoplasm foamy in young sporocysts, endopolygenous development into intrasporocystic sporozoites. Mostly intraoocystic, could be free in haemolymph.	Murata et al. (1995) Mathew et al. (1999) Baneth et al. (2007) Kubo et al. (2008) Giannelli et al. (2013a, 2017)
Sporozoites	Elongated, narrow, curved, slightly banana shaped with foamy or lightly stained cytoplasm and dense nuclei. Thickly packed within sporocyst with no particular arrangement. Thin membrane. Mostly intrasporocystic, could be free in haemolymph.	Murata et al. (1995) Mathew et al. (1998, 1999) Forlano et al. (2005) Kubo et al. (2008) Giannelli et al. (2017)

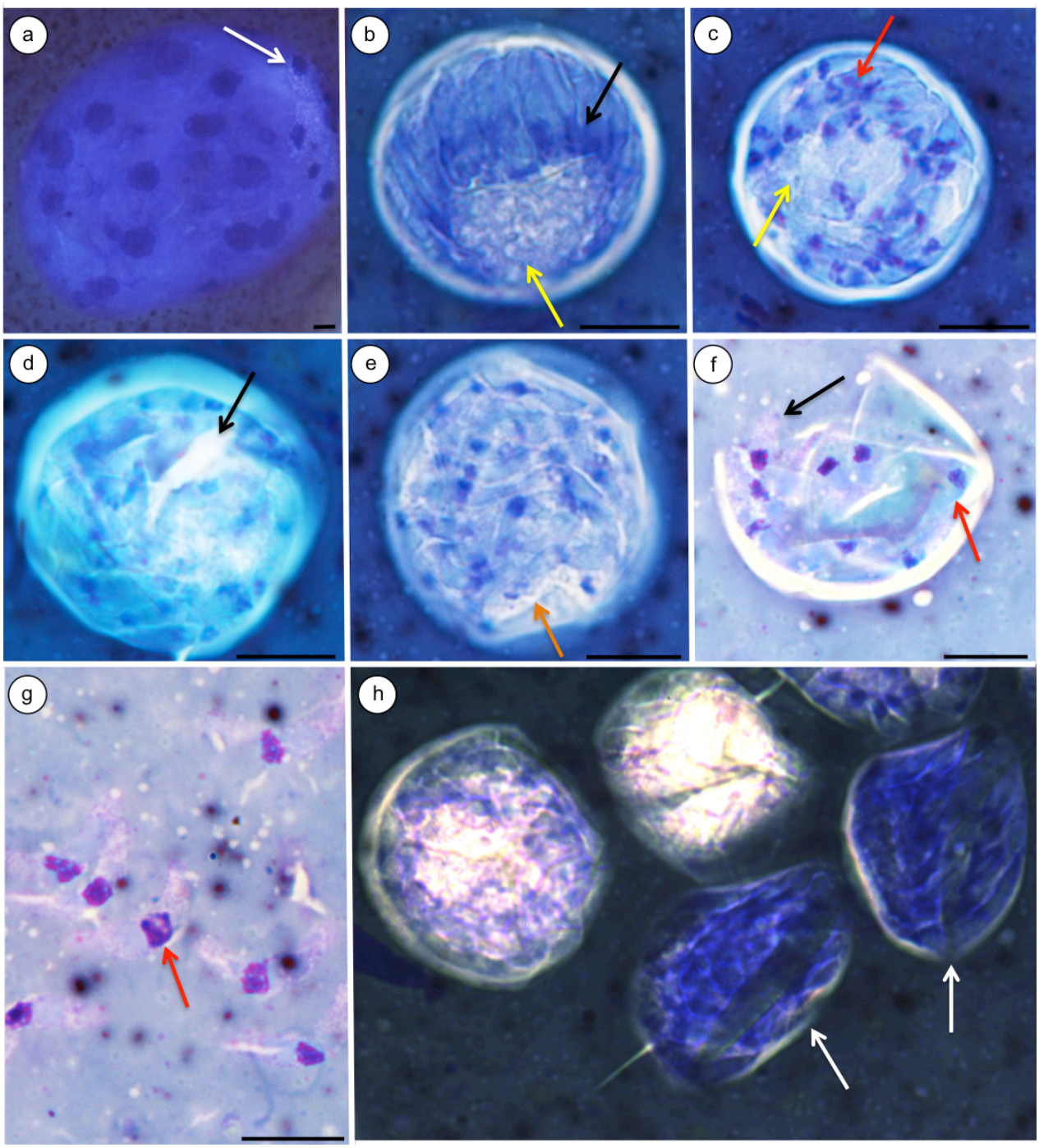

Figure 5.1 Sporogonic stages in the haemocoel of an *Ixodes* tick. **a** Mature, multinucleate sporulated oocyst with 19 nuclei. (White arrow: intracellular developing sporocysts apexally aggregating); **b–d** Maturing, free-lying sporocysts at various stages of development. Note how the contained sporozoites mature from b–d; **b** Immature, free-lying sporocyst with distinct, white membrane. (Black arrow: apical aggregation of chromatin; Yellow arrow: sporocyst basal mass). **c** Immature, intrasporocystic sporozoites. (Red arrow: dense, granulated chromatin; Yellow arrow: sporocyst basal mass). **d** Maturing intrasporocystic sporozoites. (Black arrow: no basal mass left in white central area). **e** Mature sporocyst with lysed membrane. (Orange arrow: Foamy, light blue cytoplasm). **f** Ruptured mature sporocyst with exuding mature sporozoites. (Black arrow: mature, exuding sporozoite with tapered extremities; Red arrow: mature sporozoites still intrasporocystic). **g** Free-lying, mature sporozoites in the haemocoel. (Red arrow: loosely arranged chromatin in middle of sporozoite body). **h** Free-lying sporocysts at various stages of development. (White arrows: obscured, free-lying sporocysts). Scale bar = 10 μm.

Sporulation seems to commence at the apical area of oocysts (white arrow in Fig. 5.1 a), with remaining oocyst nuclei dispersed throughout the rest of the cytoplasm. Sporulated oocysts were oval-shaped, differing from round sporulated oocysts of *H. canis* in adult *A. ovale* ticks (Forlano et al. 2005, Rubini et al. 2009). Oval-shaped sporulated oocysts have, however, been reported for *H. canis* in a naturally infected *Rhipicephalus microplus* (Canestrini, 1888) tick (de Miranda et al. 2011), and in adult *R. sanguineus* (Giannelli et al. 2013b) and *R. turanicus* (Giannelli et al. 2017). Sporulated oocysts' thin membranes were similar to those of *H. canis* in *R. sanguineus* (Baneth et al. 2007) and *Hs. longicornis* (Murata et al. 1995).

Sporulated oocysts were morphometrically most similar to immature oocysts of *H. canis* in *R. sanguineus* (Giannelli et al. 2013b), and smaller in size than that of *H. canis* in *A. ovale* (Forlano et al. 2005, Rubini et al. 2009), *R. microplus* (de Miranda et al. 2011), *R. sanguineus* (Vincent-Johnson et al. 1997, Baneth et al. 2007, Giannelli et al. 2013b), *R. turanicus* (Giannelli et al. 2017), *Hs. longicornis* (Murata et al. 1995), *H. americanum* in *A. maculatum* (Vincent-Johnson et al. 1997, Mathew et al. 1998, 1999, Ewing et al. 2002), and *H. ursi* in *Hs. flava* (Kubo et al. 2008) (Table 5.1). Oocysts tend to enlarge as they mature (Bashtar et al. 1991, Mathew et al. 1999, Baneth et al. 2007), which is evident in the relatively large variation in the sporulated oocyst size in the present study. Thin, faint sporulated oocyst membranes observed in this study were comparable with observations by Baneth et al. (2007).

Maturing sporocysts and sporozoites at various stages of development (Figure 5.1 b to g and Figure 5.2 g and h): Multiple sporocysts were seen, suspended free in the haemocoel, round in shape, at various stages of development (Fig. 5.1 h). Sporocysts measured 30.34 ± 2.00 (26.48–34.19) x 29.8 ± 2.31 (24.03–32.76) μm, area 714.30 ± 73.00 (532.95–825.976) μm^2 (n=20) and contained 30 ± 6 (21–41) (n=14) sporozoites. Distinct white membranes encapsulated immature sporocysts (Fig. 5.1 b). Intrasporocystic sporozoites seen at various stages of development (Fig. 5.1 b–f). Immature sporocysts contained separating, immature sporozoites in the process of differentiating (Fig. 5.1 b). Maturing sporocysts containing developing sporozoites undergo membrane lysis (Fig. 5.1 d), membrane turquoise colour and looser around sporocyst than Fig. 5.1 c.

Intrasporocystic sporozoites were elongated and measured 12.86 ± 4.22 (6.56–19.92) x 2.00 ± 0.60 (1.17–2.81) μm (n=9). Nuclei measured 1.72 ± 0.56 (0.93–3.4) x 1.65 ± 0.46 (2.00–2.60) μm (n=23). Immature sporozoites differentiated at apical area of sporocysts, invaginated by thin, dark blue membranes. Immature intrasporocystic sporozoites, with uniformly blue cytoplasm (Fig. 5.1 b), crowned the sporocyst basal mass, light blue and foamy (Fig. 5.1 b yellow arrow).

Apical aggregation of dense chromatin observed within immature sporozoites, dark blue and spherical (Fig. 5.1 b black arrow). Immature intrasporocystic sporozoites develop to have

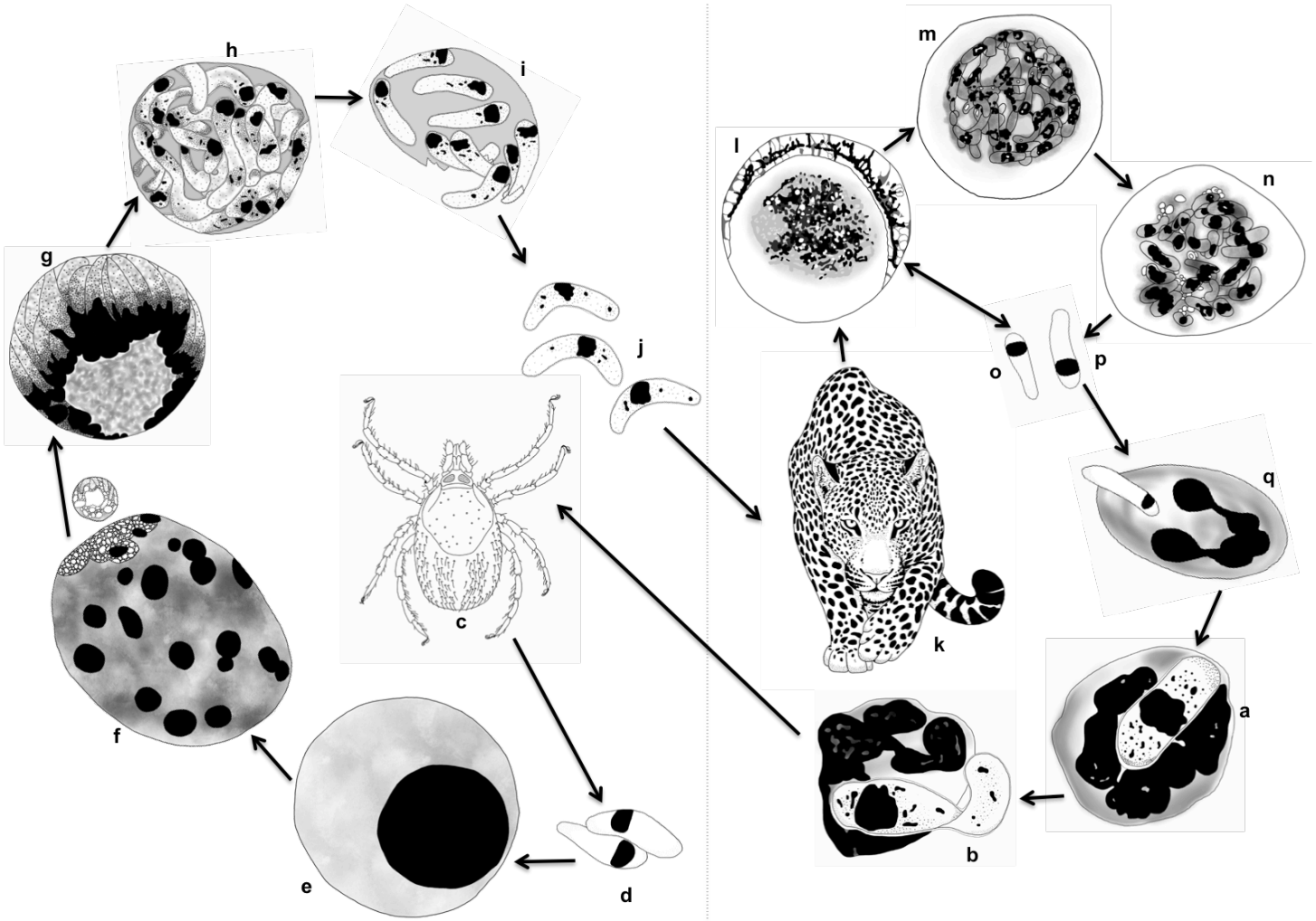

Figure 5.2 Diagram of the possible life cycle of *Hepatozoon luiperdjie* in its intermediate and definitive hosts. **a–b** Mature and extracellular gamonts in peripheral blood of the African leopard are ingested with a blood meal by **c** an *Ixodes* species tick. **d** Male microgamete and female macrogametes fuse to form a zygote (redrawn from Baneth et al. 2007). **e** Zygote or early oocyst (redrawn from Baneth et al. 2007 and Giannelli et al. 2017). **f** Multi-nucleate sporulating oocyst with immature sporocysts exiting from apexal area. **g** Young, free-lying sporocyst at the onset of sporozoite formation. **h** Mature, free-lying sporocyst containing fully developed sporozoites. **i** Mature sporozoites break out of sporocyst. **j** Free-lying extracellular sporozoites. **k** African leopard ingests *Ixodes* sp. tick containing infective stages in its haemocoel. **l** Young meront (Baneth et al. 2013). **m** Maturing meront (Baneth et al. 2013). **n** Mature meront (Baneth et al. 2013). **o** Macromerozoite undergoes secondary morogeny (O'Dwyer 2011). **p** Micromerozoite (O'Dwyer 2011). **q** Micromerozoite invades neutrophil and grows into immature gamont.

turquoise, grainy cytoplasm (Fig. 5.1 c) and dense, dark blue chromatin, sometimes with purple and magenta granules (Fig. 5.1 c red arrow). Immature intrasporocystic sporozoite membranes in Fig. 5.1 c were white and more distinguishable than Fig. 5.1 b. Small area of light blue, foamy, basal mass was still visible in maturing sporocyst (Fig. 5.1 c yellow arrow). Maturing intrasporocystic sporozoites were more evenly distributed throughout sporocyst (Fig. 5.1 d) than Fig. 5.1 c, the cytoplasm of some stained grainy turquoise (Fig. 5.1 d). Immature intrasporocystic sporozoites were more distributed throughout the maturing sporocyst (Fig. 5.1 c), relaxing their previous crown formation around sporocyst basal mass (Fig. 5.1 b). Spongy basal mass of mature sporocyst completely absorbed, leaving only a white central area (Fig. 5.1 d black arrow), subsequently completely absent and replaced by mature sporozoites (Fig. 5.1 e).

Mature sporocyst with a lysed membrane was seen (Fig. 5.1 e), containing mature intrasporocystic sporozoites with distinct, white membranes and grainy light blue cytoplasm (sometimes foamy light blue (Fig. 5.1 e). Mature intrasporocystic sporozoite nuclei were dense, dark blue chromatin with purple granules, rounded, and more centralized than Fig. 5.1 c and d. Ruptured mature sporocyst with distinct, white membrane (Fig. 5.1 f) and exuding mature sporozoites (Fig. 5.1 f black arrow) observed. Mature, exuding sporozoite cytoplasms were grainy light pink and light purple, no visible membrane, and tapered towards both extremities (Fig. 5.1 f black arrow). Chromatin was less densely packed (Fig. 5.1 f) than Figure 5.1 b to e, purple with magenta and dark purple granules, and close to middle of mature sporozoite. Mature intrasporocystic sporozoites, still visible through sporocyst membranes (Fig. 5.1 f red arrow), had overall bluer hue than exuding mature sporozoites from the same sporocyst (Fig. 5.1 f black arrow), similar to staining properties in Figure 5.1 c and e.

Free-swimming mature sporozoites were suspended in tick's haemocoel (Fig. 5.1 f and g; Figure 5.2 i and j), with staining properties similar to exuding mature sporozoites (Fig. 5.1 f black arrow) (see description above). Mature sporozoites tapered towards both extremities (Fig. 5.1 f), with no visible membranes or capsules. Measured 17.27 ± 0.66 (16.22–18.30) x 3.29 ± 0.53 (2.71–4.24) μm, area 46.51 ± 4.36 (39.88–51.65) μm^2 (n=7). Chromatin was loosely concentrated in rectangular shapes, in middle of mature sporozoites (Fig. 5.1 g red arrow), with more magenta granules than exuding mature sporozoites (Fig. 5.1 f black arrow).

Remarks (sporocysts and sporozoites): Intraoocystic sporocysts were rarely encountered during this study (Fig. 5.1 a), thus measurements were not taken. Vincent-Johnson et al. (1997) also reported this for *H. americanum* sporogonic stages in *A. maculatum*. Intraoocystic sporocysts of *Hepatozoon* species associated with carnivores seem to range in size from 18 – 34 μm long by 13 – 39 μm wide (Table 5.1). We did observe, however, that these intraoocystic sporocysts had a lightly blue-stained, foamy appearance and were rounded (Fig. 5.1 a). This shape was similar to that of *H. americanum* in *A. maculatum* (Vincent-Johnson et al. 1997, Mathew et al. 1999) and *H. canis* in *Hs. longicornis* (Murata et al. 1995), but different from the oval shaped intraoocystic sporocysts of *H. canis* in *R. sanguineus* ticks (Mathew et al. 1999,

Baneth et al. 2007) and *H. ursi* in *Hs. flava* ticks (Kubo et al. 2008). Some reports on intraoocystic sporocysts from other *Hepatozoon* species indicate that these sporocysts could also be ellipsoidal (Furman 1966, Redington & Jachowski 1971), subspherical (Kubo et al. 2008) or round (Murata et al. 1995, Vincent-Johnson et al. 1997). Wenyon (1911) specifically reported *H. canis* intraoocystic sporocysts to also be ellipsoidal. Unlike the thick membranous walls of intraoocystic sporocysts reported for *H. americanum* in *A. maculatum* (Mathew et al. 1999), but similar to Murata et al. (1995)'s description of *H. canis* in *Hs. longicornis,* intraoocystic sporocysts from the present study had thin membranes. Morphometrically, the free sporocysts from this study were larger than that of *H. americanum* in *A. maculatum* (Vincent-Johnson et al. 1997, Mathew et al. 1998, Ewing et al. 2002), but smaller than that of *H. canis* in *R. sanguineus* (Vincent-Johnson et al. 1997, Giannelli et al. 2013b). The number of intrasporocystic sporozoites in free-lying sporocysts from the current study exceeded that of *H. americanum* in *A. maculatum* (Mathew et al. 1998, Ewing et al. 2002) and *H. canis* in *Hs. longicornis* (Murata et al. 1995) (Table 5.1).

Sporocyst formation appeared similar to that of other hepatozoans (Wenyon 1911, Allison & Desser 1981, Van As et al. 2015). Intraoocystic sporocysts in the *Ixodes* sp. tick from the current study seemed to aggregate at the apical area of the encompassing oocyst and subsequently break out of the oocyst from there (Fig. 5.1 a (white arrow) and Fig. 5.2 f). From a mammalian carnivore hepatozoonosis point of view, reports of mature sporocysts lying free in the haemocoel of a tick vector are relatively scarce in comparison with reports on mature oocysts. Existing records include that of *H. canis* and *H. americanum* in an assortment of tick species (Murata et al. 1995, Vincent-Johnson et al. 1997, Mathew et al. 1998, Ewing et al. 2002, Giannelli et al. 2013a) (Table 5.1). McCully et al. (1975) found free lying sporocysts of an unknown *Hepatozoon* species in haemolymph smears of a *R. simus* tick collected from a Spotted hyaena in the same geographical area where this study's leopard was sampled.

Free-lying sporocysts sometimes stained very dark indigo and tended to occur in thicker smear areas (Fig. 5.1 h), making them difficult to see on the microscope slide (Fig. 5.1 h white arrows). In several sporocysts, minimal intrasporocystic detail could be seen (Fig. 5.1 h), impeding collection of intrasporocystic, and subsequently, immature sporozoite data. Younger, immature sporocysts had more basal mass, giving it a foamy appearance (Fig. 5.1 a white arrow; 5.1 b yellow arrow). Sporocystic basal mass (seen as degree of foaminess) seemingly decrease as intrasporocystic sporozoite counts increase (compare Fig. 5.1 b (yellow arrow), c (yellow arrow) and 5.1 e). Lysis of sporocyst membranes was seen as the dense, distinctly white membranes (Fig. 5.1 b and c) seemingly loosens into faintly turquoise (Fig. 5.1 d) and light blue (Fig. 5.1 e) membranes as sporocyst matures.

Visible intrasporocystic sporozoites in free-lying sporocysts from the present study was longer, but thinner than that reported by Murata et al. (1995) for *H. canis* in *Hs. longicornis* (Table 5.1). Intrasporocystic sporozoites from the current study varied in size and were curved and elongated, similar to that of *H. canis* in *R. sanguineus* (Baneth et al. 2007) (Table 5.1). The

author observed staining properties of intrasporocystic sporozoites to change during maturation. The staining of intrasporocystic sporozoites was very light at early development, with barely visible membranes and a smooth, uniform cytoplasm with no staining granules (Fig. 5.1 b). Intrasporocystic sporozoites seemingly become more granulated as they mature, with more pronounced membranes (compare Fig. 5.1 b and e). Sporozoite formation seemed to take place through endopolygeny at the apex of each sporozoite (Fig. 5.1 b), with the nucleus migrating towards the middle of the intrasporocystic sporozoite as it matures (Fig. 5.1 c to e). Immature intrasporocystic sporozoite nuclei were rounded and similar to that described by Baneth et al. (2007) and Van As et al. (2015), but changed in shape as sporozoites matured to become more rectangular (compare Fig. 5.1 b black arrow and 5.1 f) (Table 5.1). Similar to these findings, concerning decreasing basal mass as intrasporocystic sporozoites mature, Mathew et al. (1999) reported decreased foaminess for *H. americanum* in *A. maculatum*, while the number of definably shaped sporozoites increase.

The free-lying, exuded sporozoites from the current study (Fig. 5.1 f and g) had similar staining properties to that of *H. canis* in *R. turanicus* (Giannelli et al. 2017), with the nuclei positioned in the middle of the sporozoite body, similar to those documented for *H. americanum* by Mathew et al. (1998). However, the sporozoites from the present study were less rounded and tapered more towards its extremities, than that reported by Mathew et al. (1998) (*H. americanum*) and Giannelli et al. (2017) (*H. canis*). Free-lying sporozoites from the current study were elongated and fell morphometrically within the range of *H. americanum* in *A. maculatum* (Mathew et al. 1998), but were shorter than that of *H. canis* (Giannelli et al. 2017) (Table 5.1).

5.4. Discussion

Transmission of species of *Hepatozoon* in leopards may occur due to ingestion of infected vectors, as is the case for *H. americanum* (Vincent-Johnson et al. 1997), *H. canis* (Baneth et al. 2007, Giannelli et al. 2013a, 2013b), *H. ursi* (Kubo et al. 2008) and possibly *H. felis* (Baneth et al. 2013). This may happen during feeding on infected prey or grooming, with infection between mother and cubs highly probable. Most ticks of southern African wildlife are multi-host species, found on both predator and potential prey species alike (Walker et al. 2003), and may act as vectors between these two parties. Leopards are known to pluck feathers and fur with their incisors from their prey before feeding (Bothma 1998, Carnaby 2006); also evident in the large quantity of hair usually present in leopard scat (Walker 2007). This plucking behaviour may easily dislodge ticks from prey hosts, to be swallowed by the leopard. Due to the multiple-host species nature of wildlife associated ticks in southern Africa, another potential path of transmission may be when a leopard feeds on another infected vertebrate, ingesting tissue-associated merogonic cyst stages (Smith 1996, Baneth et al. 2007, Baneth 2011). Reports on *Hepatozoon* species from leopards are relatively scarce and usually in the form of molecular or microscopic detection in peripheral blood smears (Brocklesby & Vidler 1963, 1965, Keymer

1971, Khoshnegah et al. 2012, Pawar et al. 2012, Van As et al. 2020). McCully et al. (1975) reported on merogonic stages of an unspecified *Hepatozoon* species in an African leopard, from the same geographical region where the current study's leopard was sampled, but did not mention associated stages in ticks from that leopard. It should be noted that engorged ticks from several genera were collected from an infected leopard and were given the opportunity to digest blood meals before dissection. The current study, read together with Van As et al. (2020), links both the development in the definitive and intermediate hosts of this hepatozoan, assumably *H. luiperdjie*, since this engorged tick was collected off an infected leopard.

By allowing engorged ticks to be in a fasting state for seven days after collection from an infected leopard, the author aimed to ensure that the haemogregarine already present in the potential vector's gut may undergo all possible developmental stages, if any. By doing so, the present study's method differed from that of Kubo et al. (2008) and Bhusri et al. (2017), who preserved engorged ticks in 70% ethanol immediately after collection. As Bhusri et al. (2017) did not screen their intermediate carnivore hosts (captive lions) for haemogregarine infections, their conclusion that *R. sanguineus* is a potential vector for *H. felis,* is in the author's mind, not conclusive as that tick may have fed on another host before collection.

The hepatozoan from this study had the typical phases of sporogonic development reported for vectors that needs to be ingested by an intermediate host for the life cycle to be completed (Smith 1996). As only adult ticks were found during this study, there would be value in future examinations of development in nymphs. A high variation with regards to the site of early sporogonic development of mammalian *Hepatozoon* species in their invertebrate hosts has been reported (Furman 1966, Redington & Jachowski 1971, Krampitz 1981, Mathew et al. 1999, Baneth et al. 2007), this potentially indicating that there may be no definite migration path that is followed by all species. The author acknowledges that tissue samples from the intermediate feline carnivore host are necessary to enable the description of a complete life cycle. Nevertheless, it is accepted that a species of tick can be regarded as a successful vector for a *Hepatozoon* species if developing and sporulated oocysts are present in its haemocoel (Ewing et al. 2002), which is what the author found in this study.

It has been suggested that mammalian carnivore *Hepatozoon* species may have a much narrower variety of definitive hosts than intermediate hosts (Ewing et al. 2002). However, some species, such as *H. americanum* and *H. canis,* are successfully transmitted by more than one tick species (Murata et al. 1995, O'Dwyer et al. 2001, Forlano et al. 2005, Rubini et al. 2009, de Miranda et al. 2011, Demoner et al. 2013). Firstly, only two studies report the presence of a *Hepatozoon* species, in the form of molecular detection, in engorged ticks collected from a feline carnivore. Firstly, *Hepatozoon felis* was reported from *Ixodes tanuki*, collected from a Tsushima leopard cat *Prionailurus bengalensis* in Japan (Tateno et al. 2015). Secondly, Bhusri et al. (2017) reported the presence of *H. felis* in engorged *R. sanguineus* ticks infesting captive lions in Thailand. A study by McCully et al. (1975) on lions, spotted hyaenas, black-backed jackals, cheetahs and a leopard from the same geographical region as this study's sampled

leopard, reported hepatozoonosis in all of these carnivores. Furthermore, McCully et al. (1975) observed sporogonic development of an unspecified *Hepatozoon* species in engorged adult *R. simus* ticks from an infected Spotted hyaena, simultaneous with no development in *R. sanguineus* or *Hs. leachi* collected from the same hyaena. Similarly, the present study also found no developmental stages in *R. sanguineus* and *H. leachi* ticks. Moreover, McCully et al. (1975) did find that *R. sanguineus* are successful vectors for a *Hepatozoon* sp. infecting Black-backed jackals. The above reports may suggest that arthropod vectors may be more species-specific to transmit hepatozoans than previously assumed, especially in the wild. Since sporogony is evident from the current study's results, it can be assumed that oogenesis occurred in this specific *Ixodes* tick (Siddall 1995, Smith 1996), but not in the other screened ticks. The absence of gametogenesis, syzygy and subsequent fertilization may indicate these stages to have been completed by the time the tick was dissected, as these stages may be observed as early as 24 hours after a blood meal (Baneth et al. 2007). This, together with reports of other leopards infected with *H. luiperdjie* (Van As et al. 2020), may indicate that, at least for this specific hepatozoan, naturally occurring *Ixodes* ticks may be successful vectors between different leopards in the same areas.

Oocyst and sporocyst shape seems to show intraspecific variation, dependent on the tick vector species. This can be seen in *H. canis*, which has round oocysts in *A. ovale* (Forlano et al. 2005, Rubini et al. 2009) and oval oocysts in *R. microplus* (de Miranda et al. 2011), *R. sanguineus* (Giannelli et al. 2013b) and *R. turanicus* (Giannelli et al. 2017). *Hepatozoon canis* sporocysts could be elongated, oval or ellipsoidal in different tick vectors (Forlano et al. 2005, Baneth et al. 2007, Giannelli et al. 2013a, 2013b). Round sporocysts, similar to that from the present study, were seen in *R. simus* (unknown *Hepatozoon* species) (McCully et al. 1975).

Most mammalian carnivore life cycle studies suggest that oocysts in the haematophagous vector are the infective stage to the vertebrate host that ingests that vector (Baneth et al. 2001, 2007, Baneth 2011), and rarely go beyond morphological description of oocysts. The current study adds value by morphologically describing sporocysts and sporozoites and various stages of development. It was shown that contact with bile induces oocysts to rupture, which led several authors to conclude that mature sporulated oocysts are the infective stage of carnivore hepatozoans to their intermediate hosts (Redington & Jachowski 1971; Mathew et al. 1999). Thin, faint sporulated oocyst membranes observed in the current study may indicate a readiness to tear easily so that sporocysts may be released (Baneth et al. 2007). However, the presence of free mature sporocysts and free sporozoites found in the present study may indicate that more immediate infective stages may also be ingested with the haematophagous vector. The author proposes that this may lead to faster, more successful infection in the intermediate carnivore host.

No intrasporocystic sporozoites were seen in intraoocystic sporocysts during the present study, but details from other carnivore-associated hepatozoans can be seen in Table 5.1. Other studies did report on their morphological characteristics and it seems that this developmental stage in

carnivore-associated *Hepatozoon* species measures 10 – 15 μm long by 3 – 5 μm wide (Mathew et al. 1999, Kubo et al. 2008). According to available reports, intraoocystic sporocysts of carnivore-associated hepatozoans may contain 8 – 26 sporozoites each (Table 5.1).

Sporocysts from the present study seemed to follow a path of maturation in line with descriptions by Mathew et al. (1999) and Baneth et al. (2007), and the author proposes that degree of maturation can be classified from immature to mature according to the following characteristics: As immature sporocysts develop further, the amount of sporocystic basal material (seen as foaminess) decrease while the number of immature, differentiating intrasporocystic sporozoites increase. As this process continues, lysis of the sporocyst membrane increases, seen in changing staining properties and membranes seemingly loosening and becoming fainter. Thus, immature sporocysts will have an overall foamy appearance with a thick membrane, while mature sporocysts will have sporozoites that are distributed throughout the whole cytoplasm, with no basal material and a lysed, faint membrane. The author proposes that these characteristics could dependably be used to distinguish between different sporocysts at different stages of maturation, within one vector.

The current study is the first to report intrasporocystic sporozoite nuclei morphometrics of a carnivore-associated hepatozoan, as well as free-lying sporozoites in the haemocoel of a naturally infected tick collected from a naturally infected carnivore host. Sporozoite development followed the same path/trend/pattern as that of *H. americanum* (Mathew et al. 1999), with nuclei changing from rounded, densely chromatinized areas in immature sporozoites to rectangular, loosely chromatinized areas with purple and magenta granules in mature, exuded sporozoites. The author proposes this to be a good diagnostic means of determining the degree of development of sporozoites within a tick vector.

5.5 Conclusions

In the case of this study, and due to the current conservation status of the African leopard in South Africa, the author was not permitted to kill and dissect a specimen and during the study, no specimens became available that either died from natural causes or as a result of human-wildlife conflict. Subsequently the author cannot definitively conclude that the *Hepatozoon* species from this study only exists between a tick and leopard host. Reports on life cycle stages from wild ticks collected off wild animals are quite rare (Ewing et al. 2002, de Miranda et al. 2011), which is where the present study adds value to existing knowledge.

The definitive answer as to which ticks may act as vectors for feline hepatozoonosis remains relatively unanswered for big cats in southern Africa, and this report provides some valuable morphological data in this regard. The current study further sheds some light on a possible tick vector in which almost all developmental and infective stages of a *Hepatozoon* species was

observed, but further in-depth studies are necessary to determine the complete life cycle of this haemogregarine. Understanding the transmission process of hepatozoonosis in large carnivores provides a good basis for further research into the ecological and health impacts of this infection, ultimately aiding in the conservation management of large carnivores.

5.6 References

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CHAPTER 6
Organochlorine pesticides in African leopard blood serum

Sections of this chapter has been published in the following peer-reviewed publication:

VAN AS M., SMIT N. J., WOLMARANS N. J. & WEPENER V. 2022. First record of organochlorine pesticides in blood of wild and captive African leopards, *Panthera pardus pardus* (Linnaeus, 1758). *Frontiers in Environmental Science* 10:938453.

6.1 Introduction

Persistent Organic Pollutants (POPs), including organochlorine pesticides (OCPs), are resistant to biological, physical and chemical breakdown and as a result they have been banned world-wide through the Stockholm Convention in 2004 (Bouwman 2004). Despite their banning, OCPs are still widely distributed in African ecosystems (Bouwman et al. 2014; Volschenk et al. 2019, Gerber et al. 2021). South Africa, a known user of OCPs in the agricultural sector (Wepener and Chapman 2012), is the largest pesticide user south of the Sahara (Dabrowski et al. 2014). According to Dabrowski et al. (2014) pesticides are widely used for veterinary and crop protection purposes in South Africa, which helps to promote food security. Coupled with the increasing development in rural malaria-endemic areas, increased reliance on insecticides has become the trend in South Africa (Wepener and Chapman 2012). South Africa has used Dichlorodiphenyltrichloroethane (DDT) since the late 1940s as a means to control malaria (Bouwman 2004) and residues have been measured in human breast milk (Bouwman et al. 1990) and blood (Gaspar et al. 2015) of residents of malaria endemic areas. The semi-volatile nature of OCPs results in their long-distance distribution to regions where there has been no direct use of these chemicals, e.g. ending up in coastal regions and remote river headwaters (Viljoen et al. 2016, Verhaert et al. 2017, Erasmus et al. 2020, Rose et al. 2020). This has led to a recent increase in studies addressing levels of OCPs in terrestrial and aquatic environments in South Africa (Thompson et al. 2017a, Volschenck et al. 2019, Gerber et al. 2021, Wolmarans et al. 2021). Due to trophic migration of compounds along the food web, the highest concentrations are usually reached at the top trophic level of a chain (Skaare et al. 2000), as seen in the study on trophic biomagnification of perfluorinated compounds in lichen, Barren-

ground caribou *Rangifer tarandus groenlandicus* (Borowski, 1780) and wolves *Canis lupus* Linnaeus, 1758 (Müller et al. 2011). This results in the highest OCP concentrations usually ending up in apex predators and therefore the study of biomagnification is particularly relevant in species that are at the top of the food web.

In contrast to aquatic apex predators, reports on the incidence of POPs in terrestrial apex predators are surprisingly limited and Rodríguez-Jorquera et al. (2017) found that there is biased coverage of this subject, with most literature originating from Europe and North America (see Table 6.1). Most work done to date has been on marine mammals, concentrated in Asia, western Europe and North America. The majority of studies on mammalian carnivores focused on Polar bears *Ursus maritimus* Phipps, 1774 (e.g. Bentzen et al. 2008a, 2008b), North American river otter *Lontra canadensis* (Schreber, 1777) (e.g. Carpenter et al. 2014) and mink (genus *Neogale* Gray 1865) (e.g. Romanić et al. 2015), which are all apex predators in aquatic systems. Very few reports on mammalian apex predators from Africa exist. Cockroft et al. (1989) determined the concentrations of Polychlorinated biphenyls (PCBs), DDTs and dieldrin in blubber samples of Bottlenose dolphins *Tursiops aduncus* Montagu, 1821 along the east coast of South Africa and recently Malarvannan et al. (2020) published the first record of POPs in serum of lion *Panthera leo*, spotted hyaena *Crocuta crocuta* and cheetah *Acinonyx jubatus* from conservation areas in South Africa and the Antwerp Zoological Gardens in Belgium, while Leighton et al. (2022) recorded organochlorines in plasma and adipose tissue of caracal *Caracal caracal* from peri-urban areas in the Western Cape peninsula.

Notwithstanding their important ecological role, the distribution of some of the most wide-ranging African predators has diminished by more than 76% (Ray et al. 2005). The African leopard *Panthera pardus pardus* is the least studied of all big cats (Bailey 1993, Maheshwari 2006). They are highly adapted to live in diverse habitats, can persist regardless of various pressures and utilize food resources not favoured by other large carnivores (Norton et al. 1986, Bailey 1993, Spong et al. 2000). These cats are generalists and will eat whatever food is easiest to obtain, ranging from beetles to large ungulate species (Bailey 1993, Hayward et al. 2006). Leopards have the ability to live in human-modified habitats whereas other large felids such as lions and tigers cannot (Ray et al. 2005, Athreya and Belsare 2007), with the largest part of their population occurring outside formally protected areas (Daly et al. 2005). This puts them more frequently in contact with anthropogenic factors than any other big cats in Africa. Nationally protected areas where leopards are still known to exist in viable populations include the Kgalagadi Transfrontier Park, Kruger National Park and Addo Elephant National Park (Daly et al. 2005). The constraints in capturing and working with large, dangerous apex predators make it problematic to investigate the occurrence and effects of toxic chemicals in these animals. As Rodríguez-Jorquera et al. (2017) pointed out, the most convenient way of obtaining data in this subject area is to process carcasses and rely on post-mortem results, which does not necessarily provide information on the effects of sub-lethal concentrations in live animals.

Table 6.1 Summary of global studies conducted on terrestrial mammalian carnivores. All values are presented as Mean, or Mean±Standard Deviation (Range) ng/g ww unless otherwise indicated. -: data not reported. ND: not detected.. Exceptions are noted and explained at the footnote of this table. Multiple records for the same tissue-samples in the same species indicate sampling at different localities in the same country. reported in the same paper. -: data not reported. ND: not detected. LOD: limit of detection.

Species	Common Name	Location	Tissue	*p,p'*-DDE	*o,p'*-DDD	*p,p'*-DDD	*o,p'*-DDT	*p,p'*-DDT	ΣDDTs	ΣPCBs	ΣCHLs	HCB	α-HCH	β-HCH	γ-HCH	ΣHCHs	ΣChlordane	Dieldrin	Endrin	Reference
Canidae																				
Canis familiaris	Domestic dog	Japan	Blood	-	-	-	-	-	-	(<0.1-0.3)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
		Greenland	Fat	-	-	-	-	-	167 (18-358)	2996 (936-6740)	-	67 (38-103)	-	-	-	61 (28-127)	1164 (809-1658)	531 (13-948)	-	(Sonne 2010)[b]
			Liver	-	-	-	-	-	666 (284-1018)	43111 (20188-62655)	-	ND	-	-	-	ND	72798 (42550-86725)	5111 (4000-8030)	-	
			Blood	-	-	-	-	-	29 (3-74)	1854 (943-3801)	-	61 (38-90)	-	-	-	ND	770 (474-1414)	415 (149-654)	-	
Canis lupus	Grey wolf	Croatia	Fat	-	-	-	-	-	(0.2-2.9)	-	-	(0.2-2.2)	(<LOD-0.3)	(0.2-2.5)	-	(0.7-3.2)	-	-	-	(Romanić et al. 2015)[d,f]
		Spain	Spleen	539000	-	-	218000	1028000	-	-	-	-	-	-	-	-	-	15000	3000	(Carril González-Barros et al. 2000)[b,e]
			Liver	350000	-	-	89000	47000	-	-	-	-	-	-	-	-	-	6000	8000	
			Muscle	16000	-	-	ND	4000	-	-	-	-	-	-	-	-	-	3000	ND	
			Kidney	1325000	-	-	292000	2278000	-	-	-	-	-	-	-	-	-	2067000	26000	
			Suprarenal	48000	-	-	29000	46000	-	-	-	-	-	-	-	-	-	107000	11000	
Nyctereutes procyonoides	Raccoon Dogs	Japan	Liver	28±58 (0.5-270)	-	17±29 (<0.2-120)	-	1.6±6.8 (<0.2-31)	46±73 (0.5-290)	200±370 (15-1700)	4400±9100 (180-43000)	4.5±13 (0.8-61)	0.5±0.7 (<0.2-2.5)	41±59 (5.4-250)	-	41±59 (6.5-250)	-	-	-	(Kunisue et al. 2008)
			Liver	58±42 (5.3-150)	-	150±240 (0.8-650)	-	0.6±0.9 (<0.2-2.3)	210±270 (6.4-800)	390±410 (24-1200)	8200±7600 (170-22000)	2.7±2 (0.5-6.2)	0.2±0.7 (<0.2-2.3)	67±74 (6.3-230)	-	67±74 (6.3-230)	-	-	-	
			Liver	18±18 (3.7-60)	-	8.8±8.5 (1.2-27)	-	2.1±4.1 (<0.2-12)	29±25 (5.1-84)	130±100 (38-350)	8700±6300 (2000-20000)	1.4±0.8 (0.6-2.5)	0.3±0.2 (<0.2-0.6)	94±68 (24-220)	-	94±68 (25-220)	-	-	-	
			Fat	16±27 (1.0-82)	-	<0.2 (<0.2-0.62)	-	3±3.7 (0.1-9.2)	19±30 (1.2-92)	240±170 (59-600)	590±330 (210-1000)	1.1±0.5 (0.7-2.0)	0.3±0.2 (<0.2-0.7)	62±51 (6.4-150)	-	62±51 (6.5-150)	-	-	-	
			Blood	-	-	-	-	-	-	(0.02-1.1)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
			Liver	-	-	-	-	-	(0.8–33)	(26–330)	(33–1200)	(0.2–1.0)	-	-	-	(1.2–45)	-	-	-	(Yamamoto et al. 2012)[d]
Vulpes lagopus	Arctic Foxes	Norway	Liver	-	-	-	-	-	98 (35-203)	5486 (1535-13603)	-	-	-	-	-	-	8656 (1406-30850)	-	-	(Sonne 2010)[b]
			Liver	(1-19633)	-	-	-	-	-	(76-53129)	(121-48722)	(9-1082)	-	(3.1-527)	-	-	-	-	-	(Andersen et al. 2015)[c]
	Females	Alaska	Blood	-	-	-	-	-	-	1.9±1.9 (0.0-4.7)	-	-	-	-	-	-	-	-	-	(Harley et al. 2016)
	Males		Blood	-	-	-	-	-	-	0.7±1 (0-2.5)	-	-	-	-	-	-	-	-	-	
Vulpes vulpes	Red fox	Spain	Plasma	<0.01	-	-	-	-	-	7.7±4.4	-	-	-	-	-	-	-	-	-	(Mateo et al. 2012)[g]
			Liver	4.1±1.3	-	1.4±0.50	-	-	-	1321±402	-	0.8±0.4	-	0.4±0.7	-	-	-	-	-	
			Fat	100±31	-	0.1±0.1	-	0.5±0.3	-	4579±1318	-	5.5±1.3	0.3±0.2	27.4±11.4	0.6±0.2	-	-	-	-	
		Japan	Blood	-	-	-	-	-	-	(100-680)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
		Norway	Plasma	(0.0-0.1)	-	-	-	-	-	(6.8-8.8)	(3.4-5.6)	(0.3-0.8)	-	(0.1-0.2)	-	-	-	-	-	(Polder et al. 2009)[c]
Felidae																				
Acinonyx jubatus	Cheetah	Belgium	Plasma	-	-	-	-	-	7.3±2.11 (3.75-7.51)	16.17±4.77 (14.91-23.73)	0.43±0.16 (0.29-0.61)	0.13±0.02 (0.10-0.14)	-	-	-	0.5±0.1 (0.4-0.7)	-	-	-	(Malarvannan et al. 2020)
Felis catus	Domestic cat	Spain	Liver	37.3±37.3	-	0.9±0.9	-	-	-	221±173	-	0.43±0.31	-	0.2±0.2	-	-	-	-	-	(Mateo et al. 2012)[g]
			Fat	1079±922	-	3.4±3.4	-	16.3±13.8	-	1055±917	-	5.1±3.0	<0.01	3.1±0.7	0.6±0.6	-	-	-	-	
		Japan	Blood	-	-	-	-	-	-	(0.0-16)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
Lynx lynx	Eurasian lynx	Sweden	Plasma	(0.1-0.1)	-	-	-	-	-	(1.2-3.1)	<LOD	(0.1-0.2)	-	(0.02-0.03)	-	-	-	-	-	(Polder et al. 2009)[c]

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Felidae																				
Lynx pardinus	Iberian Lynx	Spain	Plasma	1.3±0.5	-	-	-	-	-	0.4±0.3	-	-	-	-	-	-	-	-	-	(Mateo et al. 2012)[g]
			Plasma	0.3±0.3	-	-	-	-	-	0.8±0.8	-	-	-	-	-	-	-	-	-	
			Liver	55.1±29.6	-	<0.01	-	-	-	101±44	-	0.4±0.4	-	0.3±0.2	-	-	-	-	-	
			Liver	25.9±18.4	-	<0.01	-	-	-	37±28	-	<0.01	-	1.1±0.5	-	-	-	-	-	
			Fat	455±229	-	<0.01	-	<0.01	-	510±398	-	1.6±0.4	<0.01	4.3±1.2	<0.01	-	-	-	-	
			Fat	740±234	-	<0.01	-	<0.01	-	259±29	-	2.3±0.9	<0.01	6.3±4.7	<0.01	-	-	-	-	
Panthera leo	African lion	South Africa	Plasma	-	-	-	-	-	0.3±0.3 (0.7-2.0)	0.00±0.00 (0.00-0.03)	0.01±0.01 (0.00-0.07)	0.00±0.00 (0.00-0.03)	-	-	-	0.04±0.09 (0.01-0.42)	-	-	-	(Malarvannan et al. 2020)
		Belgium	Plasma	-	-	-	-	-	0.3±0.6 (0.1-1.7)	0.5±1.1 (0.3-3.2)	0.02±0.04 (0.02-0.11)	0.03±0.02 (0.03-0.09)	-	-	-	0.07±0.04 (0.04-0.15)	-	-	-	
Hyaenidae																				
Crocuta crocuta	Spotted hyaena	South Africa	Plasma	-	-	-	-	-	1.6±5.7 (0.2-16.9)	0.0±0.1 (0.0-0.4)	0.05±0.93 (0.01-2.77)	0.04±0.04 (0.01-0.15)	-	-	-	0.1±0.1 (0.1-0.4)	-	-	-	(Malarvannan et al. 2020)[h]
Herpestidae																				
Herpestes ichneumon	Egyptian mongoose	Spain	Plasma	4.2±3.2	-	-	-	-	-	15.4±6.4	-	-	-	-	-	-	-	-	-	(Mateo et al. 2012)[g]
			Liver	19.3±6.9	-	7.7±5.4	-	-	-	486±256	-	0.08±0.03	-	<0.01	-	-	-	-	-	
			Fat	276±125	-	1.7±1.7	-	3.3±0.6	-	789±292	-	2.9±0.3	<0.01	2.6±0.5	1.1±0.4	-	-	-	-	
Herpestes javanicus	Javan Mongoose	Japan	Blood	-	-	-	-	-	-	(24-29)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
	Females		Liver	880	-	ND	-	ND	880	190	510	2.6	ND	ND	-	<0.60	ND	-	-	
	Males		Liver	1390±1433 (180-3400)	-	ND	-	ND	1390±1433 (180-3400)	2065±2873 (200-6300)	118±145 (9.4-330)	1.1±0.7 (<0.3-1.8)	ND	ND	-	<0.60	ND	-	-	
Mustelidae																				
Gulo gulo	Wolverine	Norway	Plasma	(<LOD-0.02)	-	-	-	-	-	(1.7-3.6)	(0.05-0.06)	(0.33-0.39)	-	(0.04-0.07)	-	-	-	-	-	(Polder et al. 2009)[c]
Lutra lutra	Eurasian otters	Spain	Liver	130±66	-	24.5±11.2	-	-	-	3324±1399	-	28.6±18.1	-	1.1±0.6	-	-	-	-	-	(Mateo et al. 2012)[g]
		England	Liver	23300000	-	-	-	-	-	60510000	-	-	-	-	-	-	-	10400000	-	(Mason & Macdonald 1994)[b,e]
Meles meles	Badger	Spain	Liver	36.3	-	84.2	-	-	-	213	-	<0.01	-	0.5	-	-	-	-	-	(Mateo et al. 2012)[g]
			Fat	738	-	13.2	-	9.9	-	924	-	1.3	<0.01	17.1	<0.01	-	-	-	-	
		Japan	Blood	-	-	-	-	-	-	(<0.1-0.8)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
Procyonidae																				
Procyon lotor	Raccoon	Japan	Blood	-	-	-	-	-	-	(<0.1-2.6)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]

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Ursidae																				
Ursus arctos	Brown bear	Sweden	Plasma	<LOD	-	-	-	-	-	(0.2-0.3)	<LOD	(0.3-0.3)	-	(0.01-0.03)	-	-	-	-	-	(Polder et al. 2009)[c]
		Croatia	Fat						(0.1-1.2)			(0.1-2.3)	(<LOD-0.2)	(<LOD-0.6)		(0.2-1.7)				(Romanić et al. 2015)[d,f]
				-	-	-	-	-		-	-				-		-	-	-	
Viverridae																				
Genetta genetta	Common genet	Spain	Liver	754±661	-	33.9±15.5	-	-	-	2874±2685	-	0.4±0.1	-	1.1±0.5	-	-	-	-	-	(Mateo et al. 2012)[g]
			Fat	17531±15260		3.5±3.5		4.2±4.2		17326±15632		6.8±1.7	0.8±0.8	25.6±15.9	1.3±1.1					
					-		-		-		-					-	-	-	-	
Paguma larvata	Masked palm civet	Japan	Blood	-	-	-	-	-	-	(0.26-95)	-	-	-	-	-	-	-	-	-	(Mizukawa et al. 2013)[b,c]
			Liver						(84–1200)	(29–1700)	(47–590)	(0.3–1.3)				(1.1–44)				(Yamamoto et al. 2012)[d]
				-	-	-	-	-					-	-	-		-	-	-	

[a] Data from one individual
[b] Only mean values reported
[c] Range calculated from two individuals
[d] Only range reported
[e] Concentrations given in dry weight
[f] No mean values reported
[g] No range reported
[h] Corrected species name (personal communication Covaci 2021)

The aim of this section of the study was to see whether African leopards have detectable OCP concentrations in their blood serum and to see if there were differences between captive and wild leopards. This would be achieved by establishing baseline concentrations of OCPs; (ii) comparing differences between captive and wild leopards; and (iii) doing a preliminary assessment of individual and group category differences to account for anticipated life history effects of sex, age class, phenotypes and conservation management approaches.

6.2 Materials and methods

Description of study area, characterisation of wild and captive leopards and blood collection is presented in Chapter 2 of this thesis.

6.2.1 Blood and serum sample preparation

Peripheral blood was usually collected from the jugular or the cephalic vein by venipuncture with the use of a sterile Vacutainer system, in BD Vacutainer® (Franklin Lakes, USA) CAT (Clot Activator Tubes). Samples were left overnight in a cooler box with an ice pack to keep temperatures stable, allowing blood components to separate. Early the following morning the separated serum was aliquoted into smaller Eppendorf vials (approximately 1 ml each), labelled and stored at -20 °C and transported to the analytical laboratories of the North-West University, South Africa for further analyses. Blood samples were obtained from representative leopards of three core wild populations as identified by Daly et al. (2005). Samples from captive leopards represented two colour variations, six had the regular colour variation and three leopards were melanistic. Four captive males and five females were used for this study. Although two age classes, as defined by Fattebert et al. (2015), were noted, the effect of age was not analysed due to the small sample size (n=3) for the subadults. Two captive leopards, a regular female and a melanistic male, from the same facility, were sampled twice throughout the study, with at least one year between the first and second sampling.

6.2.2 Organochlorine analysis

Levels of *α*–Hexachlorocyclohexane (*α*–HCH), *β*–Hexachlorocyclohexane (*β*–HCH), *γ*–Hexachlorocyclohexane (*γ*–HCH), *δ*–Hexachlorocyclohexane (*δ*–HCH), Hexachlorobenzene (HCB), aldrin, dieldrin, endrin, Heptachlor, *oxy*-chlordane, *cis*-heptachlor-epoxide, *trans*-heptachlor-epoxide, *trans*-chlordane, *trans*-nonachlor, *cis*-chlordane, *cis*-nonachlor, *o,p'*-Dichlorodiphenyldichloroethylene (*o,p'*-DDE), *p,p'*-Dichlorodiphenyldichloroethylene (*p,p'*-DDE), *o,p'*-Dichlorodiphenyldichloroethane (*o,p'*-DDD), *o,p'*-DDT, *p,p'*-DDD and *p,p'*-DDT in serum were determined using adaptations of a method by Keller et al. (2004). A known volume of serum (approximately 1 ml) of serum from each leopard was used for extraction. Prior to extraction 100 μl of 100 μg/l chlorinatedbiphenyl (PCB #143) and 100 μl of 100 μg/l epsilon-

hexachlorocyclohexane (*ε*–HCH) was added to each sample as internal standard. PCB #143 was used as an internal standard for the organochlorine pesticides as it has similar functional groups, Polarity, and molecular size to the larger OCPs but is not expected in environmental samples. Unfortunately, no suitable OCP was found with no probability of environmental presence to serve as the perfect Internal standard for larger OCPs in this analysis. The use of certain PCBs as internal standards for OCPs is a long-standing accepted practice for this reason (see Covaci et al. 2001, Covaci 2006, López et al. 2007). Sample extraction consisted of liquid-liquid extraction using 2 ml formic acid (> 88%) and 4 ml of amethyl tert-butyl ether (MTBE) and hexane mix (MTBE:hexane = 1:1 v/v) in 15 ml polypropylene tubes. Samples were briefly vortexed (Vortex-Genie 2; Scientific Industries, Inc.) to ensure solvent interaction, followed by sonication for 20 min in an ultrasonic bath (Scientech) at 25 °C. Following sonication samples were centrifuged for 5 min at 2 000 rpm in a Centrifuge 5430 (Eppendorf), and the organic phase collected. The extraction process was repeated three times in total, with the second and third extraction performed only with 4 ml MTBE:hexane (1:1 v/v). The collective organic extract sample volume was reduced to < 10 ml under gentle N_2 at 36 °C in a Turbo Vap® Classic (Biotage) evaporator. Sample clean-up consisted of elution through a 40 cm glass column tightly packed with 4 g Florisil (5% deactivated), topped with (≈ 1 cm) sodium-sulfate, and prepared with hexane. The sample was eluted with 35 ml of dichloromethane:hexane (3:7 v/v) after which it was evaporated to near dryness (≈ 5 μl) under a gentle N2 stream at 36 °C. The sample was reconstituted in 100 μl n-decane containing tetrachloro-m-xylene (100 μg/l) as surrogate marker and analysed using gas chromatography (Hewlett Packard®6890) coupled with a ^{63}Ni micro-electroncapture detector (GC-μECD).

The Gas Chromatography with an Electron Capture Detector (GC-ECD) analysis was performed using a splitless injection (1 μl) at inlet temperature of 225 °C. This was specifically lowered from initial methods of 260 °C during the method development phase to prevent thermal degradation of DDT during chromatographic analysis, which was confirmed have no significant impact on analysis repeatability at 225 °C through the method validation process. The oven program initiated at 100 °C held for 1 min, followed by a ramp of 20 °C/min to 200 °C, then changing to a ramp of 6 °C/min until 260 °C held for 4 min. Separation of compounds was achieved using a 30 m length x 0.25 mm I.D. x 0.25 μm film HT8-MS (SGE) column and H_2 carrier gas at a constant flow of 1.5 ml/min. The detector temperature was set at 310 °C with N_2 as the make-up gas (60 ml/min). Calibration of the 22 OCP peaks was performed using a 7-point calibration curve of the Dr. Ehrenstorfer® pesticide mix 1023 (LGC Standards) ranging between 5 and 1000 μg/l with a linear regression R^2 > 0.998 for all analytes. The instrumental limit of detection (LOD) and limit of quantification (LOQ) was calculated based on 3 times and 10 times (respectively) the standard deviation of the gradient slope of the calibration curve of each compound. The instrumental LODs were as follows: *α*–HCH = 1 μg/l, HCB = 10 μg/l, *β*–HCH = 5 μg/l, *γ*–HCH = 3 μg/l, *δ*–HCH = 0.8 μg/l, heptachlor = 12 μg/l, aldrin = 9 μg/l, *oxy*-chlordane = 5 μg/l, *cis*-heptachlor-epoxide = 6 μg/l, *trans*-heptachlor-epoxide = 8 μg/l, *o,p'*-DDE = 11 μg/l, *trans*-chlordane = 11 μg/l, *trans*-nonachlor = 11 μg/l, *cis*-chlordane = 14 μg/l, *p,p'*-DDE = 6 μg/l, dieldrin = 14 μg/l, *o,p'*-DDD = 17 μg/l, endrin = 13 μg/l, *o,p'*-DDT = 26 μg/l, *cis*-nonachlor = 5

μg/l, *p,p'*-DDD = 30 μg/l, *p,p'*-DDT = 34 μg/l. The mean internal standard recovery was > 80% for ε–HCH and slightly lower and more variable, 66 ± 20%, for PCB #143.The mean internal standard recovery was 66 ± 20% and all concentrations reported were adjusted according to recovery.

The extraction and GC analysis method was validated using Bovine blood as a matrix substitute spiked with the same pesticide mix used for calibration. Spiked samples were left to equilibrate for 24 h at 4 °C prior to extraction. No matrix interference was observed within the retention time range of interest for any of the pesticides (tested through Blank Bovine blood analysis). The spiked bovine blood method validation samples had an average recovery of 78.8 ± 17% for all pesticides in 15 samples extracted and analysed in three batches over several days.

The analytical batches contained a blank after every two samples to assess the potential for carry-over. A quality control (QC) check for system stability was analysed with every 10th sample. Quality control standards had RSD < 10 % for all pesticides except *p,p*-DDT that showed an RSD of 11.8%.

6.2.3 Statistical analysis

All OCP concentrations are expressed as pg/ml and OCP profiles represent the relative percentage contribution of the detected compounds to the *Σ*OCP concentrations. To allow for statistical analysis, all concentrations that were below the LOQ were transformed to a value by multiplying the detection frequency with the LOQ (Wepener et al. 2012). The respective frequencies of detection for detected compounds were as follows: *α*–HCH 100%, HCB 91.7%, *β*–HCH 8.3%, *γ*–HCH 75%, *δ*–HCH 66.7%, heptachlor 100%, aldrin 58.3%, *cis*-heptachlor-epoxide 16.7%, *trans*-heptachlor-epoxide 25%, *o,p'*-DDE 33.3%, *trans*-chlordane 66.7%, *trans*-nonachlor 8.3%, *cis*-chlordane 33.3%, *p,p'*-DDE 50%, dieldrin 0%, *o,p'*-DDD 83.3%, endrin 100%, *o,p'*-DDT 33.3%, *cis*-nonachlor 58.3%, *p,p'*-DDD 100%, and *p,p'*-DDT 25%.

Data were analyzed with GraphPad Prism version 7 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com). All data were checked for normality and homogeneity of variance prior to analyses using the D'Augustino-Pearson omnibus normality test and test, respectively. If data met the normality and homogeneity of variance assumptions, differences between the OCP levels in the selected cases were determined using the unpaired t test with Welch correction. Means of data that did not meet the normality and homogeneity of variance assumption were compared using a Mann-Whitney U test. Significant differences were regarded as $p < 0.05$.

6.3 Results

Refer to Tables 2.3 and 2.4 to see which samples were obtained from which captive and wild leopards in this study.

6.3.1 Organochlorine pesticide contamination in South African leopards

To the author's knowledge these are the first data on OCPs in wild and captive African leopards. Varying concentrations of OCPs were detected in all leopards from this study. All the OCPs tested for, with the exception of *cis*- and *trans*-heptachlor-epoxide, *o,p'*-DDT, *p,p'*-DDT and *o,p'*-DDE were recorded in leopard serum samples during the present study (Table 6.2). The average total OCP concentrations in the blood serum of leopards measured were 770 ± 230 (range 434–1383) pg/ml. The OCP contamination pattern was in the decreasing order of DDTs (27%) > HCHs (21%) > heptachlors (15%) > CHLs (15%) > drins (14%) > HCB (8%) (Fig. 6.1).

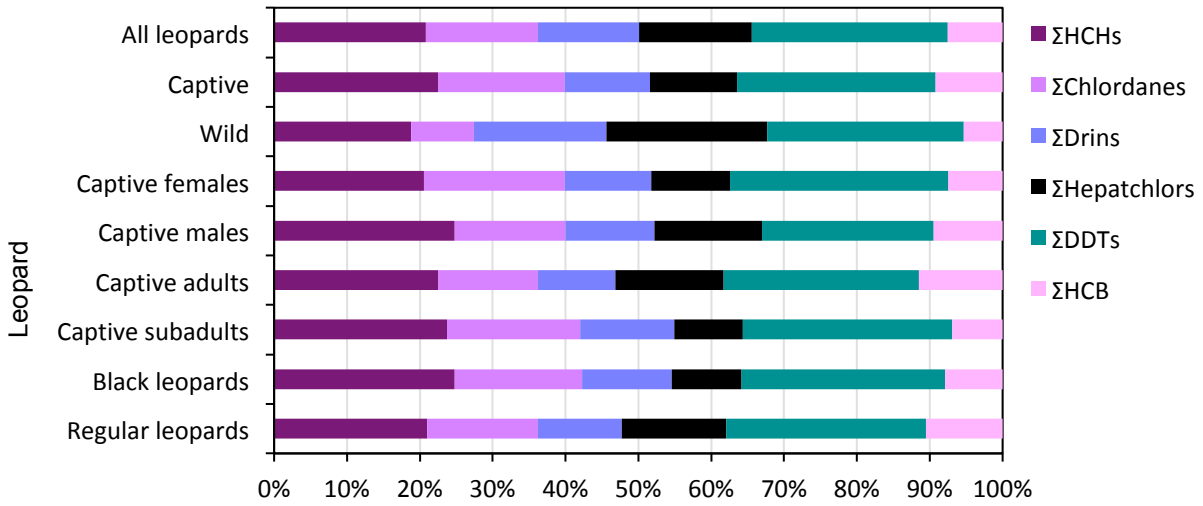

Figure 6.1 The relative contribution of main compound groups (ΣHCHs; ΣChlordanes; ΣDrins; ΣHeptachlors; ΣDDTs; ΣHCB) to ΣOCP concentrations as analyzed in all captive and wild leopards.

Concentrations of *α*–HCH, HCB, endrin, heptachlor and *p,p'*-DDD were present in all fourteen leopards, at varying concentrations.

The OCP profiles of captive and wild leopards differed in that the contribution of heptachlors and drins were greater in wild leopards (Fig. 6.1). Although the total OCP concentrations in wild leopards were lower than captive leopards it was not significantly different (Table 6.2). There were no significant differences in DDTs between wild and captive leopards with *p,p'*-DDD making up the highest concentration of the isomers (Table 6.2). Similarly, the HCHs did not differ significantly between wild and captive leopards with *α*–HCH being the predominant isomer. The heptachlors and chlordanes also did not differ significantly between wild and

captive leopards. Only endrin was significantly higher in wild leopards ($t = 2.3$, $df = 9$, $p = 0.042$), while HCB were significantly higher in captive leopards ($t = 2.9$, $df = 9$, $p = 0.015$) (Table 6.2).

It was not possible to statistically compare the wild female and male OCP levels due to the small sample size for females (n=2) (Table 6.2). However, based on the mean concentrations, total OCP concentrations appeared to be similar in wild males and females. Wild males had higher concentrations of HCB and heptachlors, while wild females had higher concentrations of HCHs and drins. Small insignificant differences between concentrations of chlordanes and DDTs were found.

6.3.2 Organochloride pesticide contamination in captive leopards

The author acknowledges that sample sizes are too small to obtain meaningful inter- and intra-group comparisons, but in some cases statistical analyses was done to obtain preliminary insights into e.g. differences between life stages (sub-adult vs adult) and colouration (normal vs melanistic) in captive leopards. In captive leopards only the HCHs were significantly higher in males than females ($t = 2.6$, $df = 8$, $p = 0.032$) (Table 6.2). Notable, although not significant, was that all the OCP compounds/isomers were higher in captive males than in females (Table 6.2). Captive adult leopards had higher mean OCP serum concentrations than subadults, albeit not significant. Adults also had higher mean concentrations of hepatchlor and drins, while subadults had higher mean concentrations of HCHs, HCB and significantly higher concentration of chlordanes ($U = 8$, $df = 11$, $p = 0.049$) (Table 6.2). Except for heptachlor, the concentrations of OCP compounds/isomers were higher in captive melanistic leopards compared to the regular colouration leopards (Table 6.1).

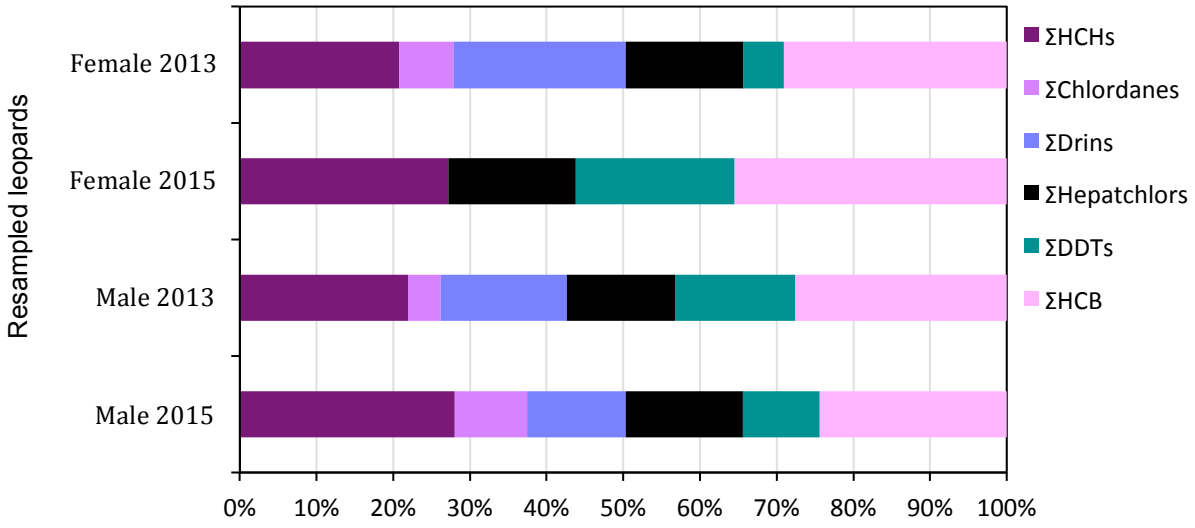

Figure 6.2 The relative contribution of main compound groups (*Σ*HCHs; *Σ*Chlordanes; *Σ*Drins; *Σ*Heptachlors; *Σ*DDTs; *Σ*HCB) to *Σ*OCP concentrations as analyzed in the two resampled captive leopards.

Two captive leopards, CF2 (Fig. 2.3 c,e,g,k) and CM2 (Fig. 2.3 d,f,j,k), from the same facility in the Free State were sampled twice with at least two years between the sampling events (see Table 2.2). Total OCP concentrations in serum of the regular female decreased by 35% from 666 to 434 pg/ml between the two sampling periods. Notable changes in her OCP contamination profile were observed between samplings (Fig. 6.2), which showed that heptachlors were higher during the second sampling but both the HCB and chlordanes decreased to below LOD. Total OCP concentrations in the melanistic male serum also decreased (39%) between the two sampling periods from 1383 to 842 pg/ml.

A notable change in the male's OCP contamination profile was decreased chlordane and heptachlor concentrations from the second sampling event. The HCHs were the predominant compound in the male's 2015 sample compared to DDTs in the 2013 sample (Fig. 6.2). The only compounds that increased between sampling was heptachlors in the female (156%) and HCB in the male (34%). Unlike the female, no compounds in the male decreased to below LOD between samplings.

6.4 Discussion

There are clear gaps in knowledge with regards to ecotoxicological work on apex predators. The constraints in capturing and working with large, dangerous carnivores make it problematic to investigate the occurrence and effects of toxic chemicals in these animals. According to Rodríguez-Jorquera et al. (2017) it is convenient to obtain tissue from carcasses for chemical residue analysis, but it does not necessarily provide information on sub-lethal concentrations in live animals. In contrast, the advantages of using blood serum samples include the possibility of resampling individuals in future studies, and stress inducement on study subjects are less due to its lower invasiveness. Few studies report on OCP concentrations from blood or plasma samples of apex terrestrial carnivores (Table 6.1), and these authors only report on a few OCP compounds. Whilst it is acknowledged that the sample size of this study was relatively small and precludes from undertaking meaningful statistical analyses, it is still worthwhile commenting on some of the findings. In addition, the author does acknowledge that for some compounds the recovery rates were below 80% and for these the reported concentrations were adapted correspondingly. Despite the levels of OCPs being low in the serum of leopards, the author is certain that the data are relatively accurate since they are all within the same order of magnitude as OCPs reported in blood/plasma/serum of mammalian carnivores elsewhere in the literature (see Table 6.1).

6.4.1 Contextualising organochlorines in leopards

This study found limited significant differences in *Σ*OCPs, individual compounds and isomers among many of the groups tested. The OCP contamination pattern found in wild African leopards was similar to that found in wild lions, spotted hyaenas (Malarvannan et al. 2020) and

caracal (Leighton et al. 2022) in South Africa. The only group in which *Σ*DDTs was not the main contributing compound was in captive males, where like reports from brown bears *Ursus arctos* Linnaeus, 1758 and grey wolves *Canis lupus* in Croatia (Romanić et al. 2015), *Σ*HCHs were the main compounds. *Σ*DDTs concentrations from leopards (0.22 ng/ml) were lower than that of domestic sled dogs (29 ng/ml) from Greenland (Sonne, 2010), wild spotted hyaenas (1.6 ng/ml) from South Africa (Malarvannan et al. 2020) and wild caracal (2.5 ng/ml) from South Africa (Leightin et al. 2022). The *Σ*DDTs concentrations in leopards were however similar to that of wild lions (0.27 ng/ml) from South Africa (Malarvannan et al. 2020). Other mammals in which *Σ*DDTs was the main contributing compound in plasma or blood samples include red foxes *Vulpes vulpes* and wolverines *Gulo gulo* (Linnaeus, 1758) from Sweden (Polder et al. 2009), Eurasian lynxes *Lynx lynx* (Linnaeus, 1758) from Norway (Polder et al. 2009), Masked palm civets *Paguma larvata* (Smith, 1827) from Japan (Yamamoto et al. 2012), wild lions and hyaenas from South Africa (Malarvannan et al. 2020) and caracal from South Africa (Leighton et al. 2022).

Traces of *p,p'*-DDE, *o,p'*-DDD and *p,p'*-DDD were detected in wild and captive leopards from this study. Comparatively higher concentrations of *p,p'*-DDE have been reported from the blood of other terrestrial mammalian carnivores such as red foxes, wolverines and Eurasian and Iberian lynxes *Lynx pardinus* (Temminck, 1827) (Polder et al. 2009, Mateo et al. 2012), lions, spotted hyaenas and cheetahs (Malarvannan et al. 2020), and caracal (Leighton et al. 2022). Leighton et al. (2022) and Malarvannan et al. (2020) also found traces of *p,p'*-DDT in wild lions, spotted hyaenas and caracal from South Africa, respectively, and captive lions and cheetahs in Belgium. Reports on *o,p'*-DDD and *p,p'*-DDD from other tissues such as liver and muscle of mammalian terrestrial carnivores are more frequent (see Table 6.1), but not comparable to the results of this study due to the lipophilic nature of the tissues reported on. There is an anomaly in the current study's data that is difficult to explain; where the concentrations of *p,p'*-DDD in wild leopards from this study, were much higher than the concentrations reported from other studies in the northern hemisphere (i.e. red foxes in Sweden, Norway (Polder et al. 2009) and Spain (Mateo et al. 2012), polar bears in Norway (Skaare et al. 2001), wolverines in Norway (Polder et al. 2009), Iberian lynxes in Spain (Mateo et al. 2012) and Eurasian lynxes in Sweden (Polder et al. 2009)). The chromatograms were re-evaluated, and the author is confident that the peaks that were recorded were not due to any cross-contamination and due to the elution of *p,p'*-DDD. This is an aspect that requires further investigation. The *p,p'*-DDE in wild and captive leopards were similar to levels reported elsewhere in the literature and is usually the predominant metabolite of DDT encountered in terrestrial mammals (Romanić et al. 2015, Mateo et al. 2012, Bernhoft et al. 1997, Hoshi et al. 1998, Malarvannan et al. 2020). Its prevalence is indicative of historical contamination from DDT. It is therefore likely that the DDT contamination in leopards from this study can be attributed to the historic use of DDT in South Africa.

Table 6.2 Organochlorine concentrations (pg/ml) in serum samples from wild and captive leopards. The sample size (n) is presented in brackets. Bold p-values indicate a significant difference p<0.05. <LOD: below limit of detection. na: not applicable. Asterisk (*): detected in one individual only. Hashtag (#): mean of two individuals.

							Captive leopards						Wild leopards		
	Captive	**Wild**	**p-value**	**Males**	**Females**	**p-value**	**Melanistic**	**Regular**	**p-value**	**Adults**	**Subadults**	**p-value**	**Males**	**Females**	**p-value**
OCPs	(10)	(6)		(4)	(6)		(4)	(6)		(7)	(3)		(4)	(2)	
							ΣHCHs								
α-HCH	113 ± 74	75 ± 16	0.118	151 ± 54	88 ± 14	0.209	91 ± 11	127 ± 38.8	0.484	125 ± 33	82 ± 12	0.436	95 ± 33	85#	na
δ-HCH	33 ±12	17 ± 11	0.408	51 ± 42	20 ± 33	0.235	73 ± 21	6 ±13	**0.009**	25 ± 42	51 ± 24	0.383	41*	62*	na
γ-HCH	48 ± 8	48 ± 13	0.998	52 ± 43	46 ± 34	0.998	48 ± 8	48 ± 13	0.998	48 ± 8	48 ± 13	0.998	28 ± 29	79#	na
ΣHCHs	196 ± 2	143 ± 24	0.168	256 ± 35	156 ± 21	**0.032**	242 ± 54	165 ± 76	0.114	174 ± 24	181 ± 24	0.874	131 ± 34	194#	na
							ΣHCB								
HCB	74 ±14	33.7 ± 3	**0.015**	96 ± 27	60 ± 13	0.205	76 ± 6	73 ± 23	0.905	72 ± 20	79 ± 5	0.829	48 ± 21	34#	na
							ΣHeptachlors								
Heptachlor	131 ± 36	142 ± 8	0.823	157 ± 108	114 ± 124	0.762	99 ± 43	152 ± 54	0.506	157 ± 26	35 ± 2	0.104	198 ± 104	137#	na
							ΣDrins								
Aldrin	31 ± 29	17 ± 29	0.383	47 ± 29	24 ± 28	0.325	51 ± 31	18 ± 21	0.088	31 ± 12	41 ± 19	0.642	<LOD	76*	Na
Endrin	79 ± 48	116 ± 25	**0.042**	79 ±33	80 ± 59	0.610	69 ± 46	86 ± 51	1.000	93 ± 49	48 ± 30	0.183	134 ± 39	138#	Na
ΣDrins	118 ± 16	140 ± 21	0.407	133 ± 53	108 ± 48	0.610	127 ± 28	112 ± 20	0.659	133 ±16	69 ± 11	0.316	134 ± 39	214#	na
							ΣChlordanes								
trans-Chlordane	70 ± 25	24 ± 12	0.185	107 ± 92	45 ± 63	0.331	146 ± 58	19 ± 34	**0.009**	52 ± 86	113 ± 31	0.253	71*	71*	na
trans-Nonane	100*	68*	na	<LOD	100*	na	<LOD	100*	na	100*	<LOD	na	68*	<LOD	na
cis-Nanochlor	20 ± 22	25 ± 0.2	0.654	18 ± 9	25 ± 10	0.385	24 ± 25	17 ± 22.0	0.719	9 ± 18	44 ± 4	0.110	<LOD	<LOD	na
ΣChlordanes	114 ± 27	51 ± 13	0.109	134 ± 46	101 ± 36	0.580	186 ± 52	66 ± 68	0.062	71 ± 73	151 ± 50	**0.049**	139#	71*	na
							ΣDDTs								
o.p'-DDD	35 ± 7	38 ± 7	0.799	40 ± 13	32 ± 8	0.623	52 ± 6	24 ± 7	**0.018**	31± 9	45 ± 5	0.342	38 ± 18	38*	na
p.p'-DDD	190 ± 53	172 ± 24	0.713	203 ± 75	181 ± 38	0.762	209 ± 35	177 ± 17	0.393	192 ± 65	185 ± 8	0.383	178 ± 52	186#	na
p.p'-DDE	8 ± 5	13 ± 12	0.692	<LOD	18#	na	18#	<LOD	na	17*	18*	na	28*	230*	na
ΣDDTs	269 ± 20	258 ± 13	0.723	284 ± 49	259 ± 16	0.578	308 ± 39	242 ± 17	0.113	264 ± 18	266 ± 14	0.965	218 ± 30	220#	na
							ΣOCPs								
ΣOCPs	901 ± 84	768 ± 48	0.269	1058 ± 142	797 ± 88	0.135	1038 ± 142	810 ± 95	0.201	856 ± 76	838 ± 40	0.899	764 ± 186	761#	na

To the author's best knowledge, the detection of HCH-isomers from blood or plasma samples are rarely reported (see Table 6.1). Unlike other studies, specifically the study on wild lions and spotted hyaenas by Malarvannan et al. (2020) in South Africa, the current study did not find traces of *β*–HCH in any leopards. This is surprising, as Willett et al. (1998), Bernhoft et al. (1997) and Bentzen et al. (2008b) suggested that *β*–HCH is far more persistent in mammals than *α*–HCH and *γ*–HCH isomers. In the present study, *α*–HCH was the largest of the HCHs, with concentrations higher in captive than in wild leopards. Interestingly, this contrasts with the results from Malarvannan et al. (2020) on lions and hyaenas, who found that *γ*–HCH was the most prevalent HCH isomer. These authors suggested that this result might reflect a preferential usage of lindane, which consists of purified *γ*–HCH, in the regions their animals were sampled. The predominance of *α*–HCH in leopards from the current study corresponds to the suggestion of Tomza-Marciniak et al. (2014), who reported that this is usually highest in animals near agricultural areas where mixtures of HCHs were recently used. This may suggest that leopards from this study are either continuously exposed to these mixtures of HCHs in agricultural areas, or that the leopard metabolism is better at metabolizing HCHs than that of other terrestrial mammalian carnivores. Contrary to the information generated in the present study, no records of blood/plasma concentrations of *α*–HCH could be found in literature, even though studies by Romanić et al. (2015) suggest that these isomers constitute substantially to the OCP profiles of carnivores such as Brown bears and Grey wolves.

The comparison of OCP contamination between species and intraspecific groups must always be approached with caution (Skaare et al. 2000). Numerous factors such as age, sex, seasonality, feeding habits and reproductive status can influence levels of concentration within individuals. Differences in diet can result in variation within and between species (see Hop et al. 2002, Borgå et al. 2004, McKinney et al. 2009, 2013).

6.4.2 Diet and organochlorine accumulation

Although no necropsies and actual stomach content identifications were undertaken during this study, dietary regimes were obtained for captive leopards via interviews with their ex-situ managers and general information on the generalist diet of leopards and their feeding ecology is relatively common (Norton et al. 1986, Bailey 1993, Hayward et al. 2006). So even though the home ranges of wild leopards from this study included agricultural areas, where pesticides are used on a regular basis, the captive leopards had higher *Σ*OCP concentrations in their blood. Data collected during visits to ex-situ facilities showed that captive leopards are regularly treated for ecto- and endoparasites with products such as Frontline® Boehringer Ingelheim. The portion of chicken meat in the diet of captive leopards, obtained from commercial broilers, is higher than that of red meat. It has been reported that commercial chicken meat in South Africa is contaminated with OCPs such as Chlordanes and DDTs (Quinn et al. 2011, Thompson et al. 2017a, 2017b). Keeping in mind that wild leopards may not have regular access to chicken

meat but would rely more on whatever food source they can find, chicken meat may therefore be a contributing factor to increased OCP exposure among captive leopards in South Africa.

The only significant differences between captive and wild leopards were in their burdens of HCB and endrin. Captive leopards (74 pg/ml) had higher HCB levels in their blood serum than wild leopards (34 pg/ml), while wild leopards (116 pg/ml) had almost twice as much endrin in their blood than captive leopards (79 pg/ml). This is indicative of the differences in exposure routes and concentrations of these two groups. Wild leopards in South Africa are known to roam agricultural areas, where insecticides/avicides are regularly used for pest management. Reports on OCP contamination in livers of ground birds such as Guinea fowl *Numida meleagris* (Linnaeus, 1758) (e.g. *p,p'*-DDD – 57 ng/g) and francolins *Pternistes natalensis* (Smith, 1833) (e.g. *p,p'*-DDD – 345 ng/g) from these agricultural areas (Barnhoorn et al. 2009) may already be cause for concern as these birds are known to constitute quite a large part of the leopard diet. Contrary to the author's expectations, captive leopards had slightly higher concentrations of *Σ*OCPs, *Σ*HCHs, *Σ*HCB, *Σ*Chlordanes and *Σ*DDTs than wild leopards. However, this result does correspond with levels reported by Malarvannan et al. (2020) from wild and captive lions. This is supported by the findings of Leighton et al. (2022) who attributed higher OCP bioaccumulation in caracal drawn to urbanised areas to the consumption of "exotic" prey (i.e. prey that would not form part of their natural diet in the wild). This results in a co-incidental increase in exposure to OCPs.

6.4.3 Life history and organochlorine accumulation

All leopards from this study had unique OCP profiles. Captive males had significantly higher *Σ*HCHs and HCH isomer (*α*–, *β*– and *γ*–HCH) concentrations than females, with all concentrations higher than that reported from the plasma of Polar bears in Norway (Skaare et al. 2001) and lions and spotted hyaena (Malarvannan et al. 2020). Apart from *cis*-nonachlor and endrin, captive males had higher OCP contamination of every compound and isomer tested, and an overall higher *Σ*OCP concentration than females. However, this was not the case for all mammalian carnivores, as can be seen in the study on Alaskan Arctic foxes *Vulpes lagopus* (Linnaeus, 1758) (Harley et al. 2016) and British Eurasian otters *Lutra lutra* (Linnaeus, 1758) (Mason et al. 1986), where the OCP burdens are higher in the blood of females than males. On the other hand, Polder et al. (2009) and Mateo et al. (2012) reported that sex may not be a factor in OCP contamination in Iberian lynx and red foxes from Europe, and similar findings were documented by Shore et al. (2001) in Russian wolves. These findings coincide with the conclusion of Romanić et al. (2015), indicating that the effect of gender on OCP contamination may be species specific and that little is understood about how felids accumulate and metabolize OCPs. From these results it appears that in the case of leopards that gender differences in OCP contamination exist and it is most probably due to the unloading effect that pregnancies and lactation might have on females' OCP burdens (also see below).

This study is the first to report on intraspecific differences in *Σ*Chlordane concentrations among captive adult and subadult carnivores. Captive adult leopards had significantly lower *Σ*Chlordane contamination than captive subadults. *Σ*Chlordane concentrations from the current study (0.13 ng/ml) was much lower than that reported from plasma of domestic sled dogs (770 ng/ml) from Greenland (Sonne 2010), but much higher than that reported from the blood of caracal (0.03 ng/ml) (Leighton et al. 2022). These findings concur with that of Bytingsvik et al. (2012), who reported that polar bear cubs had higher OCP concentrations in their blood plasma than their mothers. On the other hand, Mateo et al. (2012) found that age does not influence OCP concentrations in wild terrestrial carnivores such as the Iberian lynx and red foxes. The present study's results suggest that leopards may be born with OCP contamination obtained during the foetal stage from the female or has been exposed to contamination during suckling (Skaare et al. 2000, Jaspers et al. 2010, Bernhoft et al. 1997, Greig et al. 2007).

There were some interesting observations related to OCP accumulation in captive melanistic and regular leopards that are worth mentioning. In general, most of the OCPs measured during this study were higher in the melanistic leopards when compared to e.g. the regular leopards. Notably, the *δ*–HCH were 14 times higher, *trans*-chlordane were seven times higher and the *o,p'*-DDD concentrations were double than regular leopards. This is while both groups are fed similar diets and have similar parasite treatment regimes. These data may indicate that black leopards are not able to metabolize these isomers as efficiently as their regular counterparts. It is, therefore, interesting to speculate that these findings may be attributed to the physiological effects accompanying melanism in mammals since it is known that melanin-producing cells have been reported to be effective at isolating potentially toxic metal ions (Sarna et al. 2022). Since the overall *Σ*Chlordane concentrations are very similar between captive males and females from this study, it may be deducted that melanism in leopard males may influence the accumulation of *Σ*Chlordanes, especially *trans*-chlordane. However, more sampling is needed to come to a definitive conclusion.

6.4.4 Organochlorine accumulation in resampled leopards

The OCP composition profiles of both resampled individuals changed between samples and *Σ*OCP concentrations decreased in both leopards. However, the *Σ*Heptachlor concentrations increased in the female and the *Σ*Chlordanes levels increased in the male. Since this female had a litter of cubs between sampling, the female seemed to have either completely metabolized HCB, *trans*-nonane, *cis*-nonachlor, aldrin and *o,p'*-DDD from her blood or transferred it to her cubs through lactation, while there was a build-up of heptachlor and endrin, with very little change in the concentration of *p,p'*-DDD. These findings further support the phenomenon of maternal transfer of OCPs from the female to cubs, as discussed previously. It would thus stand to reason that the decrease in the abovementioned OCP compounds and isomers may be attributed to a number of factors: that the body is able to metabolize these compounds over time; levels decrease due to maternal transfer directly to the foetus and later lactation, or that exposure to these compounds diminish as the diet of leopards are changed as they get older.

6.4.5 Management implications

Concurring with Hall et al. (2006), the consideration of environmental pollutants should be a highly important factor in conservation and management concerns. There is growing literature that shows that, despite the banning of many OCPs, there is still a considerable amount that enter South African conservation areas (Volschenk et al. 2019, Gerber et al. 2021, Wolmarans et al. 2021). Currently, no specific guidelines for managing ex-situ leopard populations exist in South Africa. A successful conservation strategy promotes the awareness of the importance of leopard conservation, which includes an increased emphasis on ex-situ conservation. Effective conservation efforts should take a holistic approach, with all-inclusive evaluation of the occurrence of contaminants and the possible threats posed to apex predators.

6.5 Conclusions

The global leopard population is increasingly exposed to intensifying pressures due to habitat loss and impacts of chemical pollution on apex predator populations are seldom considered. Reports on OCP contamination in living terrestrial apex predators, with the possibility of resampling, are rare. As the first report establishing important base-line data for OCP contamination in South African leopards, this study's results showed similar OCP serum concentrations compared to decade-old data reported for lion and hyaena by Malarvannan et al. (2020) and more recently for caracal (Leighton et al., 2022) in South Africa. This study found that dietary factors have a greater influence on OCP accumulation than other intraspecific factors such as age and sex. Attention should be paid to implications for population growth, especially during harsh periods such as droughts when leopards may expand their home ranges, subsequently increasing their presence in agricultural areas where pesticides are used. Importantly, this study shows the need for future, continuous monitoring of POPs in terrestrial predators, especially in countries that are known users of OCPs in their agricultural sector and combating malaria, such as South Africa.

6.6 References

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CHAPTER 7
An integrated leopard health philosophy

7.1 Introduction

An integrated philosophy of health provides a framework that integrates existing models and concepts into a tool of health assessment. Health is defined in diverse inferences across various research disciplines, but according to Saad & Prochaska (2020) health could be defined as an individual's ability to function in a way vital to its survival, despite having diseases and pathological infections. This view of health will be followed in this concluding chapter in relation to leopard health.

Discussions about wildlife health measures are few (Stephen 2014) but have recently gained interest among researchers. Integrated research on toxicology, haematology, parasites and ecology helps to create detailed knowledge of the physiological responses of vertebrates to various stress factors (Wikelski & Cooke 2006, Cooke et al. 2012, Maceda-Veiga et al. 2015). From the extensive literature review on leopards presented in Chapter 1 of this thesis, it is clear that, although there is a lot of available knowledge on leopard taxonomy, behaviour, habitat preferences, diet, feeding ecology and conflict with humans, there still exists large gaps in knowledge on leopard health and the parameters that can be used to assess it. To work towards filling these gaps, the main aim of this Ph.D. project was to use a multi-disciplinary approach that integrates relatively diverse parameters, to establish a workable, repeatable, integrated approach to the health assessment of South African leopards, the one aspect of this large cat that has remained largely unstudied. To achieve this aim, this study focused on 3 main aspects namely haematology (Chapter 3), haematozoan blood parasites (Chapters 4 and 5) and blood OCP levels (Chapter 6). Therefore, in this final concluding chapter, the objectives are to highlight and evaluate the interaction of the internal and environmental factors from a health parameter perspective and to present a detailed discussion of leopard health, using an integrated approach whereby all the results from this study are combined and analysed in order to point the way forward for research on and management of leopard health, the latter more specifically related to captive leopards.

7.2 Environmental Parasitology

Environmental parasitology, an interdisciplinary field that combines toxicology, parasitology and environmental chemistry (Sanchez-Ramírez et al. 2007), concerns the interplay between parasites and pollutants in their shared environment (Lafferty et al. 2004, Goater et al. 2013, Sures et al. 2017). The effects of co-ocurring pollutants and parasites cannot be easily foreseen (Sures 2008), for example, some publications specifically report that an organism's immune response can be suppressed by pollutants, leading to increased, more intense parasite infections (see for example Morley et al. 2006). On the flip side, it has also been reported that parasite infections may alter a host's physiological response to pollutants in different directions, as these two parameters can interact in unpredictable 'additive, synergistic or antagonistic' manners (Sures 2004, 2008, Morley et al. 2006, Sanchez-Ramírez et al. 2007). Spencer & Zuk (2016) subsequently reported that, although it is widely believed that parasites reduce a host's biological fitness (Roberts & Janvoy 2008), conservation biologists have come to realise that parasites of endangered host species should be seen and conserved as part of their host's natural environment, as parasitic exposure might be pivotal to developing a healthy immune system. This is ascribed to these co-existing parasites co-evolving with their hosts for such a long time, that parasites have become a part of a host's natural environment. Therefore, host populations can develop weak phenotypes in the absence of their co-evolved parasites.

The recent growing trend of integrated research on parasite-pollution interactions is still mostly focused on the aquatic perspective (Selbach et al. 2022), leaving a gap in this type of research in terrestrial environments (Sures 2008, Sures et al. 2017). Current available terrestrial environmental parasitology studies mostly focus on helminthic endoparasites (see Sures 2004) and according to the author's best knowledge, no studies thus far had combined environmental stressors (i.e. OCPs) and haemoparasite loads as variables in large, terrestrial carnivores. A better grasp of terrestrial environmental parasitology can help to reduce the clear research gap on wildlife and environmental health.

7.3 Thesis summation and highlights of health research

As discussed in Chapter 2, blood was specifically chosen as a biological source for data as it can be collected from live leopards, offers the option of future resampling and can be sustainably used as a method of research on leopard health. Several advantages of using this tissue have been considered in Chapter 2, including the ease of sample management in the field and the value of blood as a health assessment tool. Chapter 3 also touches on this subject.

One of the novel contributions of this study is the design and introduction of a five-point body condition score (BCS) system for leopards to quantify physical/body condition (see Chapter 2 Table 2.2). All leopards were given a BCS score (1=emaciated; 2=underweight; 3=ideal; 4=overweight; 5=obese) upon sight in the field. Using the BCS it was no surprise that the final

scores of wild and captive leopards differed from each other (Chapter 2, Tables 2.3 and 2.4). The BCS for captive leopards ranged from 3 – 5, indicating that these animals are mostly overweight, while the BCS of wild leopards were lower and ranged from 2 – 3, thus either underweight or in an ideal condition. A key advantage of the BCS system was that it allowed the researcher to evaluate a leopard on sight, even before sedation, which left more time for other data gathering under strenuous field conditions.

The internal and external variables used for the integrative analysis presented in this chapter have already been discussed in detail in Chapters 3, 4 and 6 of this thesis. In summary, the haematological characteristics of captive and wild leopards differ from each other, as well as from other felids. In Chapter 3 a detailed guide for morphological characterization of blood cells and haematological characteristics for African leopards was provided, the first of its kind for a leopard subspecies using captive and wild populations. Results presented in Chapter 3 highlighted the haematological differences among big cats, stressing that so-called 'normal' haematological values for one species should not be applied to assess that of another species, even within the same genus. Chapter 4 presented two new *Hepatozoon* species, co-infecting the same leopard with only wild leopards being infected. These two haematozoans infected neutrophils and lymphocytes and in Chapter 4 details on how host cells were affected were provided by showing that infected neutrophils were larger and lymphocyte smaller in size, respectively. In Chapter 5 it was reported that captive leopards were mostly free of haematophagous vectors which is most probably the reason for none of the captive leopards being infected. In this chapter the possible *Hepatozoon* developmental stages in an engorged tick collected from an infected leopard were described. Following on this, Chapter 6 reported the first record of OCPs in the blood of captive and wild leopards in South Africa and provides the first baseline OCP concentrations in live captive and wild leopards. Additionally, Chapter 6 also explored the relationship between OCP levels and different conservation management strategies, and found that diet is the main contributing factor to OCP exposure in African leopards.

7.4 Integrating parasitology and toxicology

Canonical Correspondence analysis (CANOCO), using CANOCO v5.2 statistical software (Ter Braak 1994) was used in this study to analyse internal and external health variables, as described in Chapter 2. The explanatory variables of this analysis included 13 environmental variables (blood OCP concentrations), and the supplementary variables included five functional traits (Fig. 7.1). The plotted variables of this study were broadly divided into internal/health factors (differential white blood cell counts, NLR and BCS) and environmental factors (OCP concentrations).

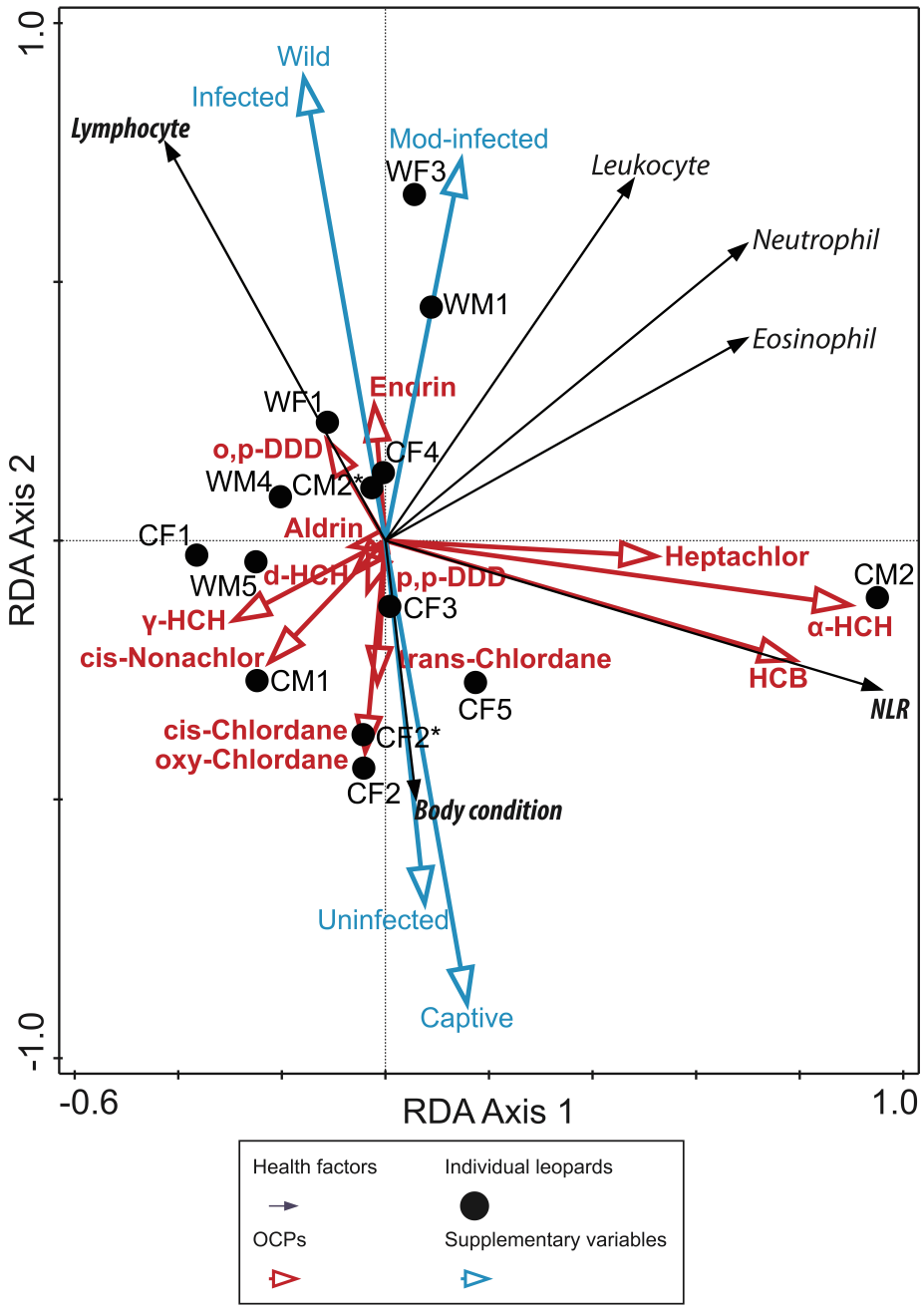

Figure 7.1 Redundancy analysis triplot of 14 leopard data points (2 from resampled leopards as indicated by *), from a captive and wild population, to examine the strength of internal (health factors represented by black arrows), supplementary (captive or wild diet and hepatozoan infections, as presented in Chapter 4, represented by blue arrows) and environmental (OCP concentrations as presented in Chapter 6 represented by red arrows) variables per leopard. A constrained redundancy analysis (RDA) was realised to assess OCP concentrations associated with health factors (BCS, NLR, lymphocyte counts, leukocyte counts, neutrophil counts, eosionophil counts) of individual leopards, with their respective diets (captive or wild) and parasite infections (uninfected, infected, moderately infected) overlayed as supplementary variables. The triplot explains 83.2% of the variation, with 55.9% on the first axis and 27.3% on the second axis. Explanatory variables: 13 environmental variables (DF = 12); Supplementary variables: 5 functional traits (DF = 3); pseudo-F = 0.3; p = 0.99. Individual leopards involved in this analysis are represented by black circles, with associated codes as can be see in Chapter 2 of this thesis. NLR: neutrophil-to-lymphocyte ratio. All differential white blood cell counts (as described in Chapter 3) are labeled per type of leukocyte. Leukocyte: leukocyte counts; Lymphocyte: lymphocyte counts; Neutrophil: neutrophil counts; Eosinophil: eosinophil counts.

Supplementary variables included haemogregarine infections (uninfected, infected, moderately infected) and leopard diet (distinguished as 'wild or 'captive' diet). Specifications of these variables were presented and detailed in Chapter 2. Findings of the multivariate statistical analysis of environmental and internal variables are graphically presented in the redundancy analyses (RDA) triplot in Figure 7.1. The purpose of this analysis was to integrate and show associations of this study's environmental and internal factors and how it may relate to leopard health.

Pesticide exposure is known to impair immune responses as Nunn et al. (2003) showed significant correlations between elevated leukocyte counts and various ecological and behavioural characteristics in carnivores. Nunn et al. (2003) also specifically reported no health effect of dietary meat content and found a positive correlation between BW (body weight) and leukocyte and neutrophil counts, as well as a negative correlation between BW and lymphocyte counts. Secondly, a recent study by Leighton et al. (2022) on caracals in South Africa, found that OCP exposure in these small felines resulted in potential immunosuppression, illustrated by elevated WBC and platelet counts. Thirdly and interestingly, Viljoen et al. (2020) reported intraleukocytic *Hepatozoon felis* from caracals from the same region as those studies by Leighton et al. (2022). However, Viljoen et al. (2020) did not investigate potential immunological effects of these haemoparasites. Thus, this current assessment is of significance since it investigates both of these parameters simultaneously in captive and wild leopards.

The most influential health factor for all leopards was NLR, followed by other WBC counts (lymphocyte, leukocyte, neutrophil, eosinophil in decreasing order of influence) and body condition. There was a strong positive association between body condition and leopards without hepatozoan infections, with a strong negative correlation between body condition and infected leopards (Fig. 7.1). The most influential OCP variable was *α*–HCH, followed by HCB and heptachlor in decreasing order of association, and was mostly associated with captive leopards. From the distribution of data it was evident that the variables selected for this analysis differentiate between the captive and wild leopard populations. Leighton et al. (2022) associated elevated WBC in caracals with a stimulated immunological response to OCPs, called OC immunotoxicity. Leighton et al. (2022) also suggested that any immunological response may increase disease susceptibility. According to Pavlova et al. (2015), a higher NLR may be indicative of a more severe pathogenic infection (see Chapter 3). Since NLR did not closely associate with wild leopards, which had hepatozoan infections, it seems that a higher NLR in leopards may be more indicative of an OC immunotoxicity response (see Leighton et al. 2022). Concentrations of *α*–HCH, HCB, endrin, heptachlor and *p,p'*-DDD were present in all fourteen leopards, at varying concentrations and each individual had a unique OCP profile (see Chapter 6). The OCP profiles of captive and wild leopards differed (Fig. 6.1) and *α*–HCH was the predominant isomer across all leopards (see Chapter 6) and in this analysis also functioned as a highly influential variable in leopard health parameters (Fig. 7.1).

7.4.1 Possible health associations for captive leopards

Body condition, NLR and OCP variables were the most influential variables for captive leopards. Captive leopards were mostly associated with these two internal and one external health variable, which imply that the health of captive leopards is mostly influenced by OCP concentrations, NLRs and body condition. As reported earlier in this chapter, captive leopards tended to be overweight and even obese (BCS). All captive leopards did not cluster together and were not influenced evenly by the same variables. Leopard individuals CM2* (resampled melanistic male) and CF4 were closely grouped and positioned among wild leopards WF1 and WM4. This grouping was closely and almost evenly associated with lymphocyte counts, *o,p'*-DDD and endrin variables, but endrin and *o,p'*-DDD were weakly influential. CF2, CM1, CF3, CF5 and CF2* (resampled regular female) grouped together and this grouping was closely associated with body condition and weakly correlated with *trans*-chlordane, *cis*-chlordane and *oxy*-chlordane.

A case study on resampled leopards

Leopard CM2 (melanistic male) was an interesting outlier in these results, with a strong positive correlation with *α*–HCH and HCB. Note that this leopard was resampled and individual leopard CM2* indicates the resampled data point 2 years after the first data collection of this leopard. As reported in Chapter 6, both CM2 and CF2 each shed more than 30% of their OCP loads in the 2 years between samplings.

As presented in Chapter 3, melanistic leopards from the current study had higher differential leukocyte counts than their regular counterparts. At the time of the first sampling of CM2, the strongest variables were *α*–HCH and HCB and NLR were strongly associated. At the 2nd sampling, this was not the case and the strongest variables were lymphocyte counts, *o,p'*-DDD and endrin. CM2's NLR was very high in 2013 and much lower in 2015 (see Chapter 3), which indicates that CM2 was experiencing some sort of immunosuppression. Several studies have shown NLR values to be good diagnostic indicators of stress or innate immune response (Davis et al. 2008, Pavlova et al. 2015, Gori et al. 2021, Petrucci et al. 2021) and an increased NLR could indicate increased stress, infection or inflammation (Maceda-Veiga et al. 2015, Gori et al. 2021, Petrucci et al. 2021). Since OCPs are the strongest influential factor among captive leopards, this high NLR may indicate that CM2 was experiencing an immunotoxicity response (see Leighton et al. 2022). At his first sampling, CM2 also had hypochromic RBCs and almost all his neutrophils were hyper-segmented (see Chapter 3). The presence of hypersegmented neutrophils may indicate anaemic conditions caused by Vitamin B12 deficiency (Singh et al. 2017), which can result in erythrocyte defects leading to an undersupply of oxygen to the body. Hypochromic RBCs are slightly haemoglobin deficient, which can indicate an iron deficiency and/or anaemia (Valenciano et al. 2014, Maceda-Veiga et al. 2015).

Contrarily, the variables associated with CF2 (resampled regular female) did not change much in the two years between samplings (compare CF2 and CF2* in Fig. 2.3 c – g,j, k). CF2 also did

not have blood cell abnormalities and her differential cell counts stayed very similar between samplings (see Chapter 3). Since CM2 and CF2 are both housed at Ex-situ Site1, it can be assumed that there was not a lot of change in their dietary regimes or other management protocols in the time between samplings. Subsequently, this may indicate that different leopard individuals have different efficiency in metabolising OCP compounds and that parameters influencing an individual's health are not constant, but fluctuates in importance throughout its life. Something that may be taken into account is the age at which individuals are sampled. CM2 was two years old at the first time of sampling and CF2 was three years old. This means that, according to the age group classification in Chapter 2 (Table 2.1), CM2 changed from being a subadult to an adult, while CF2 was an adult to start with. It may be that the rapid changes involved in maturing may have had an accelerating effect on metabolising of OCP compounds, or that immunotoxicity decreases with age in leopards. In Chapter 6 the phenomenon of maternal transfer of OCPs from the female to cubs was potentially shown, and it was speculated that exposure to these compounds diminishes as the diet of leopards is changed as they get older. This may be the reason why CM2* is associated with such different variables – as it changed from subadult to adult the content of chicken in the diet is slightly less as more red meat is incorporated. On the other hand, it is interesting to speculate that these findings may be attributed to the physiological effects accompanying melanism, confirming that melanism may cause intraspecific physiological variations (Ducrest et al. 2008, Roulin & Ducrest 2011) and melanin-producing cells have been reported to be effective at isolating potentially toxic metal ions (Sarna et al. 2021). Since these are only two case reports, conclusive deductions could not be made.

7.4.2 Possible health associations for wild leopards

Differential leukocyte counts and haemoparasie infections, all health factors, are the strongest variables for the wild leopards, which are mostly associated with these parameters as seen in Figure 7.1. All wild leopards did not cluster together and were not influenced evenly by the same variables. Leopard individuals WF1, WM4 and WM5 formed a group associated with aldrin, *δ*–HCH, *o,p'*-DDD, lymphocyte counts and hepatozoan infections, although these OCPs were weak variables. Interestingly, WF3 and WM1 grouped together, associated with moderate hepatozoan infections.

Wildlife hepatozoonosis is normally subclinical or asymptomatic (McCully et al. 1975, Mercer et al. 1988, Averbeck et al. 1990, Kocan et al. 2000, East et al. 2008, Metzger et al. 2008, Santos et al. 2013). It has been shown that immunocompromised animals may be prone to higher parasitaemia of *Hepatozoon* (Hervas et al. 1995, Baneth et al. 1997, 2001), which may in turn further affect immunological responses to other pathogens. The parasitaemia of the two *Hepatozoon* species in this study differed in such a way that one of the author's conclusions was that the leopard immune system may be better at suppressing *H. luiperdjie* than *H. ingwe* (see Chapter 4). As shown in Chapter 4, the two haemogregarine species infecting wild leopards had different effects on their respective host cells. *Hepatozoon luiperdjie*, which infected

neutrophils, compressed the lobulated nucleus of neutrophils and resulted in cell enlargement. *Hepatozoon ingwe*, which infected lymphocytes, completely absorbed the host cell cytoplasm and caused the host cells to contract in size. This study found strong positive correlations were found between leukocyte counts, neutrophil counts and eosinophil counts as wild leopard health factors. As presented here in the current chapter, wild leopards tended to be ideal or underweight (BCS).

The influence of OCP variables was much less associated with wild leopards than with captive leopards (Fig. 7.1). The only OCP variables that were associated with wild leopards were *o,p'*-DDD and endrin, and both were weak influential variables. This implies that, for wild leopards, internal health, and by implication immunocompetence, is a much more important factor and overrules environmental variables. Intraleukocytic parasites elicited a much stronger immune response in wild leopards than OCP burdens (Fig. 7.1). Leopards from this study showed disparate immune responses between the two analysed populations, concurring with studies such as those by Mori et al. (2006) and Leighton et al. (2022). In short, the overall result of this analysis showed that OC immunotoxicity is the overruling health factor in captive leopards, while parasite-related immune response is the overruling factor in wild leopards. This is an important result, as sudden severe changes in the environments of large carnivores have been shown to result in the mortality of up to a third of a carnivore population (Dybas 2009, East et al. 2008), indicating the need to correctly identify these haemoparasites.

7.4.3 Integrated health associations of parasitology and toxicology

According to the author's best knowledge, this is currently the first study that had done an integrated analysis on concurrent OCPs and haemoparasites in large, terrestrial carnivores. The newly designed BCS system of this study, specifically aimed at big cats, has worked well and provided valuable concurrent data with the other health parameters taken into consideration. This study confirms that diet, and by implication BC, is an important health factor to consider in leopards (see Bjornvad et al. 2011, Bourke et al. 2016) and that melanism may have caused intraspecific physiological variations with immune response implications (Ducrest et al. 2008, Roulin & Ducrest 2011, Sarna et al. 2021), as was also inferred in Chapters 3 and 6.

This study concurred with remarks of Sures (2004, 2008) and Morley et al. (2006) in the sense that heterogeneous relationships between pollutants and parasites directly affect a host species' health in disparate ways. The author is of the opinion that CM2's massive OCP burden decrease between samplings that were two years apart, could be an example of induced immunity in a young host which eventually led to a healthier, more efficient immune system as this leopard grew older, concurring with Spencer & Zuk (2016)'s conclusions. The fact that none of the wild leopards had clinical symptoms due to hepatozoonosis, and that parasitaemia and internally regulated variables were the most important health parameters in wild leopards, implies that these haemogregarines have become part of the leopard's natural environment.

As this chapter's results showed, the variables selected for analysis proved to be valuable in providing first insights and baseline information into the health of leopards. Thus, the author agrees with Lafferty et al. (2004) that parasitism should be researched in conjunction with environmental conditions. The current study showed a notable interplay between the importance of haemoparasite infections and OCP burdens in captive and wild leopards. The results of the integrated analysis in this chapter suggest that it is just as important to do environmental parasitology studies in terrestrial environments as in aquatic environments, which is currently lacking. Subsequently, this study concurs with Selbach et al. (2022) and calls for more focused environmental research on concurring parasites and pollutants. Ultimately, this chapter has shown that an integrated philosophy of leopard health, that integrates existing areas of research, is valuable as a future tool of health assessment.

7.5 Ex-situ leopard conservation

As discussed in Chapters 1 and 2, modern ex-situ facilities have come a long way in terms of their value to society. Modern zoos play a vital part in education, conservation awareness and ecotourism (Lindsey et al. 2007a, 2007b). However, conservation of large carnivores has reportedly failed notably with regard to some species. The literature review by Laikre (1999) on hereditary defects and genetic management of captive populations showed that hereditary disorders, such as albinism and melanism, are common in captive populations. The fact that these phenotypes are still often encouraged in the captive carnivore population by selective management, suggests that a high frequency of potentially immunosuppressive genes could be present in this population. Another problem with captive big cats is that they are often overweight (see Chapter 1), which is frequently the result of social anthropogenic pressures of ecotourism that leads to regular overfeeding for the benefit of tourists. Currently, modern enrichment is more focused on behavioural care than on the potential positive health effects of these activities. Russell et al. (2000) reported on the influence of feeding regimens on feline body condition and found that these routines, together with decreased activity levels, are definite risk factors to be considered in feline obesity. As described before in this study, maintaining an ideal BMI or body weight (BW) is an important part of good health (Bjornvad et al. 2011), as malnutrition, both under- and overnutrition, can result in immunosuppression, evident as chronic inflammation (Bourke et al. 2016).

As shown by the facilities themselves on public social media webpages (see Fig. 2.3 and 2.4), even well-meant ex-situ management strategies have the potential to cause harm to the animals. Ex-situ Site1 focused mostly on captive breeding, and according to the author's current knowledge, has yet failed to attempt to rehabilitate and release any of their big cats to the wild. Ex-situ Site2 has been shut down and Ex-situ Site3 was the only facility involved in wildlife rehabilitation and focuses more on that than on captive breeding. As Figure 2.3 shows, most enrichment activities aimed to use 'harmless' natural and man-made objects such as commercial pet-specific toys and cardboard boxes. However, toy stuffed animals and plastic pet

toys have shown the ability to accumulate semivolatile pesticides (Gurunathan et al. 1998, Chen et al. 2009) and endocrine disruptive chemicals such as phthalates and bisphenol (Wooten & Smith 2013). Cardboard boxes have also shown to be a source of chemicals that may be harmful if ingested (Albu & Buculei 2011)) and discarded vehicle tyres could be sources of at least five metals (Cd, Cr, Fe, Pb and Zn) (Shakya et al. 2006). Interestingly, it has been specifically reported that the livers of Guinea fowl (see enrichment in Fig. 2.3 g) could have high pesticide contents (Barnhoorn et al. 2009). Natural enrichment items, such as skins, tails and wool seen in Figure 2.3, are usually donated by surrounding farmers. This implies that these items may be a source of OCPs, as South African farmers regularly treat their livestock against ectoparasites with OC-rich products (see Chapter 6).

As mentioned in Chapters 5 and 6, data collected during visits to ex-situ facilities showed that captive leopards are regularly treated for ecto- and endoparasites with products such as Frontline® (Boehringer Ingelheim). This means that captive leopards were mostly free of haematophagous ectoparasites. Ex-situ managers view keeping their animals parasite-free, and in their opinion consequently healthy, as of great importance. However, as has been discussed earlier in this chapter, the 'elimination' of naturally associated parasites may prove to have unintended, adverse consequences in the long run. As indicated in Chapter 6, commercially sourced chicken meat constitutes a big part of the captive leopard diet, exceeding red meat. It has been reported that commercial chicken meat in South Africa is contaminated with OCPs such as chlordanes and DDTs (Quinn et al. 2011a, 2011b, Thompson et al. 2017a, 2017b) and may therefore be a contributing factor to increased OCP exposure among captive leopards in South Africa (see Chapter 6).

In this regard, the following potential mitigating management protocols are recommended. It is reasonable to consider the expense of keeping these animals in captivity and that they are subsequently considered property and a means of income. Therefore, having them get sick of a vector-borne pathogen is far from ideal and regular parasite treatments should be the norm. Keeping in mind that diet is a main contributing factor to OCP exposure (see Chapter 6), the author would like to suggest that ex-situ managers need to rethink the content of chicken in their leopards' diet. Organically sourced chicken meat would be a much better option than those acquired from commercial poultry farms. Additionally, ex-situ facilities should not just accept donated carcasses and other food items from surrounding farms and relay it to their animals, as these may prove to add to OCP burdens in their animals.

Addressing obesity in captive felids is of the utmost importance to their health (see Chapters 1 and 2). Since the majority of OCPs are lipophilic (see for example Polischuk et al. 2002, Tartu et al. 2017), coupled with seemingly regular OCP exposure, obesity can pose a major potential health risk to these cats. Larger, more natural enclosures with ample space for excercising would be ideal, but it is accepted that this type of expansion may not be possible for most ex-situ facilities. Some suggested strategies that can be employed include a balanced and well-monitored feeding regime from birth. Continuous monitoring through regular weighing should be prioritised, and diets should be accordingly adjusted. Regular physical activity and exercise

will also keep captive felids from becoming overweight. The author would like to suggest that attempts should be made to place tourist-focus more on physical activities, such as regular walks and runs on harnesses, than on feeding times, as this might be an economic incentive to adapt enrichment activities.

The ex-situ facilities that participated in this study included one facility that moved location, one facility that completely failed and had to be shut down, and one facility that seemed successful in its endeavours. Since the completion of data sampling at Ex-situ Site1, during March 2021, this facility and all its animals were moved to an area near Bela Bela in the Limpopo Province of South Africa, more than 500 km away. Ex-situ Site2 has been permanently closed since 2020, due to brutally shocking mismanagement that led to the starvation of many of its animals. Most of the surviving animals have been relocated to other facilities across South Africa (Steyn 2020) and as of November 2022, Ex-Situ Site2 still remains closed. Their leopards were relocated to facilities all over the country in Gauteng, the Free State, and Eastern Cape (Citizen Reporter 2020). This exchange of animals between ex-situ facilities is a common occurrence in South Africa, but what should be kept in mind, is that moving these animals across the country moves their issues with them. Linking back to how few of the contacted ex-situ facilities were willing to participate in this study, one cannot help but wonder what they are afraid of exposing to the public.

Big cat conservationists are still divided on the value of captive breeding and rewilding, as discussed by Hunter et al. (2012), and aligned with the findings of FourPaws (2022) (see Chapter 1). The current study found facilities to be quite opposed to being associated with research-related activities. The present study highlights that transparency in terms of big cat conservation management seems to be an issue among ex-situ facilities in South Africa. With this being mentioned, it is important to keep in mind that some global big cat rewilding projects seem to have at least some degree of success. India has recently had some success with the rewilding of Bengal tigers in some specially established conservation areas (Sarkar et al. 2016, Kolipaka et al. 2017). The ongoing plan for Amur leopard rewilding has been in place since 2009 (Christie 2009), but more than a decade later, is still not established as shown by Lewis et al. (2020)'s analysis of potential health risks of reintroduction. Aligning with the report by Hunter et al. (2012) on the actual role of big cat captive breeding, the current study also uncovered that promised aims of releasing captive-bred cats into the wild, is still only an aim, even though this study has been completed a decade later. Better care needs to be taken of South Africa's captive leopards if they are to add any conservation value in the future.

7.6 Concluding remarks and future work

Although the African leopard is an iconic animal among researchers and laymen alike, several avenues of its biology are, as of yet, still left unexplored. This study serves as an initial filling of these gaps in knowledge, as it adds to the growing pool of knowledge on the inner workings of big cat health, an avenue still largely left unexplored by researchers. Although this study did not analyse a large number of leopards, it adds valuable baseline information to available literature and emphasizes the need for proper regulation of ex-situ facilities in South Africa and highlights the value of resampling live animals. This would not only entail government-designed management strategies, which are already in place, but has to place focus on implementing these strategies across the board.

This study validated ecotoxicology, haematology, haemoparasites and concurring physiological factors as integrated parameters to assess the health of leopards in South Africa, and reported on these factors in Chapters 3 to 7. The overall hypothesis as stated in Chapter 1, that wild leopards are in better overall health than captive leopards, was confirmed. This makes sense as these cats are still inhabiting the environment and are still associated with the majority of environmental factors and parasites that they co-evolved with.

The data from this study support the hypothesis that haematological characteristics of captive and wild leopards will differ from each other, as well as from other felids (Chapter 3). This chapter presented detailed haematological characteristics and comparisons with existing literature and the results presented add to current knowledge of blood cell morphology and properties, and provides ranges of differential counts for different blood elements. This report is the first of its kind on African leopards from captive and wild populations.

It was hypothesized that all leopards will be infected by at least one species of intraleukocytic parasite and prevalence and parasitaemia will be higher among wild leopards. The data presented in Chapter 4, which presented two relatively new *Hepatozoon* species coinfecting the same leopard host, support this hypothesis. This is the first report of its kind concerning large felids. In addition, this chapter introduces valuable criteria, such as parasite and host cell morphology, to be considered when describing hepatozoan infections from wild carnivores, and valuable results obtained from live animals in the wild are provided.

Furthermore, it was hypothesized that higher infestations of haematophagous arthropods will be encountered on wild leopards, and possible developmental stages of intraleukocytic parasites may be found in engorged ticks collected from infected leopards. In Chapter 5, light was shed on a possible tick vector in which almost all developmental and infective stages of a *Hepatozoon* species were observed and these data support the hypoheses. This section of work showed the value of visually inspecting potential tick vectors for life cycle stages. Reports on life cycle stages from wild ticks collected from wild animals are quite rare, which is where the present study adds value to existing knowledge. This is the first description of the

morphological characteristics of different developmental stages of a big cat associated *Hepatozoon* species in both its naturally infected potential tick vector and African leopard host.

This study also utilised ecotoxicology techniques as a health assessment tool, which is a first for big cats in Africa. The hypothesis was that the serum concentrations of OCPs will be higher in the blood of ex-situ leopards than in in-situ leopards. Although Chapter 6 presented the first report on OCP concentrations from leopards in Africa showing that ex-situ leopards had higher OCP concentrations, it was not accompanied by concomitant compromised health symptoms. Thus the hypothesis is not supported by data. Chapters 6 and 7 highlighted the need for this parameter to be considered in terms of the conservation management of healthy populations. This report is the first of its kind on a large African carnivore species.

It was also hypothesized that all ex-situ facilities will manage their leopards in a different manner and that some facilities will fare better at managing these animals than others. The data and arguments presented in the current chapter support this hypothesis, where ex-situ management of the leopards from this study was described and analysed. This chapter also gave detailed baseline information on the health status of captive and wild leopards in South Africa by an integrated analysis of selected health parameters. Lastly, this chapter confirmed that different leopard populations are diversely affected by haematological characteristics, OCPs, haemoparasites and associated clinical factors, thus validating these variables as informative health parameters of leopards in South Africa.

Currently, there is a lack of health-related studies on large carnivores in Africa, which needs to be addressed. This thesis has shown the value of resampling leopard individuals in a health assessment and thus suggests that regular resampling of these cats, especially in the wild, should become an important focus in upcoming health monitoring projects. Future research should widen the scope of this type of study to other large carnivores, to see whether this model may help to show their health status and the parameters involved. Additionally, it would be ideal to broaden this research on leopards to other ex-situ facilities and leopard-inhabited areas of South Africa and to resample more individuals, ideally including wild leopards. There is also a need for in-depth studies on wild leopard-vector-haemoparasite descriptions as the definitive answer as to which ticks may act as vectors for feline hepatozoonosis remains relatively unanswered for big cats in southern Africa. The issue about the *Hepatozoon felis* species complex has been one of the results of this study, implying that several infections of feline hepatozoans may be misidentified. It is suggested that future mammalian haemogregarine research should incorporate morphological data in addition to the currently popular molecular analyses. The author also suggests that these types of studies should be expanded to other large carnivores in southern Africa.

The following questions arose after the final integrated multivariate analysis in Chapter 7 was completed:

- Is it possible that captive leopards have heavy OCP burdens partly due to the absence of haemoparasites that may result in poor immune system development in a host that has been born in captivity and never had been exposed to its natural parasites?
- Could it be important, as so many papers suggest, to stop eliminating these parasites from their leopard hosts to relieve immunosuppression that may increase their susceptibility to diseases?

These questions will need to be addressed with the help of future research.

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Acknowledgements

The first words of thanks have to go to my supervisors at NWU, Professors Nico Smit, Victor Wepener and Courtney Cook. I am incredibly thankful for the roles you played in this project and the journey we undertook together. You taught me so much. Thank you for letting me develop my research as time went on and for encouraging and helping to shape my ideas. Prof Nico, thank you so much for all the wonderful opportunities you offered, the funding, and your guidance and mentorship. Thank you for giving me the chance to do this project that involved all my passions. You enabled me to present my work at my first-ever overseas conferences and it was such amazing experiences. Thank you for your inspiration, support, patience and boosting my morale every time things got difficult. I have learnt a lot from you and will be ever grateful. Prof Victor, thank you for your mentorship and guidance in the field of ecotoxicology, which was completely new to me. Thank you for your patience and detailed inputs, it is much appreciated. Prof Courtney, we have known each other since you were yourself a PhD student. You have always been an inspiration to me and I appreciate all our talks and time spent working together. Thank you for your mentorship and guidance in this project.

What a privilege it was to be a part of the NWU Water Research group during this study. I am grateful for the opportunity to complete this study and to have been part of such an academically outstanding group of interesting people. Thank you to Dr. Ed Netherlands and Dr. Nico Wolmarans, who supported me for long hours in the lab and gave me valuable tips and tricks. To Adri Joubert, thanks for all your help behind the scenes, with all those forms and admin and arrangements. Thank you for your encouragement and willing ear, which was often needed. Thank you to all the ex-situ facilities that ended up being a part of this study, and the associated veterinarians that assisted in data collection in the field. A special word of thanks to Mr. Gerrie Camacho, who donated valuable samples, encouraged me and gave valuable inputs to the ideas and was an integral part of data sampling for this study. My appreciation to my colleagues at the University of the Free State's Department of Zoology and Entomology on the Qwaqwa and Bloemfontein campuses for giving me the time, support and encouraging me to complete my studies.

I am especially grateful to my family – I appreciate you. Dearest dad and mom, Matie and Erika, after such a long time it is time for me to say thank you. You have instilled in me a love of nature since I was a very small child. I am so grateful that you were there with me on this journey every step of the way. You have inspired me throughout my life to be the best I can be, thank you for helping me to shape my life with passion and endurance. Your unconditional love, encouragement and support carried me through this journey. I appreciated every call, every text message, and every little sign of your caring. All the sacrifices you've made for me over the years have not gone unnoticed, and I hope to have made you proud. I am truly blessed and privileged to have you as my parents. Thank you for always believing in me and never giving up on me. To my husband, Johann, I am so grateful to have you in my life. You have endured this

journey with me every step of the way and you have helped to shape me as a researcher throughout my academic career. The field trips with you as my assistant, the lab work, the academic discussions at home, your encouragement – I can not emphasize enough that your support meant the world to me. Your pep talks and encouragement carried me through all the times of despair along the road. Together we have created very special memories, from running around in the dark after darted leopards to dissecting stinky ticks in the lab and spending hours behind microscopes. Thank you for always having my back and being my biggest supporter. And thank you for bearing the last-days-of-writing-up emotions and moods, I know it could not have always been easy. To my brother Dewald. Your belief in me has always driven me to push myself further. Thank you for being such a great supporter. To Prof Liesl van As. You and Prof Jo have been an integral part of my academic, and later private life. It was so exciting to have you with me during my first sampling session! Thank you for your life lessons on the stairs and academic support and encouragement throughout this study.

Thank You Lord for your guidance and the strength You've given me to complete and overcome the obstacles on this journey. This study as originated as an internal appreciation and fascination of our natural world and You have taught me countles valuable life lessons along the way. Thank You for taking me places I could only dream of.

"……and the leopard shall lie down with the kid…..and a little child shall lead them" Isaiah 11:6