Apicomplexan diversity of selected southern African snakes and terrapins

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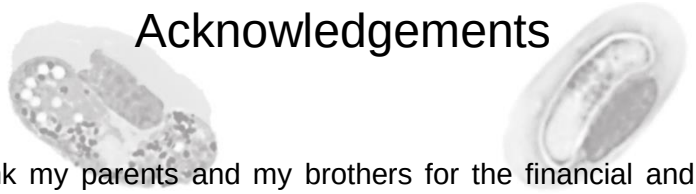

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Abstract

Apicomplexan diversity of selected southern African snakes and terrapins

Key words: Biodiversity, Chelonians, Conservation, Distribution Modelling, Genetics, Haemogregarines, Haemoparasite, Haemoproteids, Morphology, Ophidia, PCR

Southern Africa has the highest diversity of reptile fauna in Africa. Despite estimates that diversity of reptile associated haematozoans are higher compared to mammals and birds, there is a lack of research regarding Apicomplexans infecting reptiles. The overall aim of this research was to assess the diversity of apicomplexan haematozoans in a subset of snakes and terrapins of South Africa, whilst demonstrating the potential limiting effects that fragmentary studies, relating to the topic, has on progressing research. In order to achieve this aim with a sampling pool encompassing captive and wild snakes, as well as terrapins, different factors had to be taken into account that can influence the diversity of apicomplexan haematozoans infecting these hosts. Therefore, a variety of research tools, including microscopic, molecular and phylogenetic analyses as well as predictive distribution modelling were utilized to assess the diversity of these parasites. A total of 154 snakes of the Limpopo, Mpumalanga and KwaZulu-Natal provinces (82 wild, 62 captive) of five genera and 7 species were sampled and screened for the presence of species of *Hepatozoon* and *Haemocystidium*. *Hepatozoon* spp. were found infecting all of the sampled snake species (*Dendroaspis polylepis*; *Dendroaspis angusticeps*; *Dispholidus typus*; *Bitis arietans*; *Naja mossambica*; *Naja annulifera* and *Python natalensis natalensis*). Genetic analyses indicated that there were two *Hepatozoon* spp., referred to as *Hepatozoon* sp. A and *Hepatozoon* sp. B. *Hepatozoon* sp. A, comprised four morphotypes, and was found infecting 6/7 (86%) of species of snakes and 32/154 (21%) of individuals collected, while *Hepatozoon* sp. B, comprised one morphotype, and was found infecting four individuals of one snake species, *Di. typus*, as such 1/7 (14%) of snake species and 4/154 (2%) of snakes collected. Of the sampled snake species, only the two sampled cobra species (*N. mossambica* and *N. annulifera*) had positive infections with *Haemocystidium mesnili*, based on phylogenetic analysis, suggesting that this species displays host-specificity towards cobra species. The overall prevalence in *N. mossambica* was 8/28 (28.6%) and for *N. annulifera* the overall prevalence was 2/30 (6.7%). Although phylogenetic analysis indicated that this species is *Haemocystidium mesnili*, the identification of this species cannot be certain as the only available sequence of this species in GenBank is not accompanied by morphological data, even though this data was available for *H. mesnili* at the time the sequence was deposited. It was also discovered that both *Hepatozoon* and *Haemocystidium* infections in captive snakes, which were once wild, became infected with

these parasites in the wild, and that the successful infection of a host by the parasite is likely associated with the ecology of snakes in the wild. Both *Hepatozoon* and *Haemocystidium* were observed in captive snakes that were isolated from vectors and where dietary infection routes were absent, as these snakes were kept indoors in enclosed enclosures, were screened for ectoparasites before they were moved to captivity and were only fed captive bred prey items. These snakes were in captivity for up to three years, suggesting that these parasites can persist in the system of their hosts for a long duration of time. The overall prevalence and parasitaemia for *Hepatozoon* spp. was higher for wild snakes than for captive snakes, with a prevalence of 35/82 (42.7%) and parasitaemia of 0.01–9.3% recorded in wild, as compared to a prevalence of 16/62 (25.8%) and parasitaemia of 0.01–7.7% in captive snakes. There were no clear differences between the prevalence of *Haemocystidium mesnili* in wild and captive snakes, with a prevalence of 6/20 (30%) for wild and 2/8 (25%) for captive *N. mossambica* and 1/20 (5%) for wild and 1/10 (10%) for captive *N. annulifera.* The overall parasitaemia for *Haemocystidium* was higher in wild cobras compared to captive cobras, where in captive it ranged from 0.20 to 1.75% and in wild it ranged from 0.40–19.00%.

Terrapins (*Pelomedusa galeata* and *Pelusios sinuatus*), were infected with *Haemogregarina* spp., where *Pe. galeata* individuals were collected from North-West with a prevalence of 19/20 (95%) and KwaZulu-Natal 6/6 (100%) and *P. sinuatus* individuals were only collected from KwaZulu-Natal with a prevalence of 13/14 (93%). Positive infections in *Pe. galeata* were confirmed based on both microscopy and molecular analyses, whereas positive infections in *P. sinuatus* were only based on microscopy as the amplification process from the samples failed. In order to assess the distribution of *Haemogregarina* spp. infecting terrapins, predictive distribution modelling with the use of available distribution data of *Haemogregarina* spp. infecting southern African terrapins and available distribution data for the leech *Placobdella multistriata* were obtained and utilised in Maxent. The predicted distribution indicated that terrapin associated *Haemogregarina* spp. occur across a wide variety of environmental paramaters, which likely results from the wide distribution of both the vertebrate and invertebrate hosts. Therefore, it is considered that *Haemogregarina* spp. infecting terrapins have a wider distribution than what is displayed by the currently available distribution records, suggesting that the range of the predicted distribution might expand as more distribution records for *Haemogregarina* spp. are made available.

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General Introduction

If you do not know the names of things, the knowledge of them is lost too.

~ Carl Linnaeus

Chapter 1: General Introduction

Southern Africa has the highest diversity of reptile fauna in Africa. The diversity is estimated to exceed 600 species, where lizards account for the largest component, snakes the second, chelonians the third, worm lizards the fourth, and crocodiles the fifth (Branch, 1999; Branch, 2016). Many reptiles that occur in southern Africa display high levels of endemism, with highly restricted distributions. This can be seen in a number of cordylid lizards, chameleons, geckos, fossorial skinks, and lacertid lizards, as well as some snake species (Branch, 1999). Southern Africa is home to an estimated 168 snake species, of which most are harmless (Branch, 2016) and less than 10 percent have medically significant venom (Alexander & Marais, 2007; Marais, 2004). There are nine semi-aquatic terrapin species that occur in southern Africa (Boycott & Bourquin, 2000; Broadley & Boycott, 2008; Broadley, 1981), of which none of the species are endemic (Bates *et al*., 2014). The snakes and terrapins of southern Africa inhabit a wide variety of habitats, including Namib desert, fynbos, afromontane forests, lowland forest savanna, karoo and grassland (Bates *et al*., 2014; Branch, 2016).

The ecological habits of southern African snakes range from fossorial blind snakes and semi-fossorial slug eaters that feed on invertebrates to mostly terrestrial large pythons that are capable of overpowering antelope, to arboreal snakes that have a varied diet, but are restricted to arboreal prey species (Alexander & Marais 2007; Branch, 2016). Terrapins feed on a wide variety of invertebrates, fish and amphibians and some species occasionally also eat forms of vegetation (Boycott & Bourquin, 2000; Broadley, 1981; Broadley & Boycott, 2008).

Both snake and terrapin species inhabit environments where contact between reptiles and potential ectoparasitic haematophagous invertebrates are inevitable (Alexander & Marais 2007; Bates *et al*., 2014; Branch, 2016; Boycott & Bourquin 2000; Broadley, 1981; Broadley & Boycott, 2008). These haematophagous ectoparasites can be, and in some cases have been, implicated in the life cycles and the transmission of protist parasites such as species of *Trypanosoma* (which is not a focus in this study) and apicomplexan haematozoans such as species of *Haemogregarina*.

It is estimated that the diversity of reptile associated haematozoans is higher than that compared to mammals and birds, this due to their more restricted habitat ranges and lower mobility compared to that of mammals and birds (Javanbakht *et al*., 2015; Telford, 2007).

Studies suggested that the evolution of many apicomplexan haematozoans are more closely associated with the haematophagous invertebrates than they are with their vertebrate hosts,

in this case reptiles. This has been suggested for numerous apicomplexan genera that are observed in the peripheral blood of reptiles, mainly based on low host-specificity towards the reptile hosts (Barta *et al*., 2012; Cook *et al*., 2010; Dvořáková *et al.*, 2014; Gupta *et al.,* 2011; Javanbakht *et al.,* 2015; Pineda-Catalan *et al*., 2013; Telford *et al*., 2004; Sloboda *et al*., 2007; Tomé *et al*., 2012; Tomé *et al*., 2014). Yet, research is lacking to resolve the identity of potential vectors, modes, and routes of transmission, and host-specificity of these parasites towards their vertebrate hosts. Furthermore, there is also little research that approaches these parasites from an ecological perspective, for instance their role in functional ecosystems. Advances in research regarding apicomplexan haematozoans of reptiles seem to still be in the 'discovery phase', entailing the discovery and the description of species based on morphological and molecular analyses (Cook *et al*., 2010; Cook *et al.,* 2018; Dvořáková *et al*., 2014; Van As *et al*., 2013, 2015; Van As *et al*., 2016).

Morphological analyses involves the description of life stages observed in the peripheral blood of reptiles and when possible, the identification of vectors and the description of sexual life stages from these vectors, leading to potential elucidation of life cycles of these parasites. The employment of molecular analyses was anticipated to relieve a great deal of taxonomical issues regarding apicomplexan haematozoans. However, this only led to challenges at a higher phylogenetic level and species level (Kvičerová *et al*., 2014; Karadjian *et al*., 2015). In the case of apicomplexan parasites, the taxonomy, ideally, should infer phylogenetic relationships through the specific course of development in the invertebrate and then vertebrate host, and the phylogenetic findings should support the taxonomy. At presen,t this is not the case with reptile associated apicomplexan haematozoans. It is anticipated that more knowledge on these apicomplexans of reptiles, that is the diversity and ecological factors, might need to be taken into more serious consideration, particularly from an earlier stage of discovery. This may in turn assist in the resolution of the true diversity of these parasites, as ecological factors such as diet seem to have an impact on the distribution of these parasites (Tomé *et al*., 2021; Tomé *et al*., 2014).

A large component of the reported and described diversity of apicomplexan haematozoans are based on morphological descriptions of vertebrate blood stages alone. The practice of describing species based solely on morphology continued up until less than a decade ago (Cook *et al*., 2010; Van As *et al*., 2013, 2015). Even though this is not common practise today, many of these past species descriptions remain, and have not been reassessed and tested with molecular methods. These solely morphological descriptions and species accounts are concerning. This particularly so, as phenotypic characteristics of these parasites are not necessarily reflected in their genetic composition or vice versa, meaning that parasites that appear morphologically dissimilar can potentially be haplotypes of the same species and those

that appear similar can potentially be different species displaying a high degree of genetic divergence (Perkins, 2000; Perkins *et al*., 2011; Smith, 1996).

The phylum Apicomplexa is the least known of all parasite groups and the existing phylogenetic framework is unstable (Morrison, 2009). A stable taxonomy is necessary to be able to determine the phylogenetic placements of potentially new species and existing species to allow accurate interpretation regarding their genetic diversity and phylogenetic relationships (Morrison, 2009). A concern regarding the phylogeny of apicomplexan parasites is that the existing phylogeny can potentially change as new phylogenetic information is presented (Del Campo *et al*., 2019; Morrisson, 2009; Tomé *et al*., 2014). This is problematic since taxonomy should ideally serve the purpose of reflecting phylogeny which is easily understandable and is not subject to change as new evidence is presented (Morrison *et al.,* 2009). The phylogenies of apicomplexans are mainly and largely based on the 18S rRNA gene, which is considered substandard as it is proposed that phylogenetic relationships should be supported with the use of more molecular markers (Barta, 1997; Cook *et al*., 2014; Maia *et al*., 2011; Morrisson, 2009; Telford *et al*., 2004; Úngari *et al*., 2018; Tomé *et al*., 2014). The diversity of apicomplexan species that are not of veterinary or medical importance are neglected, frequently as a result of lack of funding for research (Morrison, 2009).

In general, there is a lack of sampling and research regarding reptile associated apicomplexan parasites, although some of these reptile-associated parasites are of veterinary and medical importance. An example of such a parasite is the ubiquitous reptile pathogen, *Cryptosporidium parvum* Tyzzer, 1907, which has a wide distribution, is zoonotic, and causes diarrhoea in humans (Mendoza-Roldan *et al.,* 2020). The lack of sampling is believed to be associated with human perceptions towards reptiles in general and that they display a lower aesthetic value than many other vertebrate groups (Alves *et al*., 2008; Alves *et al.,* 2009; Ceríaco, 2012).

The current stance of knowledge about the diversity of apicomplexan haematozoans infecting reptiles is sparse, specifically information regarding the number of species that exist and their genetic diversity. It is estimated that less than one percent of all Apicomplexan parasites have been described (Adl *et al.,* 2007; Morrison, 2009; Šlapeta & Morin-Adeline, 2011). This in turn hinders research that aims to address larger questions regarding the distribution and ecology of these parasites as there is currently not a stable foundation for data interpretation or enough available data to incorporate into studies regarding ecology and broad scale distribution (Del Campo *et al*., 2019; Tomé *et al*., 2021; Tomé *et al*., 2014). The international trade of reptiles is expanding and there is an increase in demand for pet reptiles (Kopecký *et al*., 2019; Mattioli *et al*., 2006). Studies have shown that captive reptiles harbour apicomplexan heamatozoans (de Vieira Santos *et al.,* 2005; O'Dwyer *et al*., 2004; Glaser *et al*., 2008; Úngari *et al.,* 2018a;

Úngari *et al.,* 2018b) and in some cases it is uncertain whether these parasites originated from the wild or were obtained in captivity (De Biasi *et al*., 1989; Úngari *et al.,* 2018a). It has also been found that wild snakes in urban environments harbour apicomplexan haematozoans (Davis *et al*., 2012). There are concerns that the translocation of reptile-associated haematozoans, resulting from the international trade in reptiles, might have unknown effects on naïve native fauna, particularly as the spread and establishment of pathogens and their vectors has occurred in the past (Halla *et al*., 2011; Harrus & Baneth, 2005; Karesh *et al*., 2005). It is, however, difficult to assess the potential impacts as the species identity of the parasites and the host ranges of both the vertebrate and invertebrate hosts are frequently unknown (Halla *et al*., 2011). The possibility of various transmission routes and the fact that some apicomplexan haematozoans display a low host-specificity towards reptiles as vertebrate hosts (Telford *et al*., 2004, 2008; Sloboda *et al*., 2007; Tomé *et al*., 2014) further complicates studies that aim to assess the ecology and true distribution of these parasites.

The overall aim of this research is to assess the diversity of apicomplexan haematozoans in a subset of snakes and terrapins of South Africa, whilst demonstrating the potential limiting effects that fragmentary studies, relating to the topic, has on progressing research.

1.1 Aims of the study

The aims of this study are to assess:

i. the diversity of apicomplexan haematozoans infecting captive and wild South African snakes with the use of molecular and microscopic analyses;
ii. the potential influence of snake host ecology and diet on the diversity of apicomplexan haematozoans, identifying potential primary hosts;
iii. the possible longevity of apicomplexan haematozoans in wild-caught snakes in captivity;
iv. the infection status of terrapins from the North-West and KwaZulu-Natal provinces for *Haemogregarina* spp.;
v. the distribution of southern African terrapins and their associated *Haemogregarina* spp. and *Placobdella multistriata* leech vectors.

1.2 Objectives of the study

The following objectives were formulated in order to achieve the above mentioned aims:

i. to collect blood from captive (short- to long-term) and wild snakes of several species from southern Africa for microscopic and molecular analyses;
ii. to obtain data on the localities where captive (but once wild) snakes were originally collected, as well as their captive husbandry and diet, and the period spent in captivity;
iii. to utilize BioBank samples in the form of blood spots collected from southern African snakes with the use FTA cards for molecular analyses, as well as blood smears for microscopic analyses;
iv. to collect blood from terrapins from the North-West province, South Africa, for microscopic and molecular analyses;
v. to utilize blood samples collected from terrapins in the KwaZulu-Natal province, South Africa, for microscopic and molecular analyses;
vi. to obtain the distribution of *Haemogregarina* species from field data of previous studies for prediction modelling with the use of maximum entropy modelling;
vii. To utilize georeferenced distribution data of the *Haemogregarina* leech vectors *Placobdella multistriata* for predictive modelling with the use of maximum entropy modelling.

1.3 Dissertation outline:

The dissertation consists out of the general introduction (**Chapter 1**), which provides brief background information about reptile diversity in southern Africa, as well as drawbacks affecting the diversity assessments of apicomplexan haematozoans of reptiles, along with the overall aims and objectives of the study. The literature review (**Chapter 2**) provides general information regarding reptile and parasite conservation. This chapter also provides general information about apicomplexan haematozoans recorded from southern African snake and terrapin species. **Chapter 3** assesses the *Hepatozoon* prevalence, parasitaemia, and diversity in captive and wild snakes by providing morphotype and genotype data, while taking ecological behaviours and dietary habits of the different snake hosts into consideration. **Chapter 4** assesses the prevalence, parasitaemia and diversity of haemoproteids in captive and wild cobras by providing morphological and molecular data, while also taking ecological factors into account. **Chapter 5** shows the known distribution of southern African terrapins, terrapin associated *Haemogregarina* spp., as well as the leech vector *Placobdella multistriata*. Additionally, Chapter 5 also provides the predicted distribution of *Haemogregarina* spp. and *P. multistriata* based on available distribution data. Morphological and molecular results for terrapin associated *Haemogregarina* spp., obtained from the North-West and KwaZulu-Natal provinces, are also shown in Chapter 5. **Chapter 6** encompasses a general discussion and conclusion, summarizing the findings of the aforementioned chapters, emphasising findings that were repeatedly observed in the literature of the different apicomplexan genera extracted from different hosts as well as recommendations for future research. A reference list according to the NWU Harvard style is provided and appendices containing supplementary information. **Appendix A** contains supplementary information of Chapter 3 while **Appendix B** and **Appendix C** contains supplementary information of Chapter 4 and Chapter 5 respectively. Additionally, **Appendix D** contains the permits applicable to this study.

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Chapter 2

Literature Review

Human values and folklore strongly influence the effectiveness of conservation efforts

~ Ceriaco (2012)

Chapter 2: Literature Review

2.1 Reptile conservation

Reptiles are considered an ethnozoological entity since humans and reptiles have interacted with each other throughout history whenever they occur in the same location (Ceríaco, 2012). Over time, these interactions became associated with folklore. The personal experience with reptiles, culture and environment of humans determine how interactions with reptiles are interpreted (Ceríaco, 2012). Studies revealed that mythical beliefs, folklore and false medicinal values are often associated with reptiles in many cultures (Alves *et al.,* 2009; Alves *et al.,* 2008; Alves & Souto, 2011; Ceríaco, 2012; Stanley, 2008). Unexpected encounters between non-enthusiasts and reptiles, especially snakes are rarely told over as pleasant experiences but rather as a plight that had to be endured. Reptiles are severely misunderstood and many people are undereducated about reptiles in general. In many cases this leads to reptiles being described and perceived as “evil and dangerous” (Ceríaco, 2012).

In South Africa, chameleons are believed to represent ancestors in Zulu and Xhosa communities and are respected animals, while geckos are believed to be associated with witchcraft and are consequently killed on sight (Simelane & Kerley, 1997). Snakes in particular, despite the fact that most southern African species are harmless, are exceedingly misunderstood and this often costs them their lives (Kontsiotis *et al*., 2022; Simelane & Kerley, 1997; Stanley, 2008; Vaughn *et al*., 2021). In modern societies, perceptions like these are often in the subconscious mind and are not necessarily a result of beliefs or religion, but rather because the demeanour and conduct of reptiles are misunderstood. The falsehood behind these fears and beliefs can only be explained and resolved if the people who hold it are willing to explore the educational field of reptiles and formulate new opinions when presented with new information. Despite the lack of factual knowledge about reptiles among human geographies (Ceríaco, 2012; Simelane & Kerley, 1997), their significance in natural ecosystems are incalculable.

Many hobbyists, enthusiasts, and researchers are awed by reptiles. Reptiles are recognised as integral parts of natural ecosystems as they are remarkable both ecologically and evolutionarily (Böhm *et al.,* 2013; Kumar *et al.,* 2014; Pincheira-Donoso *et al.,* 2013). They are highly adapted for life in temperate, tropical, and desert environments as well as terrestrial, freshwater and marine habitats (Böhm *et al.,* 2013; Etling & Schmidt, 2015). Reptiles fulfil ecological roles as prey and consumers, pollinators, and dispersers (Bower *et al.,* 2019). They often have narrower distributional ranges than other vertebrate groups and therefore they are

more frequently subjected to threat processes (Böhm *et al.,* 2013). When considering their small range and narrow niche requirements, they are regarded as a group of conservation concern (Böhm *et al.,* 2013). Worldwide, reptiles are declining in numbers (Böhm *et al.,* 2013; Gibbons *et al.,* 2000). With 11,690 described reptile species and 2,198 subspecies in November 2021 (Uetz *et al.,* 2021) and the continuous discovery of cryptic species based on molecular evidence, emphasis is placed on the fact that reptiles need to be better represented on the IUCN Red List (Böhm *et al.,* 2013; Gibbons *et al.,* 2000). The culprits responsible for the global reptile decline include: (1) habitat loss, (2) habitat degradation, (3) unsustainable trade, (4) invasive species, (5) pollution, (6) disease, and (7) climate change (Böhm *et al.,* 2013; Gibbons *et al.,* 2000; Kumar *et al.,* 2014). It is suspected that the global reptile decline is taking place on a similar scale than that of amphibians in taxonomic breadth, geographic scope and severity (Gibbons *et al*., 2000) although this has not yet been quantitatively assessed (Böhm *et al.,* 2013).

There is a shortage of fundamental research about reptiles regarding their life-histories, distribution and population sizes (Janovcovác *et al.,* 2019). Additionally, many reptile species are yet to be discovered and described, therefore their biology and ecology still need to be better studied (Ettling & Schmidt, 2015).

2.2 The conservation of reptile associated parasites

Studies revealed that reptiles are less studied than birds and mammals in terms of ecological and behavioural studies (Bonnet *et al.,* 2002). Since the ecological importance of reptiles is often misjudged, it can only be imagined how deeply misunderstood and underappreciated organisms such as parasites living in co-existence with reptiles are. It is possible that some of the most co-threatened parasites are among reptile and amphibian associated parasites (Carlson *et al.,* 2020). The success of reptile conservation is strongly influenced by human perceptions (Crump & Fenolio, 2015). While perceptions strongly influence reptile conservation, it is also applicable to parasites associated with reptiles and those that depend on reptiles at some stage of their life cycle, as it ultimately affects the success of their conservation in the future.

Parasites are frequently dismissed and for the majority, the first impression people have of them is that they cause harm and need to be overcome (Carlson *et al.,* 2020; Gómez & Nichols, 2013). It is true that some parasites act as an impediment to their hosts, but there are many whose co-existence with their hosts do not pose threats by any means. In the biological context, the word “harm” is often used subjectively where hasty conclusions are made about

the presence of organisms, especially in cases where the importance and ecological functions of organisms in ecosystems are poorly understood. A major influence as to why parasites are frequently inconspicuous is because their presence is obscured by the presence of their vertebrate hosts that are more easily observable. It is easily overlooked that almost every vertebrate harbours some sort of parasite, simply because the vertebrate is the more immediate observable presence. A daunting challenge arises, that of conserving both hosts and their parasites, where reptiles as vertebrate hosts are every so often disregarded in comparison to most birds and mammals (Bonnet *et al.,* 2002; Janovcová *et al.,* 2019; Troudet *et al.,* 2017).

2.3 The ecological significance of parasites

Until approximately 30 years ago, parasites were unnoticed and many ecologists considered them to be insignificant both individually and in ecological interactions (Bower *et al.,* 2019; Deviche *et al.,* 2001; Gómez & Nichols, 2013; Poulin, 1999). They are difficult to study because they are not easily observable, they have complicated taxonomy, and for the most part parasites have complex life cycles that involve more than one host (Gómez & Nichols, 2013). Understanding the nature and consequences of the interactions between living organisms is crucial for understanding their ecology and evolution (Brown *et al.,* 2006). Natural selection, which is the consequence of species interactions over evolutionary time, can shape species biodiversity by favouring organisms that are best suited to survive in particular environments (Poulin *et al.,* 2011). In this regard, parasitism has proved itself to be extremely successful and to be a crucial component and determinant of biodiversity, as well as the maintenance thereof (Macrogliese, 2005; Poulin & Morand, 2000). Hosts and their associated parasites usually share evolutionary histories (Krasnov *et al.,* 2016).

Some parasites are considered to impose selective pressures on host species because they have the potential to remove host resources that could have otherwise been used by the host for important life functions such as growth, maintenance and reproduction (Majláthova *et al.,* 2010; Price, 1980). However, the majority of parasitic species are considered harmless to their hosts and pose no zoonotic threats (Carlson *et al.,* 2020). It was discovered that parasites can potentially add an unexplained source of variation in biodiversity data. They play essential roles in both ecology and evolution. Environmental pressures, food web assemblage and biodiversity can be derived from parasite populations and communities in ecosystems (Macrogliese, 2005). Therefore, their significance and role in the functioning of ecosystems cannot be ignored. Some can alter the host's physiology, behaviour, development, ecology,

life-history and reproductive success. As a result, intraspecific interactions such as mate recognition, social competition, foraging and predation can potentially be influenced by parasite infections (Brown *et al.,* 2006). They also play vital roles in natural ecosystems where they shape entire community structures, form part of interactions in population processes and reduce host fitness levels (Hudson *et al.,* 2002, 2006; Pedersen & Fenton, 2007). Parasites also form a crucial element in the natural food web and the flow of biomass in natural ecosystems (Carlson *et al.,* 2020). It is possible for parasite communities to form within host organisms (Poulin, 1999), where these parasite assemblages within hosts are perhaps indicators of the host's position in the food web (Macrogliese, 2005). Ecosystem health can be estimated by determining parasite abundance and diversity, where healthy ecosystems can be associated with high parasite diversity (Hudson *et al.,* 2006). If a specific parasite is present within a host, it can also serve as an indication for the presence of other organisms in the ecosystem that are involved in the complex life cycle of the parasite (Macrogliese, 2005).

Yet, in spite of parasites being extremely diverse and important in all ecosystems, they are poorly prioritised by conservation efforts (Carlson *et al.,* 2020). In order to direct successful conservation strategies for parasites, abundance and distribution data need to be prioritized to allow a better understanding about the evolution of disease and the processes that shape parasite diversification (Carlson *et al.,* 2020). The geographic distribution of parasites at different spatial scales provides insight into the dynamics and evolution of parasite communities, consequently providing prospects to better understand the evolutionary and ecological processes that shape parasite diversification (Maia *et al.,* 2016). The different hosts in which a parasite species occur, affects its distribution and genetic diversity, where the genetic diversity and distribution of parasites are influenced by host spectrums, interactions between hosts, and different parasite traits (Maia *et al.,* 2016).

Several factors influence a parasite's ability to successfully infect a host. Among these factors are: (1) frequency of interaction between the parasite and the host; (2) the host's exposure to the parasite, and (3) the host's ability to resist infection due to specific immune responses (Maia *et al.,* 2016). In cases where parasites have complex life cycles (involving more than one host), it is considered that there are principal and auxiliary hosts. In these cases, differences in parameters such as prevalence (number of infected individuals within sample) and parasitaemia (number of parasites in an individual) can provide insight into host-specificity, where principal hosts tend to demonstrate higher levels of infection parameters (Maia *et al.,* 2016). In order to compare patterns of infection between parasite lineages and host species, both host and parasite factors should be taken into account. The need for conservation efforts are emphasized especially in cases where more than one host is involved and different developmental stages of parasites are dependent on different hosts. Many of the

vectors that are involved in the life cycles of parasites are unknown, which is problematic as they act as host in some stage of the life cycle themselves (Cook *et al.,* 2010; Cook *et al.,* 2016,2018; Van As *et al.,* 2013; Van As *et al.,* 2016). In these cases, parasites face a double threat because they are directly susceptible to anthropogenic factors as well as host co-extinction due to environmental changes (Carlson *et al.,* 2020).

2.4 Reptile associated apicomplexan haemoatozoans

Over the last few years, research has increased into reptile associated haemoparasites and it is now well known that these parasites infect reptiles across the globe (Halla *et al.,* 2014). Reptile associated haemoparasites are dependent on vectors such as haematophagous arthropods and leeches to ensure their development and transmission to suitable reptile hosts (Halla *et al.,* 2014).The vectors of many reptile haemoparasites are poorly understood as very few studies have focussed on determining specific vectors for specific parasite species. General information about vectors responsible for transmitting blood parasites to hosts are available but in many cases the exact species of vectors that are involved are unknown.

Haemoparasites are also frequently found in captive reptiles (de Viera Santos *et al.,* 2005; Glaser *et al.,* 2008; Úngari *et al.,* 2018). However, the risks these parasites pose to captive reptiles and wild reptile populations are unknown (Halla *et al.,* 2014) and research about the topic is lacking. Reptiles are hosts to families of apicomplexans that are of human health significance. These families include Cryptosporidiidae; Sacocystidae, Eimeridae and Haemosporidae (Harris *et al.,* 2012; Mendoza-Roldan *et al.,* 2020 Telford, 2009). Many studies tend to focus on clarifying the taxonomic placements and complete life cycles of these parasites, and the number of factors that need to be taken into account in order to identify the possible health risks associated with these parasites are endless.

The international trade in animals has immense consequences that threaten wildlife and ecosystems across the globe. In this case, it holds true specifically for unscreened reptiles that are shipped internationally, especially reptiles that are often not examined for ectoparasites or endoparasites (Halla *et al.,* 2014). It is these types of activities that allow pathogens and vectors to establish in geographical areas where they do not naturally occur. Many researchers fear that this problem may escalate in the future (Halla *et al.,* 2014)

2.5 Introduction to the phylum Apicomplexa Levine, 1980

2.5.1 The importance of apicomplexan parasites

There are believed to be approximately 5,000 species recognized under the phylum Apicomplexa (González *et al.,* 2019; Morrison, 2009). The phylum Apicomplexa composes a diverse range of obligate intracellular parasitic organisms (Morrison, 2009; Morissette & Sibley, 2002), and it is possible that these parasites evolved from free-living photosynthetic organisms (Gubbels & Duraisingh, 2012).

All of the members of the phylum Apicomplexa are entirely parasitic, a feature that greatly contributes to parasitologists' interest in them (Morrison, 2009). The true biodiversity of Apicomplexan parasites is poorly recorded and it is believed that only approximately 0.1% of the species belonging to this group are described (Adl *et al.,* 2007; Morrison, 2009; Šlapeta & Morin-Adeline, 2011). This emphasises the importance of their identification and the elucidation of their classification in order to assess their biodiversity, as well as their impacts on host organisms. There are several pathogenic genera in the phylum Apicomplexa that are of significance for human and animal health (Levine, 1988). Including parasites of the genus *Plasmodium* Marchiafava and Celli, 1885 which poses significant health risks to people with compromised immune systems; the coccidian genus *Eimeria* Schneider, 1875 and piroplasms such as *Theileria parva* (Koch, 1898) which is transmitted by the tick vector *Rhipicephalus appendiculatus* Neumann, 1901 and causes East Coast fever in cattle, contributing to major economic losses as it causes large scale fatalities among cattle herds (Morissette & Sibley, 2002; Thompson *et al*., 2008). The zoonotic apicomplexan parasite, *Cryptosporidium parvum* Tyzzer, 1907 was isolated from reptiles and in known to have a wide distribution and for causing diarrhoea in humans (Mendoza-Roldan *et al.,* 2020).

There is still much debate regarding the taxonomy of many apicomplexans (Bannister *et al.,* 2000), regardless of the fact that more than 4,000 species belonging to the phylum have been described (Bush *et al.,* 2001). There are several factors that contribute to the challenge of providing indisputable taxonomic schemes for apicomplexan taxa. These factors include the small size of the organisms, lack of fossil records, and their complex life cycles. The lack of differentiating morphological characteristics and the continued use of only one genetic marker, namely the highly conservative 18S rRNA gene, for molecular analysis also contributes to the challenge as it might indicate that organisms are more closely related than they truly are due to the inability of the genetic marker to indicate detailed genetic differences (Barta, 1997; Cook *et al*., 2014; Maia *et al*., 2011; Telford *et al*., 2004;

Úngari *et al*., 2018). It is becoming essential to base descriptions of apicomplexan parasites on both morphological and molecular data (Cook *et al.,* 2016; Tomé *et al.,* 2018). Recent studies revealed that there is a high diversity and abundance of apicomplexans in free living terrestrial environments through the use of high-throughput environmental sequencing surveys (HTESs), implying that the distribution of the ubiquitous apicomplexan group is more underestimated than previous estimates indicated and that currently available distribution knowledge is strictly a reflection of limited sampling, especially for species that are not of veterinary or health significance (Del Campo *et al*., 2019).

2.5.2 Form and function of Apicomplexa

There are several morphological characteristics that are considered to be unique to the members of the phylum Apicomplexa. Apicomplexan parasites have an elongate shape and contain an ultrastructural feature that is diagnostic to the phylum. This structure is known as the apical complex and is evident in the infective life stages of these parasites. It is comprised of the conoid, apical polar ring, micronemes, and the rhoptries, and it plays a role during invasion of the host cells and movement (Adl *et al.,* 2005; Barta *et al.,* 2012; Siddal, 1995; Šlapeta & Morin-Adeline, 2011). The anterior end contains the rhoptries and micronemes, while the posterior end contains the dense granules (Šlapeta & Morin Adeline, 2011).

2.5.2.1 The apical complex

2.5.2.1.1 Rhoptries and micronemes

These structures are involved in motility, the adhesion to host cells, host cell invasion, and also plays a role in generating the parasitophorous vacuole. All these functions are possible due to the secretory functions of the rhoptries and micronemes that secrete unique secretory products (Morrissette & Sibley, 2002).

2.5.2.1.2 Conoid

The conoid can be identified as a small cone-shaped structure. It also contains unspecified spiral filaments. It is considered to have a mechanical function during host cell invasion and is not present in all apicomplexans (Morissette & Sibley, 2002).

2.5.2.1.3 Apical polar ring

This organelle occurs in all apicomplexan members. It is a microtubule-organising centre (MTOCs). Other organelles of the MTOCs are the spindle pole plaques and the centrioles/basal bodies (Morissette & Sibley, 2002).

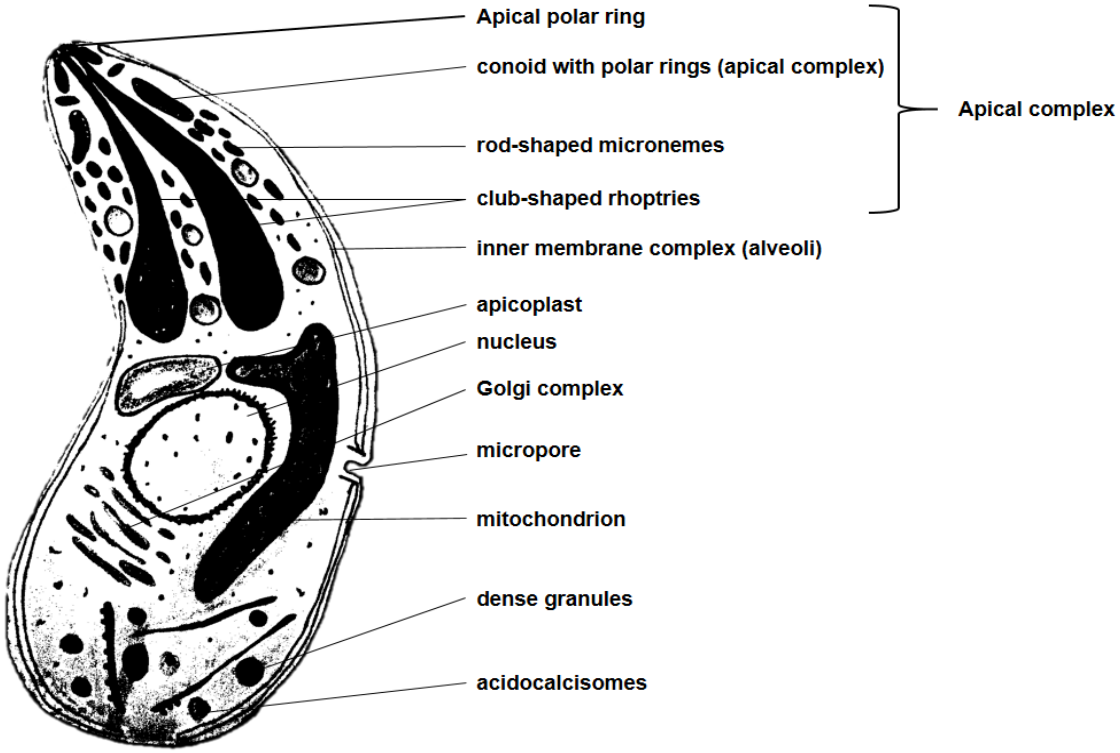


Figure 2.1: Schematic representation of general structures in an apicomplexan infective stage. Redrawn and adapted from Šlapeta & Morin-Adeline (2011).

2.5.3 Mobility and host cell invasion

Many apicomplexan parasites are motile (Morrissette & Sibley, 2002). These parasites employ three types of substrate-dependent gliding motions. These motions firstly include circular gliding, where it glides counter clockwise while lying on its right side. Secondly, upright twirling can be employed where its posterior end attaches to a substrate and clockwise rotations take place. Lastly, horizontal parasites can display helical gliding, which is similar to twirling (Morissette & Sibley, 2002).

2.6 Introduction to Haemogregarines

Haemogregarines are considered to be the most widely recorded and distributed haemoparasites in reptiles (Davis & Sterrett, 2011; Telford, 2009; Telford *et al.,* 2001). They belong to the order Adeleorina and to the family Haemogregarinidae (Alhaboubi *et al.,* 2017). The term haemogregarines in reptiles is a collective term assigned to three genera of adeleid blood parasites in the peripheral blood. These genera are *Hepatozoon* Miller, 1908, *Haemogregarina,* Danilewsky, 1885 and *Karyolysus,* Labbé, 1894 (Brown *et al.,* 2006; Cook *et al.,* 2014; Haklová-Kočíková *et al.,* 2014). They are mostly intraerythrocytic apicomplexans, are present in many environments, and are parasites of both ectothermic and endothermic vertebrates (Cook *et al.,* 2014). They are also known to occur in every order of living reptile and many terrestrial tetrapod orders (Levine, 1988).

2.6.1 Transmission of Haemogregarines

Many of the life cycles of haemogregarines need to be elucidated, but generally their life cycles are heteroxenous (Davies & Johnston, 2000), meaning that multiple hosts are needed to complete their life cycles (Brown *et al.,* 2006; Manwell, 1977; Telford, 2009). They are found in a variety of hosts such as mammals, birds, fishes, snakes, crocodilians, lizards, and chelonians, and are transmitted by a variety of haematophagous vectors such as ticks, mites, leeches, and mosquitoes (Davies & Johnston, 2000; Manwell, 1977; Telford, 2009). Haemogregarines in intermediate vertebrate hosts are mostly described from the gamonts that are observed in the leucocytes and or erythrocytes (Barta *et al.,* 2012; Brown *et al.,* 2006; Smith *et al.,* 2000). The definitive invertebrate hosts of haemogregarines are mostly leeches, acarines and insects, which also act as the vectors (Ball & Oda, 1971; Barta *et al.,* 2012).

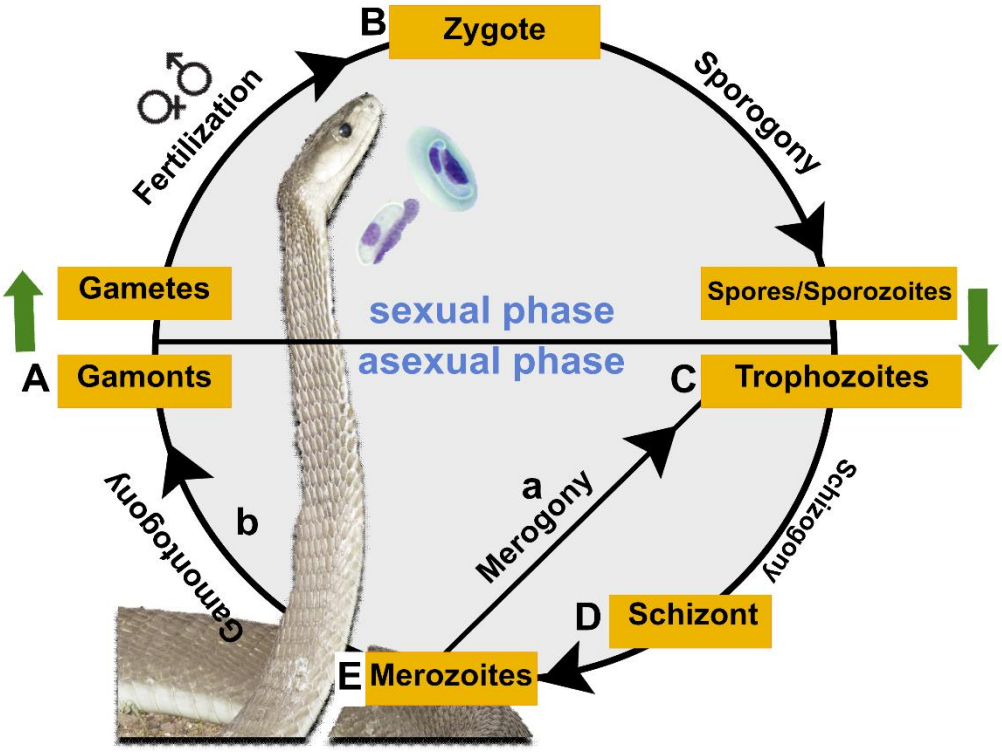
The asexual stages (merogonic and gamont development) of haemogregarine life cycles take place in the intermediate vertebrate host, whereas the sexual stages (fertilisation and sporogonic development) take place within the definitive invertebrate vector organisms. Asexual phases within hosts include merogony, gametogony, syngamy and sporogony (Davies & Johnston, 2000). However, the different infective forms are difficult to identify due to data often being insufficient for determining complete life cycle stages, vector identification and the parasite morphology within the vector (Davies & Johnston, 2000).

2.6.1.1 General life cycle of apicomplexans

The general life cycle of apicomplexans entails that the development of sexual stages take place in the definitive vector host, while asexual development takes place in the vertebrate host. During a blood meal, the vector ingests gamonts/gametocytes (Fig. 2.2 A). Fertilization takes place in the vector, where the microgamete (male) fertilizes the macrogamete (female), consequently forming a zygote (Fig. 2.2 B). Spores/sporozoites are formed when the zygote undergoes sporogony, the sporozoites are then transferred back to the vertebrate host during a blood meal, whereafter trophozoites are formed (Fig. 2.2 C). Schizonts form after trophozoites undergo schizogony (Fig. 2.2 D), where after merozoites are released by schizonts (Fig. 2.2 E). The merozoites can then follow two possible developmental routes, where they can either undergo merogony (Fig. 2.2 a) or gamotogony to form more gamonts or gametocytes (Fig. 2.2 b) (Cook, 2008).

2.6.2 Identification of Haemogregarines

Both morphological and molecular descriptions are utilized for the description of haemogregarines in order to differentiate diagnoses and taxonomy. The 18S rRNA gene has been utilized in characterization, identification, and taxonomic relationship studies among apicomplexan parasites in reptiles (Alhaboubi *et al.,* 2017). Haemogregarines are an ancient and



successful group based on the fact that they present a great taxonomic diversity, there exists

Figure 2.2: General life cycle of apicomplexan parasites. Redrawn and adapted from Cook (2008).

variations in life cycles and hosts and they have cosmopolitan distributions (Perkins & Keller,

2001). Different genera within the Apicomplexa are difficult to distinguish from each other in the peripheral blood (Smith, 1996). Hence, the general term “haemogregarine” is assigned to these parasites when the genus or species are not specified. Microscopic inspections are used to identify species based on morphological characteristics (Smith, 1996), while molecular tools are becoming essential for the sufficient identification of genera and species based on genetic characteristics. Thus far, the phylogenies of apicomplexans are mainly based on the 18S rRNA gene. However, this is considered only a basis for further work as phylogenetic relationships should ideally be supported with the use of more molecular markers to improve parasite identification (Gutiérrez-Liberato *et al*., 2021; Morrisson, 2009). However, at present, the 18S is the most widely used marker, and as such comparisons using other markers is extremely limited until such time as these other markers are available to be as widely used.

2.6.3 Pathogenicity of apicomplexan haematozoans in reptiles

Haemogragarine infections in reptile hosts rarely cause mortality or severe pathogenesis (Bouma *et al.,* 2007; Manwell, 1977). Uncertain effects are observable in reptile hosts that harbour these parasites (Majláthová *et al.,* 2010), thus the fitness and health costs in different host species are difficult to establish, although it is suspected that high levels of parasitaemia cause anaemia and other health defects that affect reptile behaviour and ultimately decreases the survival of the reptilian host (Jacobson, 2007; Knotkova *et al.,* 2005; Pierce & Adlard, 2004). The importance of the effects on host species cannot be considered insignificant, as variations in host susceptibility to disease can influence host community ecology. Studies concerning the impacts of haemogregarines on reptile fitness and overall health reported varying effects. *Hepatozoon* infections in pythons for example are believed to be linked to slower growth rates and low impacts on host fitness (Ujvari *et al.,* 2004; Madsen *et al.,* 2005), while other studies reported that there are no health effects in snakes associated with *Hepatozoon* infections (Brown *et al.,* 2006; Xuereb *et al.,* 2012). It is difficult to determine the clinical significance of haemoparasites in reptiles and infections may be enhanced when parasites with life cycles that include tissue schizonts, such as *Haemogregarina* and *Hepatozoon* if other diseases are also present (Pierce & Adlard, 2004). This may possibly enhance morbidity in hosts (Pierce & Adlard, 2004). Infections are not always reflected by immune system conditions and, overall, there is little evidence that haemogregarine infections hold visible negative health effects for reptiles (Sperry *et al.,* 2009; Wozniak *et al.,* 1996).

2.7 Apicomplexan haematozoans recorded from snakes and terrapins in southern Africa

2.7.1 Genus *Haemogregarina* Danilewsky, 1885

It was estimated that about 300 species belonging to the genus *Haemogregarina* Danilewsky, 1885 have been described from all reptilian orders however, the wide distribution of the genus was considered to be a misinterpretation (Davies & Johnston, 2000). In freshwater chelonians (terrapins), it is confirmed that haemogregarines are transmitted via leeches (Alhaboubi *et al.,* 2017; Davis & Sterrett, 2011; Paperna, 1989; Siddall, 1995). In aquatic vertebrate hosts such as terrapins, infections that are transmitted by leeches take place while leeches feed on the vertebrate host, where sporozoites or merozoites are inoculated into the vertebrate host during feeding (Davies & Johnston, 2000). Additionally, it is also possible that the vertebrate becomes infected when it somehow ingests the invertebrate (Davies & Johnston, 2000). A basic life cycle of *Haemogregarina* is depicted in Fig. 2.3, described from *Haemogregarina balli* Paterson & Desser, 1976 in the Snapping Turtle *Chelydra serpentine* Linnaeus, 1758 as the vertebrate intermediate host and *Placobdella ornata* (Verill, 1872) as the invertebrate and definitive host (Siddall & Desser 1991). Although this life cycle is specifically described for *H. balli*, and many life cycles of these parasites infecting terrapins are not fully elucidated, their life cycles are usually suspected to be highly similar, therefore the purpose is only to schematically represent a simplified *Haemogregarina* life cycle.

Haemogregarines infecting turtles and fish were assigned to the genus *Haemogregarina*, while those infecting snakes, crocodilians and lizards were assigned to the genus *Hepatozoon* (Davies & Johnston *et al.,* 2000). Species belonging to *Haemogregarina* have indirect life cycles. For chelonians, only two fully described life cycles are available. These life cycles were described from *Haemogregarina stepanowi*, Danilewsky 1885 in the European pond turtle, *Emys orbicularis* Linnaeus, 1758 (Danilewsky, 1884; Reichenow, 1910) and *Haemogragarina balli* Paterson and Desser, 1976 in Nearctic snapping turtles, *Chelydra serpentina* Linnaeus, 1758 (Dvořáková *et al.,* 2014; Paterson & Desser, 1976; Siddall & Desser, 1990; Siddall & Desser, 1992).

There are only two described species of the genus *Haemogregarina* that were recorded from *Pelusios sinuatus* Loveridge, 1941 in southern Africa, namely *Haemogregarina pelusiensi* Pienaar, 1962 from *P. sinuatus* in Mozambique, which was also recorded in in Limpopo South Africa (Pienaar, 1962) (Paperna, 1989; Pienaar, 1962) and *Haemogregarina maputensis* Dias, 1950 described from Mozambique (Dias & De Sousa, 1950). Both these species were

recorded based on only morphological descriptions. *Haemogregarina pelusiensi* is the only species with morphological descriptions accompanied by morphometric data.

2.7.1.1 *Haemogregarina* life cycle

Infective stages occur in the proboscis of leeches, which is transmitted to terrapins during feeding (Fig. 2.3 A). Pre-erythrocytic meronts containing merozoites occur in organs such as the liver, lungs, and spleen (Fig. 2.3 B). Immature meronts are elongated in appearance. Merozoites formed in erythrocytic meronts infect other erythrocytes and produce gamonts or more meronts (Fig. 2.3 C). The leeches ingest microgamonts and macrogamonts that occur in the peripheral circulation when feeding on the terrapin (Fig. 2.3 D). Gamonts associate pair up (syzygy) in the intestinal ceca (Fig. 2.3 E), where one microgamete fertilizes the associated macrogamete during microgametogenesis (Fig. 2.3 F). Monosporoblastic oocysts with sporozoites are produced during sporogony (Fig. 2.3 G). The sporozoites migrate to the anterior somites of the leech (Fig. 2.3 H) and gives rise to primary meronts containing hundreds of merozoites. The merozoites then migrate to the proboscis of the leech and is transferred to terrapins during a bloodmeal (Fig. 2.3 I) (Siddall & Desser 1991).

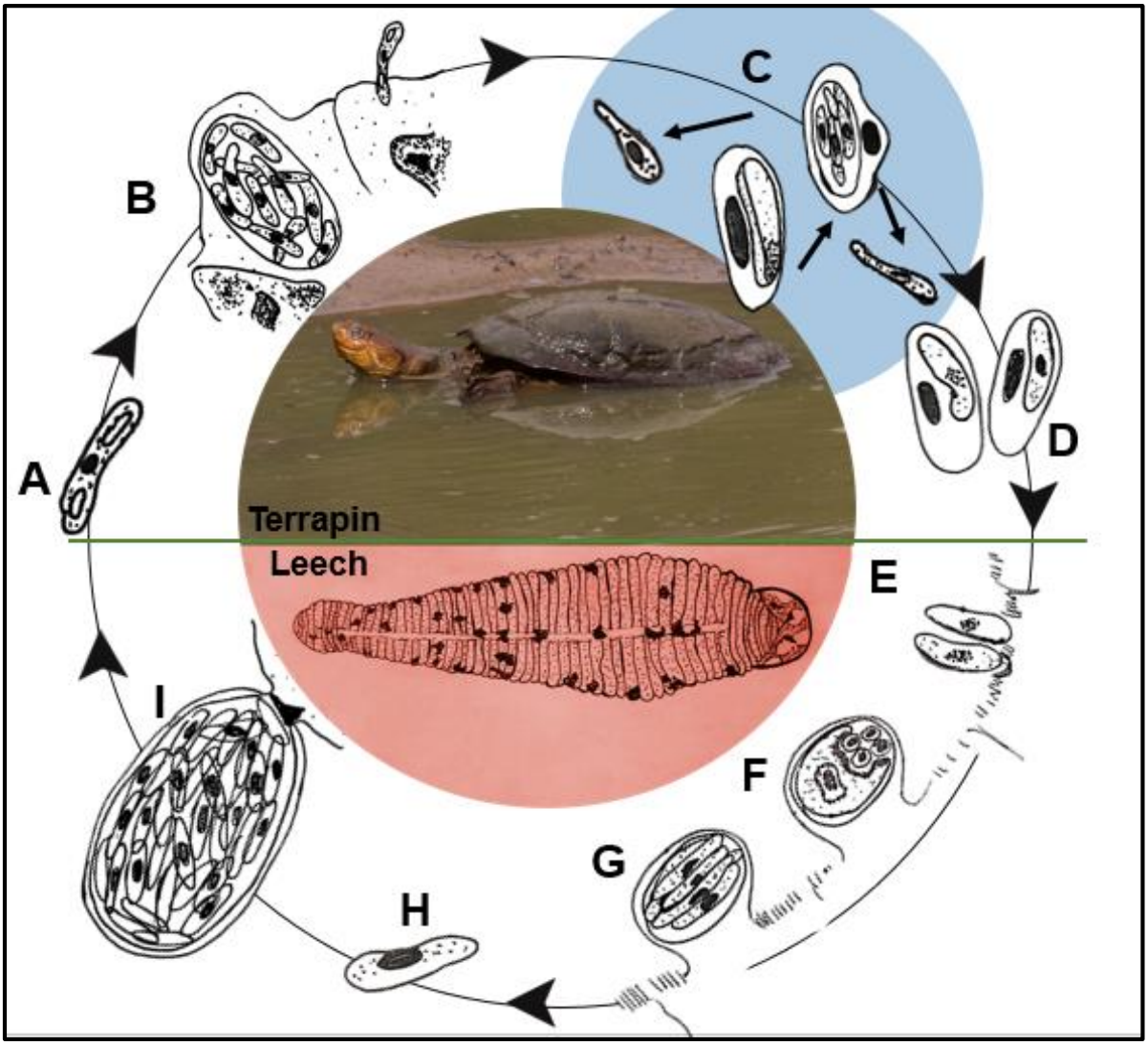


Figure 2.3: Basic life cycle of *Haemogregarina*. Redrawn and adapted from Siddal & Desser (1991), sketches redrawn from Siddal & Desser (1991). Photo (*Pelusios sinuatus*) by Darren Van Eyssen. Leech, *Placobdella multistriata* redrawn from Oosthuizen (1979). The illustrated life cycle was not described from the host species represented by the photo (terrapin) and line drawing (leech).

2.7.2 Genus *Hepatozoon* Miller, 1908

The genus *Hepatozoon* (Apicomplexa, Adeleorina, Hepatozoidae) was first described by Miller, 1908 more than 100 years ago. The genus comprises intraerythrocytic or intraleucocytic parasites that are unicellular and have a wide host range (Cook *et al.,* 2018; Smith, 1996). *Hepatozoon* have a cosmopolitan distribution and infect a wide range of invertebrate and vertebrate hosts (Baneth *et al.,* 2007; Watkins *et al*., 2006; Wozniak & Telford, 1991). In reptiles, *Hepatozoon* spp. are most commonly found in snakes and lizards (Haklová-Kočíková *et al.,* 2014).

There are more than 300 described *Hepatozoon* species, of which less than 50 have been described from mammals (Allen *et al.,* 2011; Smith, 1996). *Hepatozoon* parasites infect terrestrial vertebrates (Levine, 1988; Smith, 1996) such as birds (Merino *et al.,* 2006), mammals (Pawar *et al.,* 2012), reptiles (Maia *et al.,* 2012; Van As *et al.,* 2013, 2015) amphibians (Barta *et al.,* 2012), and possibly fishes (Davies & Johnston, 2000). There has been reports of haemogragarines from the genus *Hepatozoon* across the lizard families Agamidae, Chamaeleonidae, Gekkonidae, Lacertidae, Opluridae, Scincidae, and Varanidae (Van As *et al.,* 2013). There is uncertainty about the exact modes of transmission and the effects the parasites belonging to this genus have on their hosts in terms of health and fitness (Moreira, 2013). Molecular diagnostics aided in more frequently identifying *Hepatozoon* spp. in a wide variety of hosts, which include both wild and domestic animals. New *Hepatozoon* spp. are now frequently recorded as research concerning haemoparasites continues to expand (Desser, 1997; Harris *et al.,* 2014; O'Dwyer *et al.,* 2013; Telford *et al.,* 2001).

Not many species of *Hepatozoon* have been described from reptiles in South Africa. Three species have been described from saurians, one from chelonians, and four from ophidians (Cook *et al.,* 2018). The *Hepatozoon* spp. described from saurians were based solely on morphological descriptions (Van As *et al.,* 2013, 2015). These include *Hepatozoon langi* Van As, Davies and Smit, 2013 and *Hepatozoon vacuolatus* Van As, Davies and Smit, 2013 described from *Pseudocordylus langi*, Loveridge, 1944 (Van as *et al.,* 2013) and *Hepatozoon affluomaloti* Van As, Davies and Smit, 2015 described from *Pseudocordylus melanotus* (Smith, 1838) and *Pseudocordylus subviridis* (Smith, 1838) (Van As *et al.,* 2015). The proposed vector and host for *Hepatozoon affluomaloti* is *Culex lineata* Theobald, 1912 (Van As *et al.,* 2015); however, these conclusions were based only on morphological data of life stages observed in the vertebrate and invertebrate hosts.

Hepatozoon fitzimonsi (Dias, 1953) was initially identified as *Haemogregarina* (*senso stricto*) by Siddall (1995), but was later reassigned to *Hepatozoon* by Cook *et al*. (2014) based on

molecular analyses using the 18S rRNA molecular marker. This species was found in *Kinixis zombensis* Hewitt, 1931, *Kinixis belliana* Gray, 1831, *Stigmochelys pardalis* (Bell, 1828), and *Chersina angulata* (Schweigger, 1812) (Cook *et al.,* 2014). The parasite was additionally found in an unspecified *Kinixis* species (Mofokeng *et al.,* 2021). The life cycles of *Hepatozoon* in tortoises are not fully elucidated, but recent studies presented strong evidence that ticks are involved in the life cycle of *H. fitzsimonsi* (Mofokeng *et al.,* 2021; Omondi *et al.,* 2017).

Furthermore, the descriptions for two of the snakes were based only on morphology; these include *Hepatozoon bitis* (Fantham, 1925) described from *Bitis arietans* Merrem, 1820 and *Hepatozoon refringens* (Sambon et Seligman, 1907) described from *Pseudaspis cana* (Linnaeus, 1758). Additionally, Cook *et al*. (2018) described two *Hepatozoon* species infecting *Philothamnus natalensis natalensis* (Smith, 1848) and *Philothamnus hoplogaster* (Günter, 1863) namely *Hepatozoon cecilhoarei* and *Hepatozoon angeladaviesae* Cook, Netherlands, Van As and Smit, 2018 where the latter also infects *Philothamnus semivariegatus* (Smith, 1840). These *Hepatozoon* species were described based on both morphological and molecular analysis using the 18S rRNA gene. There are currently no identified vectors for *Hepatozoon* sp. infecting snakes in South Africa.

2.7.2.1 Life cycles of *Hepatozoon*

The major processes of *Hepatozoon* life cycles are gametogony, sporogony, and merogony (Leander *et al.,* 2003). In their life cycles, species of *Hepatozoon* are obligate heteroxenous parasites (Allen *et al.,* 2011; Smith, 1996). This means that life cycles alternate between a haematophagous invertebrate host and a vertebrate host where sexual reproduction occurs in the invertebrate and asexual reproduction occurs in the vertebrate host (Smith, 1996). Invertebrate hosts can include ticks, mites, fleas, and mosquitoes (Allen *et al.,* 2011; Majláthová *et al.,* 2010; Smith *et al.,* 1994, 1996; Smith & Desser, 1998; Telford *et al.,* 2001).

There are variations in the life cycles of different *Hepatozoon* species and some species deviate from the proposed general life cycle. One of the major attributes distinguishing this genus from other haemogregerine genera is the formation of polysporocystic oocysts within the invertebrate host (Allen *et al.,* 2011; Desser, 1990; Smith, 1996; Smith *et al.,* 1999). The second vertebrate host becomes infected by predation of the first vertebrate host, where the second vertebrate host is usually a reptile (Sloboda *et al.,* 2007; Smith et al, 1994, 1996; Smith & Desser, 1998) and the first vertebrate host a prey item such as a rodent (Sloboda *et al.,* 2007; Wiger, 1977), an amphibian (Smith *et al.,* 1994, 1996;

Smith & Desser, 1998), or a lizard such as a gecko or lacertid (Landau *et al.,* 1972; Tomé *et al.,* 2012).

There are three possible life cycle strategies that can be followed by *Hepatozoon* species, even though the exact infection process of vertebrate hosts still needs to be elucidated (Smith *et al.,* 1994; Van As *et al.,* 2013). Firstly, the two-host strategy can be followed, where the production of sporozoites (sporogony) takes place within the haematophagous invertebrate host. The first vertebrate host becomes infected with the sporozoites (infective stages) when it is fed on by the invertebrate host (Fig. 2.4 a) (Sloboda *et al.,* 2007; Viana *et al.,* 2012). Secondly, the first vertebrate host can become infected after it ingested an infected invertebrate (Smith, 1996). The sporozoites can follow different routes within the new host, where it can enter lung or liver cells and undergo cyst formation (Fig. 2.4 c and f). Consequently, one of two transmission routes can be followed where the vertebrate host acts as either the intermediate host (merogony and gametogony takes place) or the paratenic host (cyst formation occurs), which acts as the bridge between the invertebrate host and the final intermediate vertebrate host (Fig. 2.4 A). Lastly, in some instances three hosts are involved where another saurophagous lizard host can form part of the life cycle between the paratenic host and the final vertebrate host (usually a snake) (Fig. 2.4 B).

If it happens that the first lizard host is the intermediate host, both cystic development and asexual production takes place simultaneously. Merogony will then occur where sporozoites undergo multiple fission, which leads to the production of meronts (Fig. 2.4 c). When meronts mature, merozoites form, which enter the blood stream (Fig. 2.4 d), consequently infecting vertebrate erythrocytes by using their unique apical complex to penetrate them. Merozoites eventually mature into gamonts (Fig. 2.4 e) which are morphologically similar in all *Hepatozoon* spp. (Herbert *et al.,* 2010; Smith, 1996). There are two possible pathways that the gamonts can follow in order to reach the invertebrate host. Invertebrates can either become infected when feeding on infected lizards (Fig. 2.4 D), or when infected lizards are preyed upon by saurophagous vertebrates, the predatory lizard will become infected and the invertebrates will become infected during a blood meal from the infected saurophagous lizard (Fig. 2.4 C).

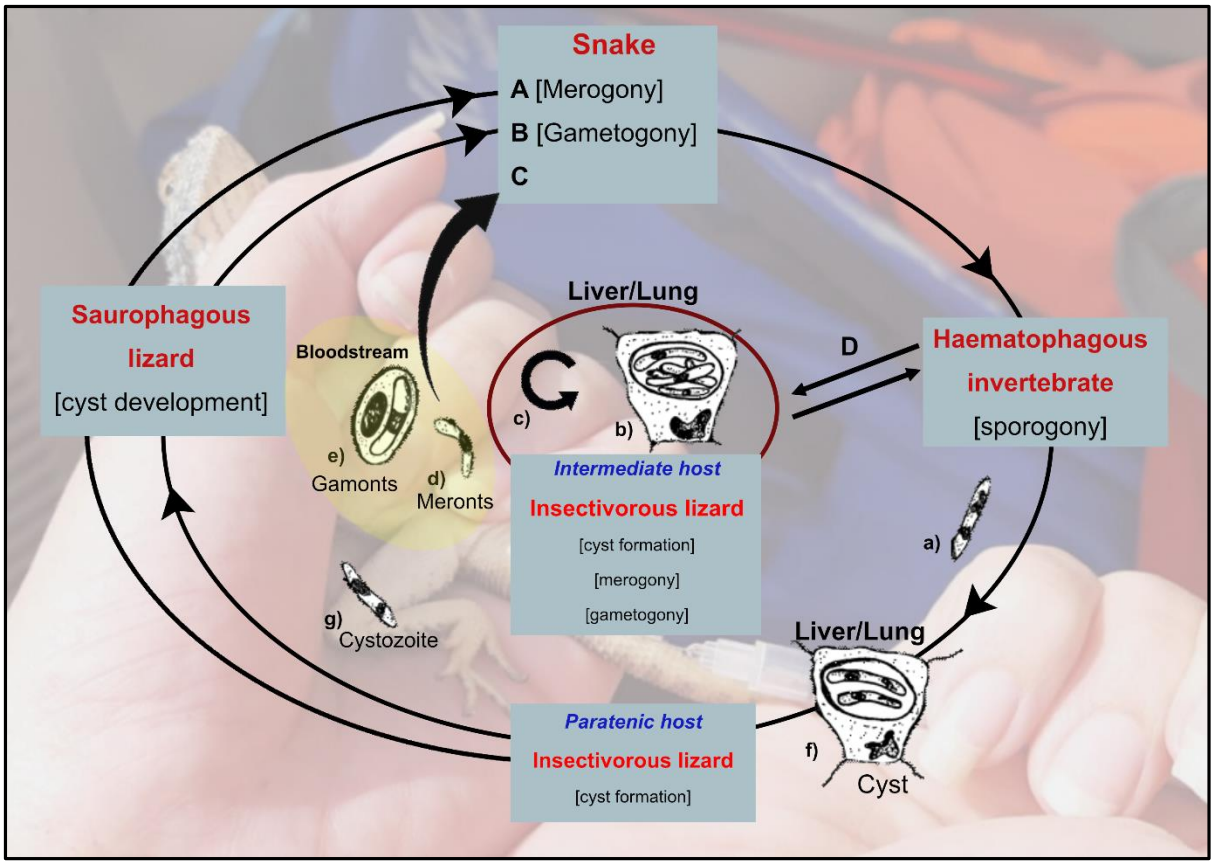


Figure 2.4: Three-host life cycle of *Hepatozoon* spp. in reptiles. Redrawn and adapted from (Moreira *et al*., 2013) sketches redrawn from Smith, 1996.

2.7.3 Genus *Haemoproteus* Kruse, 1980 (Apicomplexa: Haemoproteidae Doflein, 1916)

The taxonomic history of haemoproteid apicomplexan parasites is reasonably chaotic. Many genera were placed in the wrong genus and morphological descriptions were used as the foundation for many species' descriptions. *Haemoproteus* spp. infecting avian hosts receive by far more attention than those infecting reptiles (Telford, 2009; Valkiūnas, 2005). It was recently discovered that *Haemoproteus* species infecting birds and those infecting reptiles form two distinct monophyletic groups based on the cytochrome *b* (*cytb*) gene. Therefore, species infecting reptiles were assigned to *Haemocystidium* Castellani and Willey, 1909 (some formerly identified as *Haemoproteus* spp.) (González *et al.,* 2019; Maia *et al.,* 2016Pineda-Catalan *et al.,* 2013). Additionally, it is suggested that *Haemocystidium* infecting reptiles should further be subdivided into subgenera *Simondia* Garnham, 1966 infecting chelonians and *Haemocystidium* infecting squamata (Pineda-Catalan *et al.,* 2013; Maia *et al.,* 2016).

In southern Africa, three species of *Haemoproteus* have been recorded from tortoises. This includes *Haemoproteus testudinis* (Lavern, 1905) Wenyon, 1915, described from *Stigmochelys pardalis* Bell, 1828; *Haemoproteus balazuci* Dias, 1953 described from *K. belliana* from Mozambique (Cook *et al.,* 2010; Dias, 1953) and *Haemoproteus natalensis* Cook, Smit and Davies, 2010 described from *Kinixys natalensis* Hewitt, 1935 (Cook *et al.,* 2010). In the light of the suggested taxonomical rearrangement, the genus is now referred to as *Haemocystidium* (Simondia) for chelonians. All the species from tortoises were described based only on morphological descriptions and there is currently no available molecular information to assess the phylogenetic placements of these parasites obtained from tortoises in southern Africa.

The only species of *Haemocyctidium* found in snakes from southern Africa, is *Haemocyctidium mesnili* (Bouet 1909) Wenyon 1926 (formerly known as *Haemoproteus mesnili*), which was morphologically described from *Naja nigrocollis* Reinhardt, 1843 in Tanzania (Telford, 2007), but the first molecular information of this species was obtained from the recent molecular analysis of a blood spot that was taken from *Naja annulifera* Peters, 1854 in South Africa, which is not accompanied by morphological data (Pineda-Catalan *et al.,* 2013).

2.7.3.1 Life cycle of *Haemoproteus*

Parasites of the family Haemoproteidae such as *Haemoproteus* of reptiles and birds, do not undergo merogony within the blood and instead only circulate as gametocytes. *Haemoproteus* gametocytes that occur within reptiles and birds also do not produce symptoms associated with the disease malaria (Lainson & Naiff, 1998). Regardless of the differences between Plasmodiidae and Haemoproteidae, haemoproteids share some similarities with members of Plasmodiidae. Consequently, studying the morphology of haemoproteids has shed new light on the nature of true malaria parasites and contributed greatly to their understanding (Lainson & Naiff, 1998). Although it is now suggested that haemoproteids of snakes and chelonians should be regarded as *Haemocystidium*, they were not approached as such initially and life cycle information for reptile *Haemoproteu*s and *Haemocystidium* species is sparse. Therefore, a general description of a life cycle of avian *Haemoproteus* is provided.

Haemoproteus parasites have obligate heteroxenous life cycles and require both a vertebrate and invertebrate (vector) host for the completion of the life cycle (Valkiūnas & Mehlhorn, 2015). The vector organisms are mainly haematophagous dipterans such as Ceratopogonidae Newman, 1834 and Hippoboscidae (Valkiūnas & Mehlhorn, 2015). The sexual phase of the life cycle takes place in the vector, but the many details regarding the complete process is

unknown. Sporozoites are transferred to the vertebrate host when the haematophagous vector feeds on it. Schizonts form, which undergo asexual division in host tissues. Unicellular merozoites (asexual stage) form and spread within the vertebrate host and eventually develop into exoerythrocytic meronts that are mainly found in the lungs, but are also sometimes observed in the liver, spleen, kidneys, heart, skeletal muscle and other organs (Valkiūnas & Mehlhorn, 2015).

In *Haemoproteus* spp., merogony is absent from blood cells. Exoerythrocytic merozoites can then follow two developmental routes, where they either undergo a new cycle of merogony, or penetrate mature erythrocyte where they develop into gametocytes. Gametocytes then undergo gametogenesis, where gametes are produced. Macrogametes are produced by macrogametocytes and microgametes are produced by microgametocytes. The gametocytes are the infective stages for vector organisms with which vector organisms become infected after taking a blood meal. Within the midgut of the vector, gametocytes undergo gametogenesis and the sexual phase of the life cycle commences within the vector.

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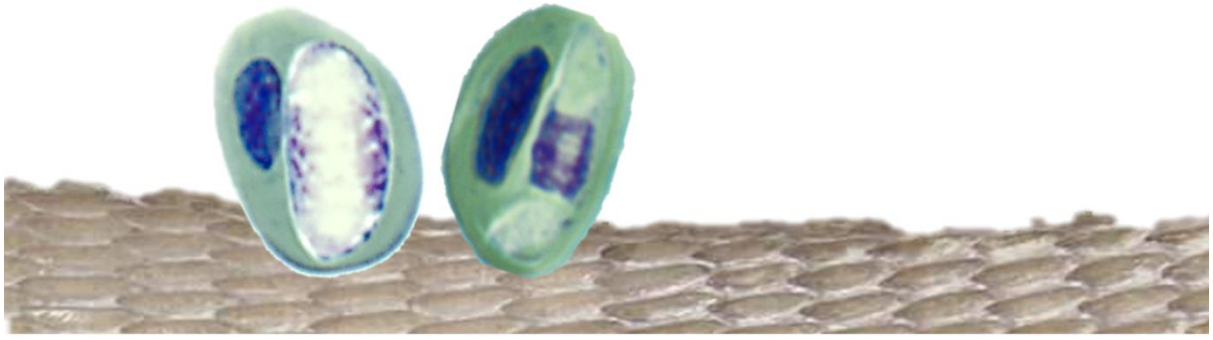
Chapter 3

Hepatozoon diversity in captive and wild snakes of Limpopo, Mpumalanga and KwaZulu-Natal

All truths are easy to understand; the point is to discover them.

~ Galileo Galilei



Chapter 3: *Hepatozoon* diversity in captive and wild snakes of Limpopo, Mpumalanga and KwaZulu-Natal

3.1 Introduction

The phylogenetic relationships of apicomplexans are poorly studied, and the elucidation of their phylogenies are uncertain as they are difficult organisms to work with (Morrison, 2009). Like many other apicomplexan parasites, the identification of the genus *Hepatozoon* Miller, 1908 was primarily based on gamont morphometrics (Sambon & Seligmann, 1909; Úngari *et al*., 2018), which rendered reliable identification difficult as the measurements of different *Hepatozoon* species are frequently very similar (Vilcins *et al*., 2009). The reptilian suborder Serpentes L., 1759 is the second largest group compared to lizards (Uetz & Hošek, 2021). Parasites belonging to the genus *Hepatozoon* are the most commonly recorded apicomplexan parasite in snakes (Jacobson, 2007; Telford, 2009; Tomé & Harris, 2012). More than 300 species of *Hepatozoon* have been identified, with less than 50 from mammals (Allen *et al*., 2011) and more than 120 from snakes (Smith *et al*., 1999). Host-specificity of *Hepatozoon* spp. is poorly understood, especially in regards to ophidian hosts, and despite recurrently similar gamont morphologies, new species were frequently described from new geographical locations based solely on the morphology of these gamont stages (Telford, 1984; Smith, 1996; Vilcins, 2009).

Experimental studies showed that there is a low ophidian host-specificity since *Hepatozoon* spp. can be transmitted between different snake families. It was found by Ball (1967) that the mosquito, *Culex tarsalis* Coquillett, 1896 can transmit *Hepatozoon rarefaciens* (Sambon and Seligmann, 1907) to a Boa Constrictor (*Boa constrictor* L., 1758) after feeding on a Western Indigo Snake *Drymarchon corais* (Boie, 1827) with a positive infection status. *Hepatozoon rarefaciens* was also successfully transmitted from *D. corais* to a Gopher Snake *Pituophis catenifer* (Bainville, 1835) and also to *B. constrictor* (Ball, 1967; Sloboda 2007). Additionally, *Hepatozoon ayorgbor* Sloboda, Kamler, Bulantová, Votýpka and Modrý, 2007 was successfully transmitted from a Ball Python (*Python regius* Shaw, 1802) to an African House Snake (*Lamprophis fuliginosus* Boie, 1827) and *B. constrictor* (Sloboda, 2007).

This supports the likelihood that the ophidian host spectrum is more dependent on the snake host ecology than the host phylogenetic relationships, as well as the possibility that *Hepatozoon* spp. are even less host-specific towards the first intermediate vertebrate hosts such as amphibians (Smith *et al*., 1994, 1996) and reptiles (Sloboda, 2007). It was found that

Hepatozoon spp. infecting colubrid snakes, the Eastern Racer (*Coluber constrictor* L., 1758) and the Southern Water Snake (*Nerodia fasciata* L., 1766 subspecies not specified) can be successfully transmitted to lizards such as the North American Green Anole (*Anolis carolinensis* Voigt, 1832) and the Cuban Brown Anole (*Anolis sagrei* Duméril & Bibron, 1837) (Telford, 1991). Consequently, the diet of snakes is brought into question as an integral component of the life cycle of *Hepatozoon* spp. as well as to why parasites that are molecularly identical, or highly similar, occur in such a wide variety of vertebrate hosts (Allen *et al*., 2011; Sloboda *et al*., 2007, 2008; Smith, 1996; Tomé *et al*., 2012, 2013, 2014). This point was further emphasized during a study by Tomé *et al.* (2014) in which the authors investigated the prevalence and phylogenetic relationships of *Hepatozoon* spp. in Mediterranean ophidians. Here, it was demonstrated that the parasites clustered with those found in lizard and gecko hosts from the same approximate location, suggesting that prey-predator relationships play an important role in *Hepatozoon* transmission routes (Tomé *et al*., 2014).

The low host specificity of *Hepatozoon* spp. was highlighted and questioned when a *Hepatozoon* sp., which was isolated from a *B. constrictor* collected from North America, was molecularly analysed and found to be most similar to a *Hepatozoon sp.* found in a Woodrat (*Neotoma micropus* Baird, 1855) from California (Allen *et al*., 2011). The life cycles of *Hepatozoon* spp. that are most often accepted are those involving frogs and lizards as intermediate or paratenic hosts, where paratenic hosts form part of the life cycle, but the parasite does not undergo any development in this host (Smith, 1996; Smith *et al*., 1999). However, there were doubts that the natural history of some snake *Hepatozoon* spp. involve rodents as intermediate hosts (Sloboda *et al*., 2008). Additionally, the infection of snakes with *Hepatozoon* spp. by direct ingestion of the invertebrate mosquito vector has been proven during laboratory assays, although it was ruled to be highly unlikely for this to happen in nature (Ball *et al*., 1967, 1969; Landau *et al*., 1972; Nadler & Miller, 1984; Lowichik *et al*., 1993; Telford, 2001, 2002, 2004). Congenital *Hepatozoon* infections via vertical transmission were also assessed in viviparous snakes, and it was found that infected wild female Garter snakes *Thamnophis elegans* (Bairds & Girard, 1853) gave birth to infected offspring (Kauffman *et al*., 2017).

Based on the low ophidian host-specificity of *Hepatozoon* spp., it was inferred that the phylogeny of host vertebrates are not reflected by the phylogenies of their adeleorinid parasites. There is a clear taxonomical separation between haematozoans that are transmitted by leeches and those transmitted by arthropods (Barta *et al*., 2012; Tomé *et al*., 2012). Therefore, it is assumed that the invertebrate hosts play a more important taxonomical

role compared to the vertebrate hosts (Barta *et al*., 2012; Tomé *et al.,* 2014), which for species of *Hepatozoon* includes various arthropods.

The vectors and definitive hosts of *Hepatozoon* spp. infecting birds, mammals, crocodilians, lizards, snakes, turtles and anurans comprise a wide variety of arthropods, which include ticks, mites, reduviid bugs, sandflies and mosquito genera such as *Culex* L., 1758; *Aedes* Meigen, 1818 and *Anopheles* Meigen, 1818 (Sloboda, 2008; Smith, 1996). It has even been suspected that leeches can potentially act as a vector for *Hepatozoon* spp. in snakes (Smith, 1996; Úngari *et al*., 2021). It became apparent that morphological and life cycle data for *Hepatozoon* spp. are inconsistent and incomplete. The consequence hereof is that the true phylogenetic relationships are not reflected accurately on a taxonomical basis (Telford *et al*., 2004; Sloboda *et al*., 2007). It is suggested that the infection patterns by *Hepatozoon* spp. in some snake species may strictly correspond with their ecology and diet (Allen *et al*., 2011; Viana *et al*., 2011). Surprisingly, it was discovered that a wild piscivorous (feeding exclusively on fish) Dice Snake *Natrix tessellata* (Laurenti, 1768) from Turkey was infected with species of *Hepatozoon*, whereas saurophagous snake species of the genus *Cornella* from Morocco and Turkey were not infected with *Hepatozoon* (Tomé *et al*., 2014). To date, there have been no reports of species of *Hepatozoon* identified from fishes. It was also found that the Sleep Snake (*Dispas mikanii* Schlegel, 1837) from Brazil, which feeds primarily on land snails and slugs, was infected with *Hepatozoon quagliattus* Úngari, Netherlands, da Silva and O'Dwyer 2021 (Úngari *et al*., 2021). In this case, the authors mention the likelihood that leeches might be unintentionally ingested by the snakes, serving as vectors due to their similar appearance to slugs. It is also stated that there have been no formal reports of leeches as a vector for *Hepatozoon* species, or that leeches feed on this particular snake species (Úngari *et al*., 2021). This demonstrates that prey-predator infection routes might not be an uncomplicated task to solve and that the importance of such transmission routes might strongly depend on snake host species and their ecology.

Hepatozoon spp. have been observed in captive reptiles (de Vieira Santos *et al.,* 2005; O'Dwyer *et al*., 2004; Glaser *et al*., 2008; Úngari *et al.,* 2018). *Hepatozoon* infections in captive animals are associated more with the parasites' potential ability to persist in the host, rather than the likelihood of reinfection in captivity by the vectors (Úngari *et al.,* 2018). A possibility that will be considered in this chapter. As infection is often unlikely in captivity, due to the lack or abscence of suitable vectors and infected prey items, the reduction of parasitaemia levels in captive snakes has been observed. This was observed when a captive Jararaca, *Bothrops jararaca* (Wied-Neuwied, 1824) was screened and found to have a high parasitaemia of species of *Hepatozoon*. After eight years in captivity, it was screened again and the

parasitaemia was reduced by two thirds (De Biasi *et al.,* 1989; Úngari *et al.,* 2018). This, it is reasoned, can possibly be due to the slowly progressive elimination of the parasites in the host (De Biasi *et al.,* 1989; Smith, 1996; Ungari *et al*., 2018). It has also been reported that factors such as sex and whether snakes are venomous or non-venomous are unlikely to have an effect on the prevalence of *Hepatozoon* spp. in snake hosts (Úngari *et al.,* 2018).

The international trade of reptiles is increasing (Halla *et al*., 2014), but the significance of apicomplexan parasites in terms of human and reptile health are mostly unknown and reptiles that are part of the international trade are usually not screened for haemoparasites (Halla *et al*., 2014). It is frequently observed that *Hepatozoon* spp. do not cause any significant health effects in their ophidian hosts, with the effects of an infection usually only observable at the cellular level, usually described in terms of the effects of gamonts on host erythrocytes (Smith, 1996; Telford, 2009). However, in general, there is insufficient knowledge about reptile health (Benn *et al.* 2019), and consequently it is possible that inaccurate assumptions are made regarding the effects of *Hepatozoon* spp. on snakes. Side effects of *Hepatozoon* infections are not observed in snakes symptomatically, as there are no visible changes in their condition (Sloboda, 2007), possibly contributing to reasons as to why this parasite group is disregarded as a health concern to reptiles. Factors such as climate change, urbanization and increasing international trade of animals are also contributing to the dispersal of wildlife diseases and translocation of vector organisms, hence the need to better understand this group and their capability of infecting such a wide variety of hosts (Harrus and Baneth, 2005; Karesh *et al*., 2005; Hallah *et al*., 2014). This is a concern in the light of the low vertebrate host-specificity of *Hepatozoon* spp. and their ability to infect snakes and lizards from different geographical locations. Considering the lack of knowledge regarding their vectors, biodiversity and distribution, this is a concerning matter since geographical translocation of these parasites is possible and the ultimate ecological consequence hereof is unknown.

The aims of this chapter were to (i) assess the diversity of *Hepatozoon* spp. in captive and wild-caught snakes based on morphological, molecular and phylogenetic analyses using the 18S rRNA gene; (ii) to determine the longevity of *Hepatozoon* spp. in snake hosts after they were removed from their natural environment, and to (iii) compare the diversity, potential prevalence and parasitaemia of *Hepatozoon* spp. between captive and wild snakes.

3.1.1 Distribution and ecology of host snakes

3.1.1.1 ***Dendrosapis polylepis*** Günther, 1864

Dendroaspis polylepis (Fig. 3.1 A) is commonly known as the Black Mamba. It occurs in Eswatini (formerly known as Swaziland), Botswana, Zimbabwe Mozambique and Namibia. In South Africa, it can be found in the Northern Cape, northern North-West, Mpumalanga, Limpopo, KwaZulu-Natal and Eastern Cape provinces.

The Black Mamba is the largest venomous snake in southern Africa. It has a slender body and is 2.4–3 m long, however, there have been reports of this snake reaching a length of up to 4.5 m. Despite its common name, the smooth scales are not black but grey to olive, with a pale grey underside and dark markings. Contrary to popular belief, the name Black Mamba is not derived from their dark outer appearance, but rather from the pitch-black colour of the inside of the mouth. They are mainly terrestrial and can be found in dry and moist savannah and lowland forests, where they prefer termite mounds, hollow tree trunks, abandoned burrows and rock crevices to stay in. This snake is active, highly responsive, and reacts quickly. They mainly prey on rats and hyraxes, which are pursued and stabbed repeatedly with the fangs, but will also occasionally climb trees in search of food. When this snake species feels threatened, it lifts its forebody from the ground and gapes widely, displaying the black inside of the mouth. It also extends a very narrow hood and gives a hollow hiss. These snakes are oviviparous and females produce clutches of about 6–17 eggs during the summer. The hatchlings have the same appearance as the adults and are 40–600 mm long, but can reach 2 m within the first year. Black Mambas are one of the most venomous snake species in the world. They have neurotoxic venom that can lead to death within 6–15 hours without treatment after a bite. The venom primarily leads to nerve paralysis particularly those controlling respiration and heart rate with suffocation as the most common cause of death in humans (Alexander, 2013; Alexander & Marais, 2007; Bates *et al*., 2014; Marais 2004, 2011).

3.1.1.2 ***Dendroaspis angusticeps*** (Smith, 1849)

Dendroaspis angusticeps (Fig. 3.1 B) is commonly known as the Eastern Green Mamba. It occurs in Mozambique and Zimbabwe, and along the coast of the KwaZulu-Natal province in South Africa.

The Green Mamba is smaller and more slender than the Black Mamba. They reach 1.8 m in length and have been reported to measure 2.5 m, but this is rarely the case. They are brilliant emerald green with a blue-white mouth lining. The underside is pale green or yellow-green. Their local distribution is restricted to KwaZulu-Natal's coastal forests and eastern Zimbabwe. They prefer the upper forest canopy, where they ambush small mammals and birds. This is a shy species that is rarely seen in the wild. Bites from these snakes are very rare, their venom is neurotoxic, but not as potent as that of the Black Mamba, and is also injected in lower amounts during a bite. They are oviviparous and females lay 6–17 eggs in a hollow log or leaf litter during the summer. The hatchlings are 750 mm long (Alexander, 2013; Alexander & Marais, 2007; Bates *et al*., 2014; Marais 2004, 2011).

3.1.1.3 *Dispholidus typus* (Smith, 1829)

Dispholidus typus (Fig. 3.1 C) is commonly known as the Boomslang. It occurs in Namibia, Botswana, Zimbabwe, Eswatini, Mozambique and throughout South Africa.

The Boomslang is a medium to large snake species, with a large blunt head and very large eyes. It has a rough appearance due to strongly keeled dorsal scales. Adults reach sizes of 1.2–2 m, and their colouration varies between sex and locality. Males are usually bright green while females remain dull olive. The males are varied in colour, they may become mottled in black and gold, uniform rust-red or powdery blue in southern regions when they reach about 1 m in length. Males that occur from Zululand north, beyond the Limpopo River are bright green. Juveniles have brown heads, emerald eyes, white throats and twig-coloured bodies. The pupils are round and lobed in the front, allowing binocular vision. Boomslangs have three large immovable fangs near the back of the maxillary bone, just below the eyes. They are oviviparous and females lay 8–27 eggs in hollow tree trunks or rotting logs in late spring. The young are between 290–380 mm in length. These snakes are docile and usually hesitant to bite, although when they feel threatened, they inflate their throats and bite readily. Their venom is haemotoxic, which prevents blood clotting, and without treatment, death can be as quick as 1–3 days (Alexander, 2013; Alexander & Marais, 2007; Bates *et al*., 2014; Marais 2004, 2011;).

3.1.1.4 *Bitis arietans* Merrem, 1820

Bitis arietans (Fig. 3.1 D) is commonly known as the Puff Adder. It occurs in Namibia, Botswana, Zimbabwe and Mozambique, and throughout South Africa.

The Puff Adder is a large snake with a thick body, slender neck, short tail, and triangular head and can reach a length of 900 mm in South Africa. They occur throughout southern Africa, except in elevated areas, true desert and dense forests. The backside is yellow-brown to pale brown, orange brown or grey with dark marks on the back and stripes across the tail. It has chevron patterns on the back and males are usually more brightly coloured than females. There is a dark mark on the crown of the head which is divided from another dark mark just above the snout by a stripe between the eyes. There are two side bands on each side of the head, one under the eye and one behind the eye. The eyes are small with vertical pupils, the snout is rounded and the nostrils large and directed upwards. The underside is yellow-white to grey and has black markings. These snakes are mainly nocturnal, but tend to bask during the day. They hide and ambush prey, usually mice, rats, hare, ground fowl, lizards, toads and occasionally other snakes. They are viviparous, with females giving live birth to 20–30 young during late summer. The hatchlings are 150–200 mm long, they are born enclosed in a membrane, which they break free from during birth. This snake has a cytotoxic venom and is responsible for many serious snake bites. People usually get bitten when they step near or on a Puff Adder. The bites are rarely fatal, but they cause serious pain, as well as blistering and swelling in the area of the bite. A bite can result in death within 24 hours without treatment, antivenom is necessary in serious cases (Alexander, 2013; Alexander & Marais, 2007; Bates *et al*., 2014; Marais 2004, 2011;).

3.1.1.5 *Naja mossambica* (Peters, 1854)

Naja mossambica (Fig. 3.1 E) is commonly known as the Mozambique Spitting Cobra. It occurs in Eswatini, North-eastern Namibia, Botswana, Zimbabwe and Mozambique. In South Africa it occurs in North-West, Limpopo, Mpumalanga, Gauteng, Free State and KwaZulu-Natal.

This is a small cobra species, with adults reaching 1–1.2 m in length. The head is fairly rectangular, their eyes are medium sized and they have round pupils. The backside is olive coloured with a black edge on each scale. The underside is salmon pink with irregular black crossbars or blotches on the throat. They prefer moist savannah and low forests and can usually be found in rocky areas, hollow trees, termite mounds and animal burrows. This snake preys on mice, lizards, amphibians, and there are also records of them eating other snakes such as Puff Adders. They are oviviparous and females lay 10–22 eggs in the summer. The hatchlings are 23–25 cm long. They are responsible for many bites in Kwazulu-Natal and Mpumalanga. The venom is cytotoxic and causes local tissue damage, usually requiring skin

transplants (Alexander, 2013; Alexander & Marais, 2007; Bates *et al*., 2014; Marais 2004, 2011;).

3.1.1.6 ***Naja annulifera*** Peters, 1854

Naja annulifera (Fig. 3.1 G) is commonly known as the Snouted Cobra. It occurs in Eswatini, Zimbabwe, Mozambique and eastern Botswana. In South Africa, it occurs in the north-eastern provinces; North-West, Limpopo, Mpumalanga, Gauteng and KwaZulu-Natal.

This is one of the largest cobra species in Africa, with adults reaching 1.2–1.8 m and on rare occasions 2.5 m. The scales on the back are yellow, grey, dark brown or blue-black, and the underside is yellow, with dark markings. There is a banded version that has 7–9 broad yellow cross bands and lighter bands only half the width of the dark bands that also occurs throughout the region which are usually males. It has a larger throat band that is usually more prominent in adults. They prefer moist savannah, and are also common in Lowveld and Bushveld areas. They are nocturnal, and prey on small vertebrates such as toads, rodents, birds and their eggs, lizards and other snakes, especially Puff Adders. They are oviviparous, females lay 8–33 large eggs in early summer. The young are on average 220–340 mm long. When this snake feels threatened, it lifts its fore-body from the ground, spreads a hood and bites readily. This cobra species does not spit and sometimes it can sham death when it is restrained. They will evade danger by escaping into the nearest hole when presented with a chance to do so. They produce a large amount of neurotoxic venom which causes respiratory failure (Alexander, 2013; Alexander & Marais 2007; Marais 2004, 2011; Bates *et al*., 2014).

3.1.1.7 ***Python natalensis natalensis*** Smith, 1840

Python natalensis natalensis (Fig. 3.1 F) is commonly known as the Southern African Python. It occurs in Eswatini, Botswana, south to north Namibia, Zimbabwe and southern Mozambique. In South Africa it occurs in the Northern Cape, North-West, Gauteng, Limpopo, Mpumalanga and KwaZulu-Natal provinces.

This is the second largest snake species in Africa, with adults reaching 3–4 m in length. The backside is dark brown with dark specks and grey markings. There are dark marks on its sides that are spread far apart. There is a dark mark on the crown of the head that is shaped like an arrowhead and the underside is white or cream coloured. There are two heat-sensitive pits on each side of the upper lips. During sampling it was found that the snakes lunge forward to

strike when they sense heat from the front. They favour rocky and bushy areas that are close to water. They prey on small mammals, but larger adults are capable of taking on antelope. They kill their prey by constricting their bodies leading to bone breakage and suffocation of their prey. They are oviviparous and females produce clutches of about 30–60 eggs, usually laid in hollow trees, termite nests or aardvark burrows. The females bask and darken to absorb heat, where after they coil around their eggs to transfer heat in order to speed up embryonic development. These snakes are not venomous, but are known to have caused fatalities and can inflict serious and painful bites (Alexander, 2013; Alexander & Marais 2007; Marais 2004, 2011; Bates *et al*., 2014).

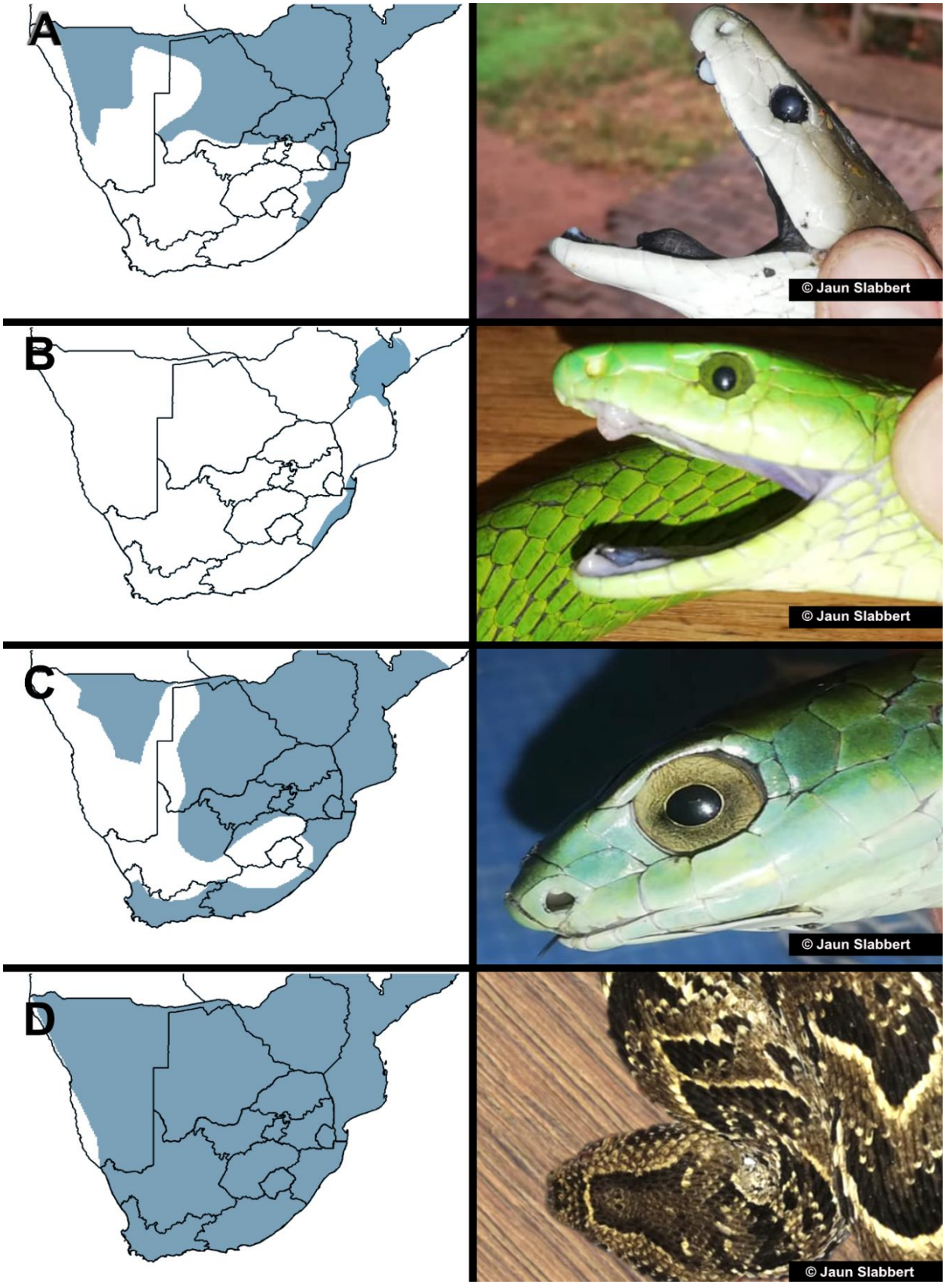


Figure 3.1: Distribution of southern African snake species sampled during this study *Dendroaspis polylepis* (A); *Dendroaspis angusticeps* (B); *Dispholidus typus* (C); *Bitis arietans* (D).

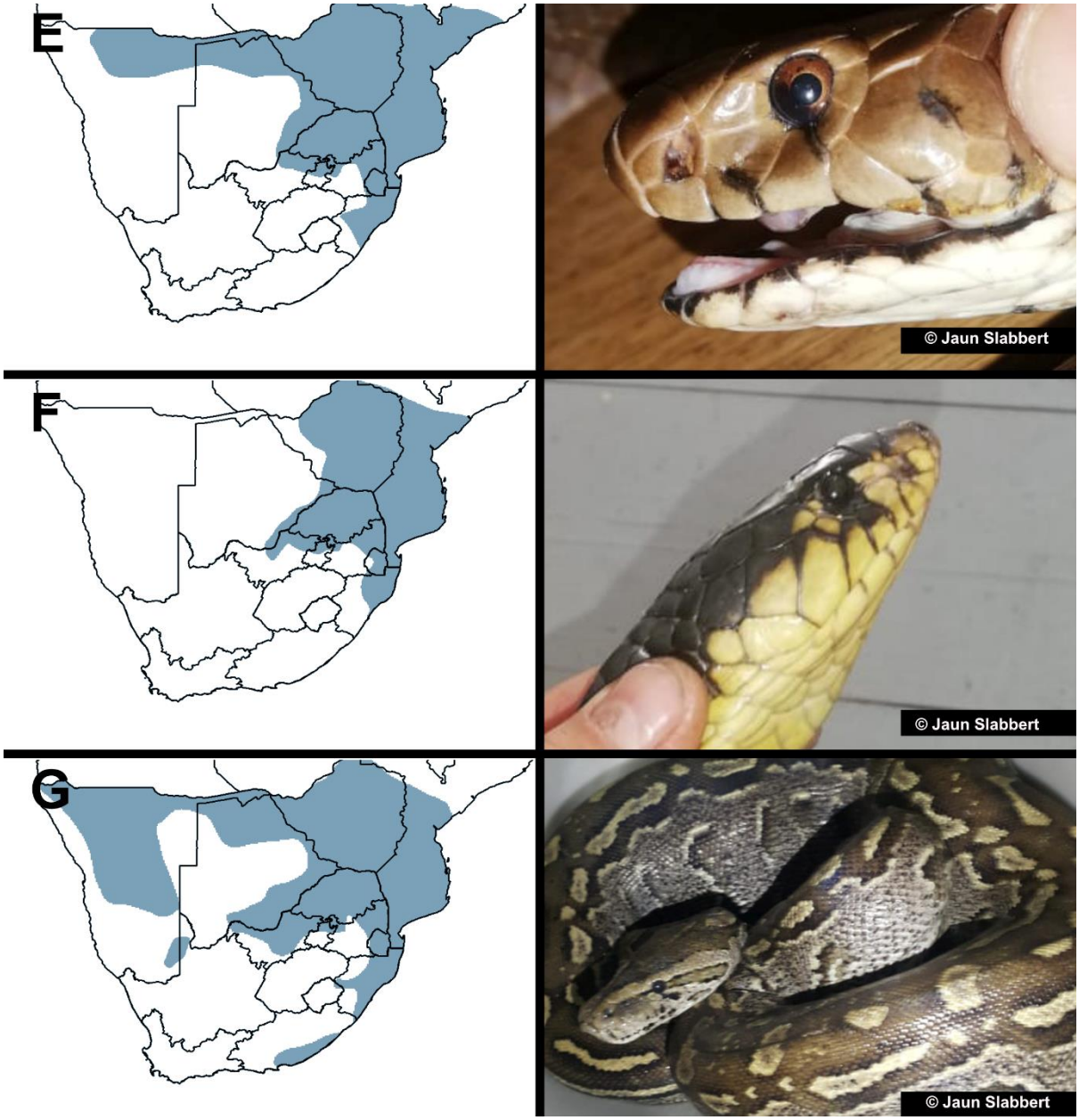


Figure 3.1 continued: Distribution of southern African snake species sampled during this study; *Naja mossambica* (E); *Naja annulifera* (F) and *Python natalensis natalensis* (G).

3.2 Materials and Methods

3.2.1 Snake hosts and husbandry

3.2.1.1 Captive snakes

Ethical approval was obtained from the North-West University (NWU), Potchefstroom Campus under the ethics number: NWU-00062-19-A5. Captive venomous snakes *Dendroaspis polylepis*, *Dendroaspis angusticeps*, *Dispholidus typus*, *Bitis arietans*; *Naja mossambica* and *Naja annulifera* were sampled at Lowveld Venom Suppliers (LVS) in Hazyview, Mpumalanga and Hoedspruit Reptile Centre (HRC) in Hoedspruit, Limpopo. Additionally, wild and captive *Python natalensis natalensis* individuals were also sampled at HRC. Snakes at LVS were collected according to permit number: MPB/V/2003. The samples at HRC were collected under veterinary supervision with an accompanying section 20 permit, reference numbers: NWU-00063-19-A5 and 12/11/1/3 (1711AC).

All of the 'captive' snakes at LVS resulted from snake removals, as such they can be considered previously wild. These snakes were selected and kept at the facility to be milked for the production of antivenom. The facility often rotates the snakes, meaning that when a larger individual of a species is caught, they are usually kept at the facility and another smaller snake of the same species is released; particularly as larger snakes produce more venom and ensure a larger venom yield (pers. comm. C. Hobkirk).

All snakes were kept in temperature-controlled rooms in appropriately sized clear plastic bins on newspaper as bedding, provided with a hide and a water dish. A few *D. polylepis* individuals were kept in spacious vegetated wire cages outside; the cage set up to mimic their natural habitat and allowing room for movement and shelter. All the captive snakes and the snakes that were sampled during this study were fed appropriately sized captive bred rats depending on the size of the snake; ranging from rat pups to adult rats.

Additionally, purchased thawed frozen day-old chicks also made up part of the snakes' diet and snakes were fed about once a week or once every other week. The feeding of captive snakes at HRC was very similar, but included larger rodents as they also house larger snake species such as *P. n. natalensis*. The snakes were kept in temperature controlled wooden or glass enclosures with appropriate bedding, water dishes and hides. The enclosures were set up as a more permanent habitation for the snakes since they were either permanent 'residents' at the facility or were used as display animals. The captive *Di. typus* and *D. polylepis*

individuals at HRC were housed in appropriate wire enclosures outside. These enclosures were vegetated and set up to mimic the natural habitat of the snakes allowing shelter and sufficient room for the snakes to move unimpeded about.

At both of these facilities, snakes have been dewormed with Panacur® and have received a general treatment with Flagyl®. Snakes receive a general health check-up at least once a year and Frontline antibacterial disinfectant hand wipes are used as mite prevention and for cleaning the snakes. Snakes that are observed to be performing badly, from a health perspective, have their routine altered according to their needs (pers. comm. Dr. J. Briner).

3.2.1.2 Wild caught snakes

Wild snakes were collected by HRC, these resulting from snake removals. Snakes were temporarily kept in plastic bins or tubs at the facility in the quarantine area before relocation. This was used as an opportunity to sample wild snakes from the local area without the exertion of finding, catching, and sampling snakes from their natural environment. Snakes were kept for a short duration of time and were temporarily provided with a comfortable enclosure. Depending on the condition of the snake and the time that they were in captivity, they were fed captive bred rats or other available suitable captive bred food items.

Additionally, biobank samples from the Hans Hoheisen Research Station were obtained and utilized during this study under the permit number: 20210329ORK-VMPI. These samples were mostly obtained from snakes from the snake removal program of Mpumalanga Tourism and Parks Agency (MPTA) from the Mpumalanga province, along with a few from the Limpopo province. From these, blood was collected by performing tail clippings (the tail clippings themselves were not utilized during this study). After a small tip of the tail was clipped, a drop of blood was dripped on a clean microscope slide and either impression slides or blood smears were made. A blood spot was also made on FTA® cards. The blood smears were not fixed or stained prior to storage and both smears and FTA® cards were stored at room temperature from 2018 to 2020. These samples were the result of opportunistic sampling by experienced snake handlers when snakes were encountered unexpectedly during daily activities, or during call-outs for snake removals and relocations.

3.2.2 Snake handling and blood sampling

Depending on the temperament of the snakes and the snake species, snakes were either restrained by 'necking' or 'tubing' by experienced snake handlers. 'Necking' entailed snake handlers grabbing the snake firmly behind the head, while being mindful of the position of the fangs of the different species (Fig. 3.2 A–B). This restraining method was comfortably used for *D. polylepis*, *Di. typus* and *P. n. natalensis*. If snakes were of a large size or resisted restraint to a degree making it difficult for the handler, they were pinned to the table by the neck while another person secured the length of the body (Fig. 3.2 D–H). Additionally, 'tubing' was used for snakes that could not be comfortably 'necked' (Fig. 3.2 C–E). This entailed the use of appropriately sized 'snake tubes', where the tube was placed over the snake's head and the snake was carefully and gently coaxed into the tube until approximately a quarter of its body length was in the tube. The tube was then secured by firmly gripping the tube and snake's body together in one hand while keeping hold of the tail with the other hand (Fig. 3.2 C). Tubing was used for *B. arietans*, *D. angusticeps*, *N. mossambica* and *N. annulifera*. During sampling of the *N. mossambica*, safety glasses were worn as eye protection. Once the snakes were securely positioned, blood was drawn from the ventral coccygeal vein with a sterile syringe and needle (Fig 3.2 I–L). A needle gauge of either 27 G or 25 G was used, depending on the size class of the snake.

3.2.3 Preparation and screening of blood smears

Blood from snakes was used to make thin blood smears in duplicate for each blood sample. Blood smears were prepared before the remaining blood in the syringe was emptied into 70% ethanol for further molecular analyses. This required placing a small drop of blood onto a clean microscope slide and dragging another slide over it to make a thin smear. The process was then repeated to make duplicate slides of each blood sample. The slides were left to air dry completely where after they were fixed with absolute methanol for 10 minutes. Slides were then stained with a 10% solution of Giemsa-stain (FLUKA, Sigma-Aldrich, Germany) for 10–15 minutes. After the staining process, excess stain was gently rinsed off under tap water. The blood smears were examined with the 60× magnification objective, equipped with immersion oil on a calibrated Nicon Eclipse N*i* (Nikon, Amsterdam, Netherlands) compound microscope for the detection of parasites. Parasitaemia was calculated per 10 000 erythrocytes, with ~10^4 examined per blood smear (Cook *et al*., 2014, 2016, 2018). Photos of the parasites were taken with 100× oil immersion objective, as such at 1000× magnification, using the accompanying digital camera and NIS-Elements BR Ver. 4.60 camera analysis software (Nikon, Tokyo,

Japan). The parasite morphometrics were measured with the aid of ImageJ software (https://imagej.nih.gov/ij/) and all the measurements were recorded in µm. Measurements included total length (TL); total width (TW); nuclear length (NL); nuclear width (NW); assumed anterior to mid-nucleus (AN) and assumed posterior to mid-nucleus length (PN).

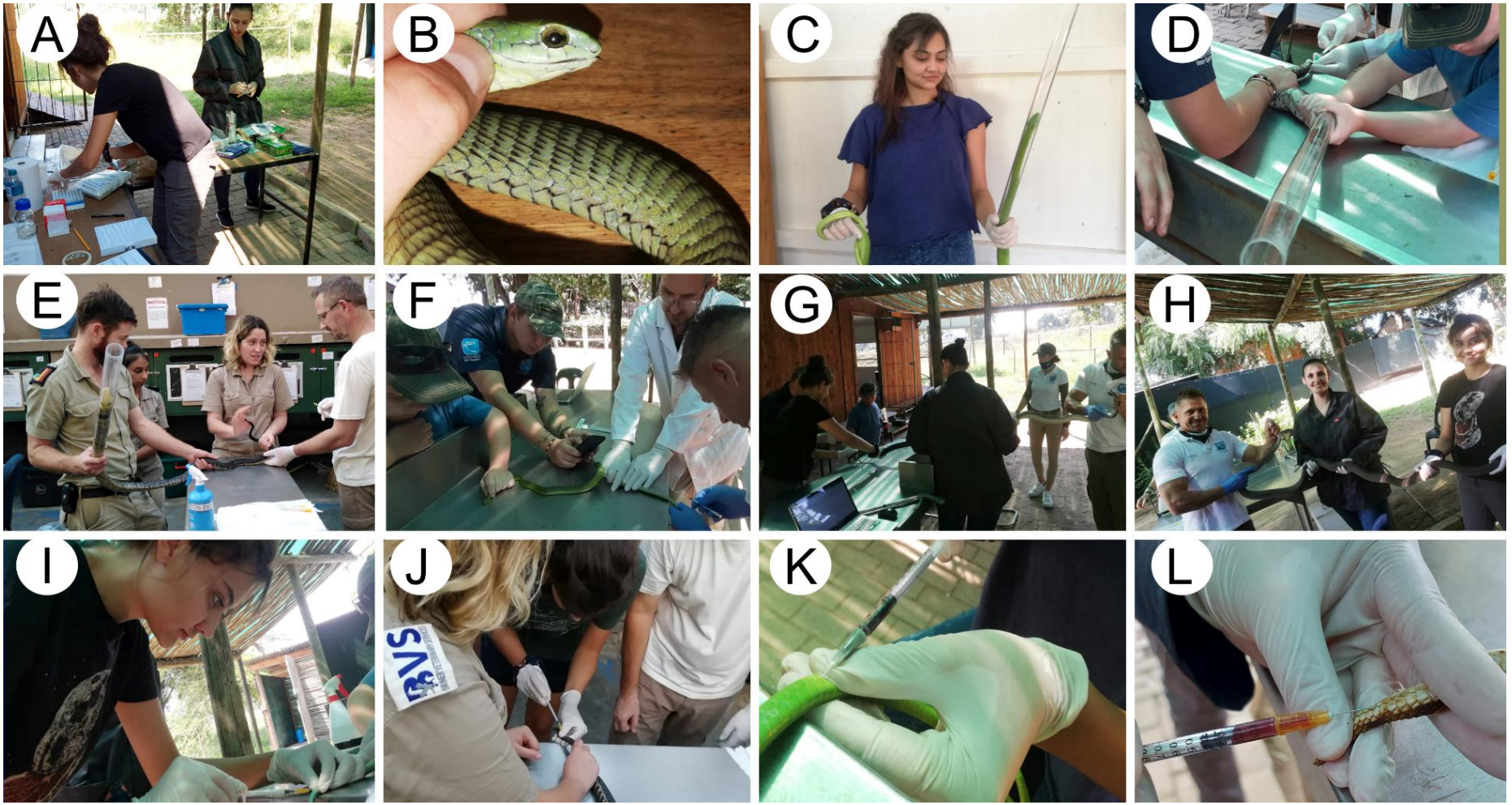


Figure 3.2: Preparation of sampling equipment (A); demonstration of snake necking of *Dispholidus typus* (B); demonstration of snake tubing (C– E); snake was pinned down during necking and body secured by extra hands if snakes resisted restraint (F–H); blood was drawn from the ventral coccygeal vein with the use of a sterile syringe and appropriate gauge needle (I–L).

Table 3.1: Sampling sites of snake species that were wild-caught, and the locations where now captive-snakes were originally collected from the wild. Wild and captive snake numbers are indicated as male/female/juvenile/not determined (m/f/juv/nd) respectively. MP=Mpumalanga, LP=Limpopo and KZN=KwaZulu-Natal. The estimated time in captivity for captive snakes is also indicated.

Host species	Sampling site	Number: m/f/juv/nd		Estimated time in captivity
		Wild	Captive	
Dendroaspis polylepis	Nelspruit (MP)[2], White River (MP)[2], Hazyview (MP)[1], Marloth Park (MP)[1], Hoedspruit (LP)[2], Jejane KNP(MP)[3]	7/6/0/3	0/0/0/10	4 days – 3 years
Dendroaspis angusticeps	KZN (Unspecified)[2]	0/0/0/1	0/0/0/10	3 months – 3 years
Dispholidus typus	Nelspruit (MP)[2], White River (MP)[3], Malelane (MP)[3], Berberton (MP)[3], Hoedspruit (LP)[2]	5/3/0/1	8/1/0/4	4 months – 2 years
Bitis arietans	Nelspruit (MP)[2], White River (MP)[2], Sterkspruit Nature Reserve (MP)[2], KZN (Unspecified)[1], Marloth Park (MP)[3], Malelane (MP)[3], Sabie (MP)[3]	6/3/0/1	0/2/0/7	1 month – 3 years
Naja mossambica	Nelspruit (MP)[2], Whiter River (MP)[1], Hazyview (MP)[2], Marloth Park (MP)[1], Malelane (MP)[3], Hoedspruit (LP)[1]	9/8/0/3	0/0/0/8	2 years
Naja annulifera	Nelspruit (MP)[2], White River (MP)[2], Hazyview (MP)[2], Malelane (MP)[2], Kanyamazane (MP)[1]	10/10/0/0	1/0/0/10	1 – 2 years
Python natalensis	Nelspruit (MP)[1], White River (MP)[1], Hazyview (MP)[1], Marloth Park (MP)[1], Schoemanskloof (MP)[1], Hoedspruit (LP)[1], Undetermined site[3]	6/10/0/0	0/0/0/1	1 year + (unspecified)

Only wild caught[1]; Captive and Wild caught[2]; Only Captive[3]

3.2.4 Molecular analyses

Genomic DNA was extracted from whole blood samples preserved in 70% ethanol and from FTA® cards. The KAPA Express Extraction Kit (Kapa Biosystems, Cape Town, South Africa) was used for the extractions of the whole blood samples by following the manufacturer's instructions for animal blood. The Machery-Nagel mini kit for DNA from cells and tissue was used for the FTA® cards, following the manufacturer's instructions for dried blood spots. The isolated DNA products for both these methods were stored at -20 °C and were used as the template for the PCR. Two separate 18S rRNA gene fragments were amplified with the use of a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Singapore). The isolated DNA product was used as PCR template for both fragments. The first fragment of the 18S rRNA gene, ~930 bp in length, was amplified using the forward primer HAMF 5'-GCCAGTAGTCATATGCTTGTC-3' (Criado-Fornelio *et al*., 2006) along with the reverse primer HepR900 5'-CAAATCTAAGAATTTCACCTCTGAC-3' (Ujvari *et al*., 2004). The second fragment, ~1,400 bp was amplified using the forward primer HepF300 5'-GTTTCTGACCTATCAGCTTTCGACG-3' (Ujvari *et al*., 2004) and the reverse primer 2868 5'-TGATCCTTCTGCAGGTTCAC-3' (Medlin *et al*., 1988; Mathew *et al*., 2000). The PCR conditions for both fragments were as follows: denaturation at 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 61 °C for 30 s with an extension at 72 °C for 2 min. Following the cycles, a final extension took place at 72 °C for 10 min (Netherlands *et al*., 2018). All PCR reaction volumes were set at 25 µl where the reagents and their respective volumes were as follows: 12.5 µl Thermo Scientific DreamTaq PCR master mix (2x) (2x DreamTaq buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each and 4 mM $MgCl_2$), 1.25 µl of forward and reverse primer (10 µM), respectively, and 25 ng of DNA (1 µl). PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania) was used to make up the reaction volume (9 µl). The PCR products were run on a 1% agarose gel aligned to a 1kb DNA ladder. The gel was run at 90 V for 30 min where after it was viewed under UV light. The fragment sizes were compared to the ladder and fragments that were about 930 bp and 1,400 bp respectively were sent to Inqaba Biotec for purification and sequencing in both directions.

3.2.5 Genetic analyses

3.2.5.1 Phylogenetic tree

The resultant forward and reverse sequences were assembled with the use of Geneious ver. 11.1.4 (http://www.geneious.com, Kearse et al. 2012) and identified using the Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990). All of the sequences used in this study had closest matches with species of *Hepatozoon* in GenBank (https://www.ncbi.nlm.nih.gov/). A total of 35 *Hepatozoon* sequences were obtained during this study. Samples of snakes that were microscopically positive were selected for molecular analysis. *Hepatozoon* sequences were obtained from all the snake species except for *N. annulifera,* which was microscopically positive but the PCR amplification process for this sample wasunsuccessful. The fragments of the forward and reverse primers from the obtained sequences were assembled and manually edited using Geneious ver. 11.1.4. The MEGA X software (https://www.megasoftware.net/) was used for the phylogenetic analyses using the obtained sequences and an additional 39 sequences that were obtained from GenBank. *Haemogregarina balli* Paterson and Desser, 1976 was used as outgroup following Cook *et al.,* (2018). The sequences were aligned using the MUSCLE tool in MEGA X where the alignment consisted of 74 sequences and was 957 bp long.

Phylogenetic analysis was performed by implementing Bayesian Inference using the CIPRES platform where the MrBayes on XSEDE (MrBayes 3.2.2 Huelsenbeck & Ronquist, 2001) tool was used. The data block was enabled and runtime was set as eight hours. The MrBayes tool entailed that the analysis was run twice over 10 million generations for the Markov Chains Monte Carlo (MCMC) algorithm. The Markov chain was sampled per each 100 generations, with the MCMC run containing 4 chains.

The output was exported, and the tree was viewed using FigTree (http://tree.bio.ed.ac.uk/software/figtree/). Further edits and shortening of the branches were made using the program Affinity Designer. Ver. 1.9. The branches were shortened according to the scale (0.005 substitutions per site) for each hash mark present on the branch. The BI values are indicated at the nodes, where values of less than 0.5 (50%) are not indicated, but values above 0.95 were considered well supported..

3.2.5.2 Evolutionary divergence

The 35 obtained 18S rRNA *Hepatozoon* sequences were aligned with the MUSCLE tool in MEGA 7 and trimmed to 548 base pairs. Estimates of evolutionary divergence between the 35 obtained sequences were determined with the use of MEGA 7. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There was a total of 548 positions in the final dataset (Kumar *et al*., 2016). These divergence values (SM: Table A2, Appendix A) were incorporated in the haplotype network (Fig. 3.3), expressed as percentage divergence.

The same analyses were performed to determine the percentage difference between some of the obtained sequences and sequences in the phylogenetic tree that were downloaded from GenBank (SM: Table A3, Appendix A). The analysis involved 11 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 560 positions in the final dataset (Kumar *et al*., 2016).

3.2.5.3 Haplotype network

In order to better visualize the results from the uncorrected p-distance tables, a haplotype network was constructed with the use of POPART ver. 1.7 (http://popart.otago.ac.nz/howtocite.shtml Leigh & Bryant, 2015). A minimum spanning network (Bandelt *et al*., 1999) was constructed to infer relationships of population genetic data where the sequences were divided into respective genotypes, with a minimum of 0.2% difference resulting in a different genotype. The number of mutations is indicated as 'hash marks' on the branches of the network, with each hash mark representing a mutation. The POPART program categorized the sequences based on nucleotide differences, forming respective genotypes. A representative sequence for each genotype was identified by the program where sequences of the same genotype are grouped with that representative sequence. The larger the circle that was generated in the haplotype network, the more sequences group with the representative sequence of a genotype.

3.3 Results

3.3.1 General findings and prevalence of *Hepatozoon* spp. in wild and captive snakes

A total of 154 snakes (82 wild, 62 captive) of five genera and seven species were sampled and screened for the presence of species of *Hepatozoon*. Overall (including both wild and captive), 49/154 (31.8%) of snakes were infected with species of *Hepatozoon*, and overall, parasitaemia ranged from 0.1–9.3%. The overall prevalence and parasitaemia was higher for wild snakes than for captive snakes, with a prevalence of 35/82 (42.7%) and parasitaemia of 0.01–9.3% recorded in wild, as compared to a prevalence of 16/62 (25.8%) and parasitaemia of 0.01–7.7% in captive snakes (Table 3.2). The overall prevalence of *Hepatozoon* spp. (which include wild and captive individuals) per species of snake were 48% for *D. polylepis*; 9% for *D. angusticeps;* 41% for *Di. typus*; 17% for *B. arietans*; 46% for *N. mossambica*; 3% for *N. annulifera* and 65% for *P. n. natalensis.* The overall prevalence per species for wild snakes was the highest in *P. n. natalensis* 10/16 (63%) and for captive it was also the highest in *P. n. natalensis* 1/1 (100%); however the sampling pool only consisted of one individual, thereby making *D. polylepis* 6/12 (50%) the species with the highest prevalence based on a sufficient number of sampled individuals. The parasitaemia, including both captive and wild snakes was the highest in *D. polylepis* (9.3%), in captive snakes the parasitaemia was the highest in *D. polylepis* (7.7%) and in wild snakes it was also the highest in *D. polylepis* (9.3%).

Table 3.2: Prevalence of *Hepatozoon* spp. among wild and captive snakes respectively, including the collective prevalence per species

Host species	Sampling site	Prevalence		
		Wild Caught	Captive	Per species
Dendroaspis polylepis	Nelspruit (MP)[2] White River (MP)[2] Hazyview (MP)[2] Marloth Park (MP)[2] Hoedspruit (Lim)[2] Jejane KNP (MP)[3]	5/11 (45%)	6/12 (50%)	11/23 (48%)

Only wild caught[1]; Captive and Wild caught[2]; Only Captive[3]

Table 3.2 continued: Prevalence of *Hepatozoon* spp. among wild and captive snakes respectively, including the collective prevalence per species

Host species	Sampling site	Prevalence		
		Wild Caught	Captive	Per species
Dendroaspis angusticeps	KZN (Unspecified)[2]	0/1 (0%)	1/10 (10%)	1/11 (9%)
Dispholidus typus	Nelspruit (MP)[2] White River (MP)[3] Malelane (MP)[3] Berberton (MP)[3] Hoedspruit (Lim)[2]	4/9 (44%)	5/13 (38%)	9/22 (41%)
Bitis arietans	Nelspruit (MP)[2] White River (MP)[2] Sterkspruit Nature Reserve (MP)[1] KZN (Unspecified)[1] Marloth Park (MP)[3] Malelane (MP)[3] Sabie (MP)[3]	2/10 (20%)	1/8 (13%)	3/18 (17%)
Naja mossambica	Nelspruit (MP)[2] Whiter River (MP)[1] Hazyview (MP)[2] Marloth Park (MP)[1] Malelane (MP)[3] Hoedspruit (Lim)[1]	11/20 (55%)	2/8 (25%)	13/28 (46%)
Naja annulifera	Nelspruit (MP)[2] White River (MP)[2] Hazyview (MP)[2] Malelane (MP)[3] Kanyamazane (MP)[1]	0/20 (0%)	1/10 (10%)	1/30 (3%)
Python natalensis	Nelspruit (MP)[1] White River (MP)[1] Hazyview (MP)[1] Marloth Park (MP)[1] Schoemanskloof (MP)[1] Hoedspruit (Lim)[1] Undetermined site[3]	10/16 (63%)	1/1 (100%)	11/17 (65%)

Only wild caught[1]; Captive and Wild caught[2]; Only Captive[3]

3.3.2 Species and morphotypes of *Hepatozoon*

Screening of the snake species revealed that they were infected with different gamont morphotypes. Typically, for present day taxonomy, species identification for species of *Hepatozoon* relies on a combined approach, using both morphometrics of peripheral blood gamont stages and molecular analyses based on the 18S marker (Barta, 1997; Cook *et al*., 2014; Maia *et al*., 2011; Telford *et al*., 2004; Úngari *et al*., 2018). Often, different morphotypes are identified and subsequently molecularly analysed to delineate species. However, in the present study, it was a challenge to accurately identify between morphotypes in the different individuals, species, and genera of snakes. It was uncertain as to whether these represented different species of *Hepatozoon* or if there may have been mixed infections, or if there was a degree of phenotypic plasticity of species between host species and genera. As such, in this study, molecular, particularly the combined use of the haplotype network (Fig. 3.3) and divergence values, assisting in linking morphotypes with their respective species. Therefore, the morphotypes were linked back to the *Hepatozoon* spp., and were presented as morphotypes: A1, A2, A3 and A4 of species A and morphotype B1 of species B, respectively. Table 3.3 contains photos of the different species, their morphotypes and these morphotype's accompanying genotypes, and the hosts from which they were isolated. Detailed descriptions of the morphotypes can be found in Appendix A. Additionally, morphotypes or potential additional species, that were observed, but of which the amplification was unsuccessful during PCR are also shown in Appendix A. This section will provide details on the morphotypes of the different species, with the following molecular section detailing the findings with regards to the species and genotypes.

Hepatozoon sp. A, comprises four morphotypes, and was found infecting 6/7 (86%) of species of snakes and 32/154 (21%) of individuals collected. Of the four morphotypes, morphotype A1 and A4 were the most prevalent, infecting 2/7 (29%) of snake species and 11/154 (7.1%) of individuals; and 6/7 (86%) of snake species and 25/154 (16.2%) individuals respectively. Morphotype A3 was observed in 3/7 (43%) snake species collected, with 6/154 (4%) individuals infected. Morphotype A2 was the least observed with only a single species and individual infected [1/7 (14%) of snake species and 1/154 (0.6%) snakes collected].

From the morphometrics, *Hepatozoon* sp. A, morphotype A1 and A3, were closely comparable in gamont size, measuring 17.8 × 4.2 µm and 16.9 × 3.2 µm respectively, with a nucleus size of 6.1 × 3.6 µm and 6.0 × 3.1 µm, respectively. This was similar for morphotypes A2 and A3, measuring 16 × 2.6 µm and 15.8 × 3.4 µm respectively, with a nucleus size of 5.4 × 1.9 µm and 5.7 × 1.1 µm, respectively.

Hepatozoon sp. B, comprises one morphotype, and was found infecting four individuals of one species *Di. typus*, as such 1/7 (14%) of snake species and 4/154 (2%) of snakes collected.

Hepatozoon sp. B, morphotype B1, had a gamont size of 17.6 × 4.4 µm, and a nucleur size of 6.4 × 4.1 µm.

The gamont morphotypes of species A had no severe effects on the host erythrocytes other than mild elongation or deformation. In most cases, the nuclei of the erythrocytes were displaced and pushed against the side of the erythrocytes' outer cell membrane. However, species B, extracted from *Di. typus*, caused severe dehaemoglobinisation of the host erythrocytes. In some occasions cell lysis was observed, mostly at one of the poles.

The morphotypes designated C,did not amplify . Morphotype C1, had a gamont size of 14.6 × 2.3 µm, and a nuclear size of 5.3 × 1.4 µm, similar to *Hepatozoon* sp. A, morphotypes A2 and A4. Morphotype C2 and C3, were similar in gamont size 15.1 × 7 µm and 16 × 7.3 µm. The nucleus was not readily visible in either C2 or C3.

3.3.3 Molecular and phylogenetic analyses

The haplotype network was interpreted using the accompanying nucleotide difference table to categorize sequences into genotypes. A minimum of 3% difference was considered acceptable for differentiating between different species. (Pretorius *et al*., 2021; Schulz *et al*., 2019; Smit *et al*., 2020; Stoeck *et al*, 2009). The genotypes and accompanying morphotypes of the sequences that were used during the analysis are indicated in Table 3.3, where the table is supportive information of the haplotype network (Fig. 3.3), with a minimum of 0.2% divergence resulting in a different genotype. From the haplotype network, species A appeared to be the dominant species observed during this study, infecting six species of snakes (Table 3.3). This species was also the most variable in both morphotype and genotype, including four morphotypes, with morphotype A3 and A4 including three genotypes respectively (Fig. 3.3, Table 3.3). Morphotype A4, genotype 5 appeared to have the highest prevalence among snake species and individuals of snakes (infecting five species of snakes and 11 individuals). This was followed by morphotype A1, genotype 1 (infecting two species of snakes and nine individuals) and morphotype A3, genotype 3 (infecting two species and five individuals). Species B (including only two genotypes), showed a low prevalence, infecting only one species of snake *Di. typus*, with three individuals infected.

From the phylogenetic tree it was observed that *Hepatozoon* sp. A, genotype 4, isolated from captive *D. angusticeps* I, groups with *Hepatozoon domerguei* (KM234646) isolated from Malagasy *Madagascarophis colubrinus* Schlegel, 1837 (Maia *et al*., 2014), with 0% divergence between these two species, with a 100% support value (Fig 3.4). It was also observed that *Hepatozoon* sp. A, genotype 6, isolated from *B. arietans* III, groups with a *Hepatozoon* sp. isolated from *Psammophis elegans* Shaw, 1802 (KC696568), which occurs north of southern Africa (Uetz & Hošek, 2021), with a divergence of 0%.

Hepatozoon sp. A, genotype 1 and 2, form a monophyletic clade, with a genetic divergence of 0.2% (Fig. 3.3, 3.4 respectively), basal to the other clades containing *Hepatozoon* spp. of snakes. Interestingly, genotype 5 of *Hepatozoon* sp. A, isolated from *N. mossambica* III, falls separately to other isolates of the same species and genotype. Isolates from *P. n. natalensis* of *Hepatozoon* sp. A, genotype 7, form a monophyletic clade, sister to an isolate from *B. irregularis* from Australia, however with low support. *Hepatozoon* sp. A, genotype 3, forms a monophyletic clade containing isolates of *Hepatozoon* sp. from the snake host *Lycognathophis seychellensis* (Schlegel, 1837), a monotypic genus of snake endemic to the Seychelles. The isolate of *Hepatozoon* sp. A, genotype 8, falls basal to that of a larger clade containing isolates of genotypes 3–7. Genotypes 1 and 2, of *Hepatozoon* sp. B, fell within a well-supported larger clade, sister to that of isolates of Species A. This clade comprised species isolated from amphibians, as well as species described from the snake species *Ph. n. natalensis* collected in South Africa.

3.3.4 Remarks

During the current study, *Hepatozoon* sp. A was found to be the most prevalent species infecting snakes. It comprised four morphotypes and eight genotypes. Of these, morphotype A4, genotype 5, showed the highest prevalence among genera and snake species screened (Table 3.3). Five genera and five species (*D. polylepis*, *Di. typus*, *B. arietans*, *N. mossambica* and *P. n. natalensis*) were found infected with this genotype. As these genera and species are known to occur sympatrically and were collected from the same larger geographical region (areas around Limpopo and Mpumalanga), this is not surprising. Furthermore, in light of the different species' varied diets, along with the proposed possible three host life cycle of *Hepatozoon* spp. of snakes (invertebrate vector, primary intermediate, and snake as secondary intermediate)(Smith, 1996), as well as the suggested low host-specificity of *Hepatozoon* spp., this again, is not all too surprising. *Dendroaspis polylepis* feeds on small rodents, birds and will prey on other snakes; *Di. typus* preys on birds, tree-dwelling lizards,

and frogs, seldomly feeding on small mammals; *B. arietans* feeds on small mammals, birds, lizards, toads and sporadically on other snakes; *N. mossambica* preys on toads, small mammals, birds, lizards and snakes, which may include *B. arietans*; and *P. n. natalensis* feeds on small to medium sized mammals, birds, larger lizards and crocodiles (when adult), as well as fishes (Alexander, 2013; Alexander & Marais, 2007; Bates *et al*., 2014; Marais 2004, 2011). With these varied diets, which overlap in prey types, along with the possibility of many of these acting as primary intermediate hosts and other snakes acting as routes of infection, the potential routes of transmission are numerous. Looking at the other morphotypes and their respective genotypes, these are represented by fewer host species. Whether this has to do with a preferred prey item or has to do with a more specific locality from which these snakes were collected, is at present speculative, but may warrant further research into at a future point.

Interestingly, the genotype isolated from *D. angusticeps* (Species A., morphotype A4, genotype 4) (Table 3.3), grouped with *H. domerguei* (KM234646) isolated from Malagasy *M. colubrinus* (Maia *et al*., 2014), with a 0% divergence. *Dendroaspis angusticeps* feeds on birds and small tree-living mammals, however, chameleons will be preyed upon by juveniles (Marais, 2004). Maia *et al*. (2014) identified the same haplotype of *H. domerguei* in both the Malagasy *M. colubrinus* and one of its natural prey items *Furcifer* sp., a genus of chameleon. The *D. angusticeps* individual in this study was captive, but was initially collected from the wild in KwaZulu-Natal. Chameleons, particularly those from Madagascar, are popular in the pet trade, especially in the illegal pet trade, and have been highly exploited (Jenkins *et al*., 2014). It cannot, at this point, be ascertained if the *D. angusticeps* ingested an infected Malagasy chameleon, which was as a result of the pet trade, here in South Africa, or that the species and genotype of *Hepatozoon* found in the *D. angusticeps*, *M. colubrinus*, and *Furcifer* sp. are as a result of this genotype being naturally distributed in both South Africa and Madagascar. No reports, as far as can be found, mention Malagasy chameleons escaping and forming established populations in South Africa. Another potential, but speculative, explanation, could be the oceanic dispersal movement of chameleons from mainland Africa to Madagascar, particularly during the Eocene (Tolley *et al*., 2013), which may have resulted in the distribution of this parasite genotype.

Hepatozoon sp. A, genotype 6, was isolated from *B. arietans* III, and grouped with a *Hepatozoon* sp. isolated from *Ps. elegans* (KC696568), from North Africa (Tomé *et al*., 2013), with a divergence of 0%. Taking into account the above potential of a genotype of *Hepatozoon* sp. A being closely related to a species of *Hepatozoon* isolated from Malagasy reptiles, this finding is not astonishing either. This particularly, as both *B. arietans* and *Psammophis* spp. feed on lizards, amphibians and birds (see Marais, 2004).

In the phylogenetic tree, *Hepatozoon* sp. A, morphotypes A1 and 2, with respective genotypes 1 and 2, clade together in a monophyletic clade, basal to the other morphotypes and genotypes of species A. This too, is represented by the haplotype network, these two morphotypes and genotypes showing at least a 2.2–2.4% divergence from the other morphotypes and genotypes of species A.

Interestingly, *Hepatozoon* sp. A, morphotype A3, genotype 7, isolated from a *P. n. natalensis*, cladded with an isolate from *B. irregularis* from Australia. The former is of the Pythonidae family (Bates *et al*., 2014) and the latter the Colubridae family (Uetz & Hošek, 2021). The latter, *B. irregularis*, has a more specialised diet of nocturnal lizards (such as geckos) (Rodda & Fritts, 1992), whilst as mentioned above, *P. n. natalensis* has a varied diet, but it too is more active at night (Marais, 2004). *Boiga irregularis* is known as an invasive species (Rodda & Fritts, 1992) and is relatively popular in the pet trade (Kopecký et al., 2019), but no reports of this species have been found, as far as possible, in South Africa. This, along with the low nodal support and the long branch length for the isolate of *B. irregularis* suggest that they are not closely related.

Hepatozoon sp. A, morphotype A3, genotype 3, isolated from *Di. typus and N. mossambica*, cladded with an isolate of a *Hepatozoon* sp. amplified from the snake host *L. seychellensis*, an endemic snake species to the Seychelles. Similar to the chameleon oceanic dispersal, ancestors of the *L. seychellensis*, may have dispersed from main land Africa, approximately 43–25 million years before present (Ma) (Deepak *et al*., 2021), along with their parasite fauna. However, once again this is speculative and will require further investigation into the morphology, genetics and potential life cycles.

Regarding *Hepatozoon* sp. B, morphotype B1, genotype 1 and 2, these cladded with species isolated from amphibians, as well as species described from the snake species, *Philothamnus natalensis natalenis* (Smith, 1848), collected in South Africa (Cook *et al*., 2018). This was observed as a larger sister clade to that of the larger clade consisting of *Hepatozoon* sp. A. *Hepatozoon* sp. B was isolated from only three individuals of *Di. typus*. This species of snake, as mentioned above, has a varied diet, but appears to prefer chameleons and other tree-dwelling lizards, along with birds and frogs, and rarely feeds on small mammals. *Ph. n. natalensis*, prefers both frogs and geckos. However, both are arboreal and diurnal snake species (Marais, 2004) and are thus likely to come across the same or similar prey items.

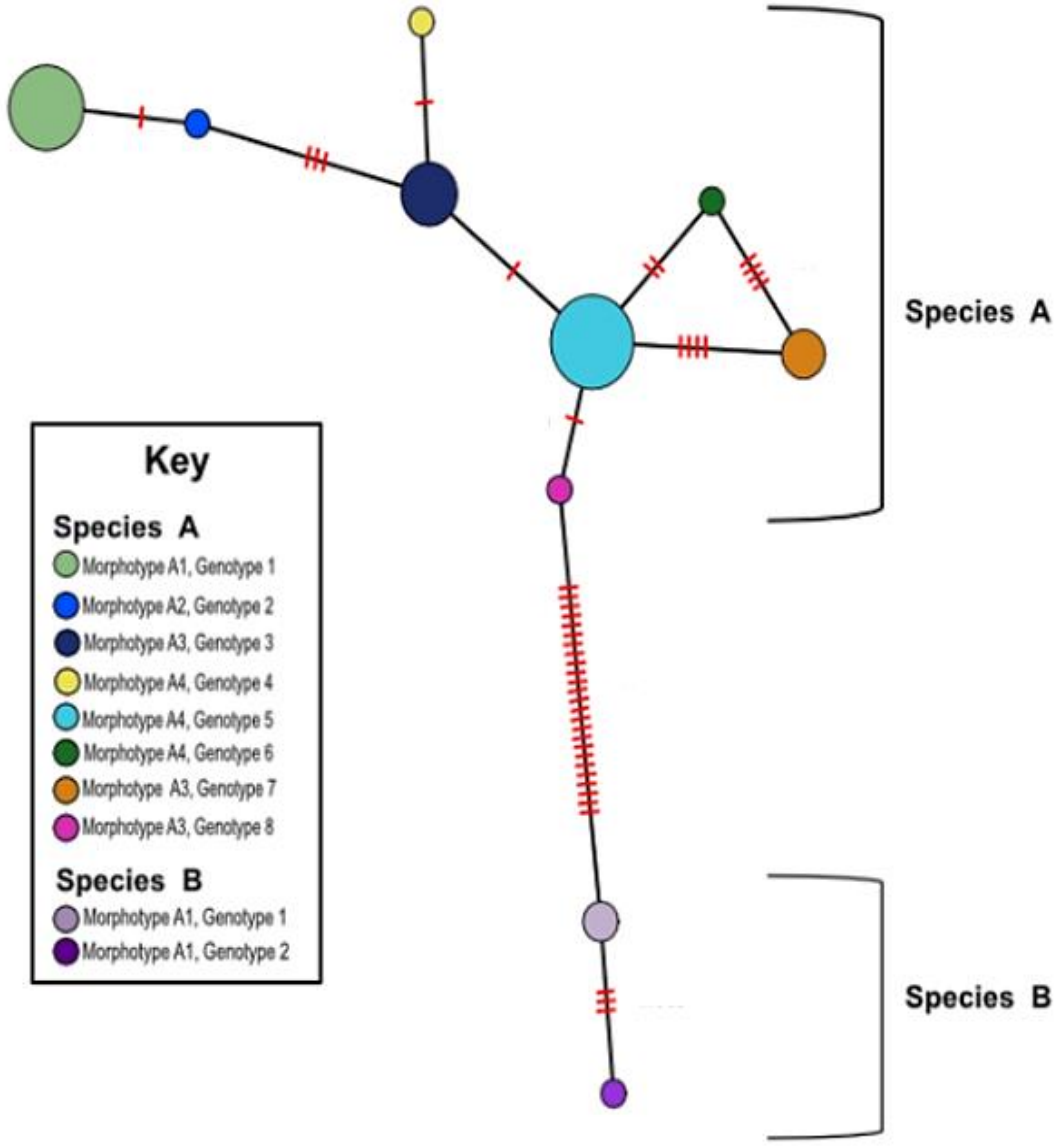


Figure 3.3: Haplotype network displaying the phylogenetic relationship between the obtained *Hepatozoon* sequences. The circles represent the genotype and the size of the circles reflect the number of sequences falling within the genotype. The percentage of divergence is indicated in red between the circles on the branches, with each hash mark representing a mutation. The sequences and their accompanying morphotypes and genotypes are summarized in the accompanying Table 3.3.

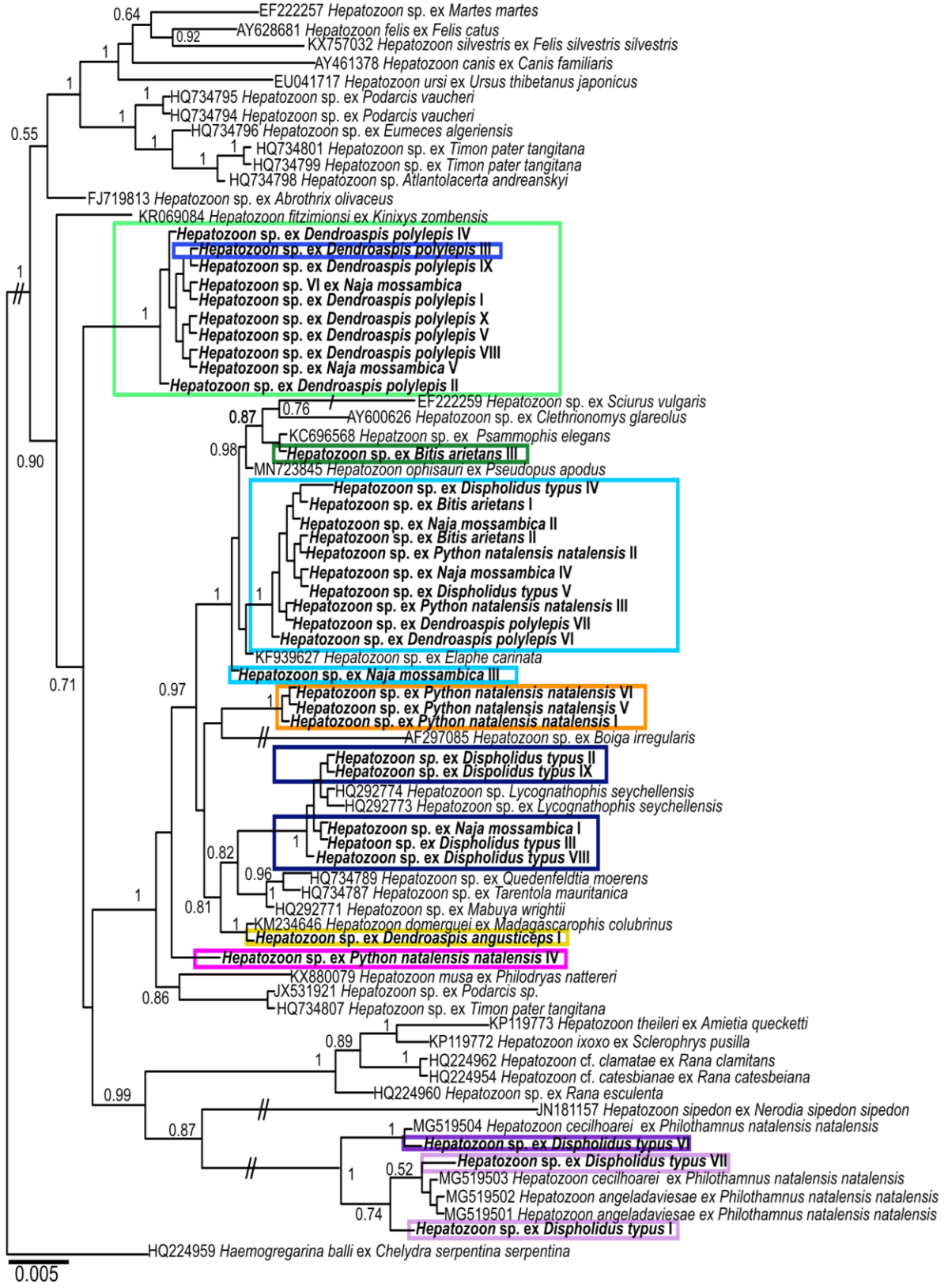
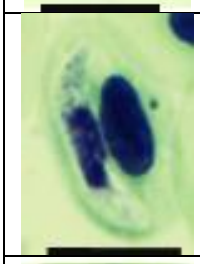
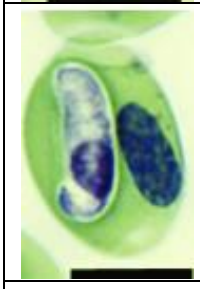


Figure 3.4: Bayesian Inference (BI) analysis of species of *Hepatozoon* extracted from snakes in Mpumalanga, Limpopo and KwaZulu-Natal provinces in South Africa and their relationships with other species of *Hepatozoon* based on 18S rDNA sequences. Node support values below 0.50 (50%) were not indicated in the tree. Branches were shortened according to the scale, each 'dash' representing a shortening of 0.005 substitutions per site. Scale = 0.005 nucleotide substitutions per site.

Table 3.3: Accompanying the haplotype network; this table shows species, genotypes and morphotypes of sequences that were obtained during this study. The colours correlate with those of the respective genotypes of the haplotype network in Figure 3.3 and the phylogenetic tree Figure 3.4.

	Photo Scale= 10 µm	Morphotype	Genotype	Hosts
Species A		A1	1	*Dendroaspis polylepis* I, II, IV, V, VIII, IX, X *Naja mossambica* V, VI
		A2	2	*Dendroaspis polylepis* III
		A3	3	*Dispholidus typus* II, III, VIII, IX *Naja mossambica* I
			7	*Python natalensis natalensis* I, V, VI
			8	*Python natalensis natalensis* IV
		A4	4	*Dendroaspis angusticeps* I
			5	*Dendroaspis polylepis* VI, VII *Dispholidus typus* IV, V *Bitis arietans* I, II *Naja mossambica* II, III, IV *Python natalesis natalensis* II, III
			6	*Bitis arietans* III
Species B		B1	1	*Dispholidus typus* I, VII
			2	*Dispholidus typus* VI

3.4 Discussion

All the captive snakes that were sampled during this study were initially collected from the wild. Once they were brought into captivity, and after spending time in quarantine, they were strictly fed captive bred rodents such as mice and rats. Additionally, day old chicken chicks were purchased and occasionally provided as food. All of the snake species that were sampled during this study were infected with species of *Hepatozoon.* There was an overlap between the parasites that were observed from captive snakes and those observed from wild snakes. Assuming that the feeder animals do not harbour *Hepatozoon* spp., it is believed that the captive snakes became infected as a result of their ecological habits prior to being placed in captivity (Glaser *et al*., 2008; Smith, 1996; Úngari *et al*., 2018). The finding that *Hepatozoon* spp. are more prevalent in some species can potentially be attributed to their typical diet preference and that larger snakes such as pythons consume a wider variety of prey throughout their lifetime, transitioning to larger mammals and reptiles when they reach larger sizes. The dietary infection route of *Hepatozoon* spp. in snakes can follow a complex life cycle. It requires the ingestion of infective stages via ingestion of infected prey animals such as lizards and is usually described as the three host life cycle, although the complete infection process still needs to be elucidated (Smith, 1996; Telford, 2009). It is also possible that some snakes are more resistant to infections and that *Hepatozoon* prevalence can reflect their basic ecology and diet (Tomé *et al*., 2012). There were few infections among snake species *B. arietans, D. angusticeps* and *N. annulifera.* This is interesting for *B. arietans* and *N. annulifera* when considering their wide distribution and that the diet of these snakes is very similar to that of *D. polylepis* and *N. mossambica*; these latter two species showing a higher *Hepatozoon* prevalence.

In contrast, *D. angusticeps* has a very small distribution range compared to all the other sampled snake species, it being largely restricted to the eastern coast coastal belt. Furthermore, it is also arboreal, feeding on small arboreal prey animals, such as chameleons (Marais, 2004; Alexander & Marais, 2007). Additionally, the sampling pool for *D. angusticeps* was also significantly smaller compared to the other snake species. As such, the finding that the *Hepatozoon* sp. in this snake showed a 0% divergence with *H. domerguei* from a Malagasy snake, which also includes chameleons in its diet, warrants further investigation. The data obtained during this study may to some degree support previous findings that the presence of *Hepatozoon* spp. in ophidian hosts is largely dependent on snake diet and ecological interactions in their natural environment (Allen *et al*., 2011 Sloboda *et al*., 2007, 2008; Smith 1996; Tomé *et al*., 2012, 2013, 2014, Úngari *et al*., 2018). However, it is difficult to assess the influence that prey-predator relationships have on *Hepatozoon* diversity without genetic

information of *Hepatozoon* species extracted from both prey and predator animals respectively. As such, there is insubstantial data to assess the impacts of prey-predator relationships on *Hepatozoon* diversity; this is due to unavailability of genetic information for species of *Hepatozoon* isolated from prey animals and snake hosts from the same geographical location. Therefore, the findings here may have identified a potential predator-prey system, which may be more feasible to study in the future.

Three *D. polylepis* individuals that were sampled were captive bred. These snakes originated from a gravid wild-caught female that laid eggs at the LVS facility (pers. comm. C. Hobkirk). The eggs were incubated, hatched and offspring were raised in captivity with their diet consisting out of appropriately sized captive bred rodents. All three of these individuals were negative for *Hepatozoon* spp. It is therefore, for the snakes from both facilities, unlikely that those found to be infected obtained these infections from their diets whilst housed at these facilities. It is more likely that infected snakes obtained these parasites as a result of their ecological behaviour in the wild prior to being placed in captivity. Most of the snakes were kept isolated from arthropod vectors and husbandry protocols at the facilities entail the screening for and removal of ectoparasites before snakes are moved to enclosures in captivity. The infection status of the parent snake of the three *D. polylepis* individuals that were raised in captivity is unknown, therefore there is insubstantial data to assess the possibility of vertical transmission for ovoviviparous (egg laying) snakes. Studies reported that vertical transmission of *Hepatozoon* spp. is possible in viviparous snakes (Kauffman *et al*., 2017). Four of the five captive *D. polylepis* individuals were positive with *Hepatozoon* spp. and two *Di. typus*, of which both were positive, were kept outdoors in wire cages that simulate their natural environment. Since it is unknown if these snakes were brought up in captivity, it is unclear if these infections were obtained prior to them being placed in captivity, or because there is a possibility of contact with possible haematophagous arthropod vectors or infected smaller reptiles, such as geckos, in their enclosures.

It appears that *Hepatozoon* spp. of snakes can survive in the system of their ophidian host for multiple years/seasons, as some of the captive snakes that were sampled during this study were in captivity for up to three years. This supports previous findings of positive *Hepatozoon* infections having been recorded after eight years in captivity in a *B. jararaca* individual (De Biasi *et al.,* 1989; Úngari *et al.,* 2018). To accurately measure the longevity of these parasites in the system of their host, and to be able to determine increased or decreased infection intensity over the years, it would be ideal to resample snakes over several years to be able to monitor these changes. There did not appear to be any clear differences between the prevalence of *Hepatozoon* spp. in captive and wild caught snakes. The overall prevalence of these parasites in captive snakes is lower than in wild snakes, but the sampling pool for wild

snakes was larger compared to captive snakes. The infection intensity was 0.01%–9.25% for wild snakes and 0.1–7.7% for captive snakes. However, this may be attributable to the possible persistence of these infections as mentioned above.

The haplotype network, in conjunction with the divergence data, indicated that the isolate from *D. angusticeps* belongs to a single species *Hepatozoon* sp. A. This species comprises eight genotypes, including that isolated from *D. angusticeps*, as well as those from five other snake species (*B. arietans, D. polylepis*, *Di. typus*, *N. mossambica* and *P. n. natalensis*). The morphotypes representing A1 and A2, and A3 and A4, may appear visually very similar respectively. However, morphometrics classes A1 and A3 as most similar, and A2 and A4 as most similar. Overall, however, the morphometrics (particularly size of gamont body and nucleus) between these morphotypes were similar, making it difficult to determine with any certainty, based on solely morphology, whether they represented the same or different species. Furthermore, the size of the gamont body and nucleus of *Hepatozoon* sp. B was similar to that of *Hepatozoon* sp. A, morphotypes A1 and A3. The morphological similarity of species of *Hepatozoon* gamont stages has been highlighted in the past (Vilcins *et al*., 2009). However, the current study also shows that there may be a degree of morphological plasticity, maybe in response to differing genotypes or the host species and/or geographical location, or potentially all three. It was noted during this study that *Hepatozoon* sp. A was observed in different hosts from completely separate geographical locations, and with different ecologies. However, even though collected from separate geographical localities, the distribution ranges of all snake species collected in this study overlap. Furthermore, even though these snakes may have different ecologies, some being strictly terrestrial such as *B. arietans* and others arboreal such as *D. angusticeps*, prey items appear to overlap overall. Similar findings were reported in previous studies where there was no apparent link between geography or host taxonomy (Tomé *et al*., 2012, 2013). This highlights the lack of knowledge about these parasites in terms of biodiversity and distribution, as well as the degree to which host-specificity or the lack thereof plays a role in the distribution of these ophidian species of *Hepatozoon*.

The true biodiversity of species of *Hepatozoon* infecting ophidian hosts are questioned, especially as many species were described based on their occurrence in a new snake host, in a new geographical location, and description was based solely on the morphology of the gamont stages (Sambon & Seligmann, 1907). Considering the low host-specificity displayed by *Hepatozoon* spp. towards the intermediate vertebrate hosts, as can be seen in this study and similarly to what has been found in other studies (Telford *et al*., 2004, 2008; Sloboda *et al*., 2007; Tomé *et al*., 2014), this was undoubtedly an erroneous approach. It remains

undeniable, however, that microscopic analyses allow for the observance of morphology, effects on host erythrocytes, and important factors, such as double infections that cannot be detected by molecular techniques alone. More importantly it allows for genetic information in the form of a nucleotide sequence to be joined with an accompanying visual representation of the parasite.

The assessment of *Hepatozoon* diversity is challenging on both a local and global scale. There is a cornucopia of *Hepatozoon* sequences in GenBank that are identified to genus level only. For the purpose of assessing *Hepatozoon* diversity with the use of the currently available phylogenetic framework, such sequences aid more as general occurrence records of *Hepatozoon* rather than support for elucidating phylogenetic relationships and species diversity. The implication is that the genetic data does not provide much, other than the confirmation of positive infections, as this data was not further analysed or combined with morphology so as to have a better understanding of species diversity. This, however, is likely the result of recent drastic changes in this field of research, such as deviating from species descriptions based on morphology alone, and the lack of molecular data for previously described species of *Hepatozoon*. The resolution of the phylogenetic framework, including the diversity and the relationships of that diversity, will likely require reassessment of past, formally described species, using a combined approach (morphology and molecular).

Besides the lack of detailed morphological assessments and life cycle/transmission route data, the majority of available sequences are based on amplification of the 18S rRNA gene. As the 18S is considered a slow evolving marker, it likely does not contain sufficient genetic information to provide a clear separation between closely related species, this resulting in the need to explore the use of additional genetic markers for molecular analyses of *Hepatozoon* (Morrison 2009; Tomé *et al*., 2014). At the same time, however, the present cut-off divergence of 3% (Schulz *et al*., 2019; Smit *et al*., 2020; Stoeck *et al*, 2009), delineating species, should be further explored for the 18S rRNA gene. Species of adeleorid apicomplexans have been delineated based on a divergence far lower than that of 3% for the 18S rRNA gene. For instance, the divergence between *Hepatozoon angeladaviesae* Cook, Netherlands, Van As, & Smit 2018 and *Hepatozoon cecilhoarei* Cook, Netherlands, Van As, & Smit 2018, collected from *Philothamnus* spp. was 1% (Cook *et al*., 2018). Between *Dactylosoma* species collected from amphibians it was 0.5%, one species from an amphibian from Europe, the other from an amphibian in South Africa. In fact, interspecific divergence values within other haemogregarine genera (*Haemogregarina*, *Hemolivia* and *Karyolysus*) is 1% and below (Netherlands *et al*., 2020). Even looking at the divergence between genera, the values are low, for instance between a species of *Hepatozoon* and *Hemolivia*, and *Haemogregarina* and

Hepatozoon, values are 2% and 4% respectively (see Cook *et al*., 2015). Considering that the various genotypes of *Hepatozoon* sp. A claded separately and often distantly from one another in the phylogenetic analysis, it further questions whether the 3% cut off divergence is entirely applicable to adeleorid parasites at the 18S rRNA gene level.

There are few studies where the reptile vertebrate host sampling pool constitutes a large number of individuals belonging to the same species or different species of the same reptilian suborder (Serpentes), much less from the same larger geographical location. In this study, the snake hosts' distribution overlapped to a large degree (excluding *D. angusticeps*), thus allowing the assessment of these parasites in sympatry, in a diversity of snakes. This consequently enabled the comparison of *Hepatozoon* spp. found in different snake hosts, allowing the exploration of some related ecological factors, while also assessing morphological and genetic data of species of *Hepatozoon* extracted from different snake host species. From what was observed in terms of host-specificity and ecology, it is clear that there are many aspects that require better understanding regarding these parasites. If species are to be described, caution should be exercised to validate these descriptions with molecular and other information such as ecological assessments to promote the resolution of phylogenetic relationships. There is also a need to better study the arthropod vectors in order to elucidate life cycles and vector ecology and distribution (Tomé *et al*., 2014).

As the international trade of reptiles is increasing, another concern is that if the translocation of these parasites escalates, it will become difficult to elucidate their true ecology inferred by their natural habitats; particularly as they appear to have such a low vertebrate host-specificity. Reptiles that are exported as part of the exotic pet trade are frequently infected with haemoparasites and it was also discovered in some cases that these reptiles are infested with ticks and mites (Burridge, 2001; Halla *et al*., 2014; Kenny *et al*., 2004; Pietzsch *et al*., 2006). There is concern that translocated ectoparasites may be able to establish new populations if conditions are favourable, consequently contributing to the spreading of haemoparasites (Halla *et al*., 2014). It was recently discovered that ticks are likely implicated in the life cycle of *Hepatozoon fitzsimonsi* (Dias, 1953) infecting tortoises (Cook *et al*., 2014; Mofokeng *et al*., 2021; Omondi *et al*., 2017). One such tick species *Amblyomma marmoreum*, the African tortoise tick, was found to have established a population in Florida, USA, most likely due to the reptile pet trade (Burridge *et al*., 2000). Considering how little is known about invertebrate vectors of *Hepatozoon* spp. associated with reptiles and the seemingly low host-specificity displayed by many snake *Hepatozoon* spp., all the reptile associated invertebrates need to be considered as potential vectors and treated as such regarding the spreading of *Hepatozoon* spp. All of the sampled snakes, on a superficial level, appeared to be in good condition and

were thus considered seemingly healthy. This is to be expected from snakes that are kept for the purpose of venom production or display, and are dewormed annually and fed regularly. The presence of blood parasites in captive snakes indicates that the medications that are generally used for deworming and other ailments in snakes are insufficient for the removal of haematozoan parasites, or in this case *Hepatozoon* spp. specifically. A few wild-caught individuals had scarring or mild skin conditions and it was difficult to determine if these were as a result of some sort of systemic infection or physical injuries obtained in the wild. No health data was available for the BioBank samples obtained from the Hans Hoheisen Research Station. However, even with the seemingly sustained infections of these haemoparasites, no major impacts were evidently visible first hand. The most distinct effects were only apparent when observed on a cellular level by the displacement of the host erythrocyte nucleus by the parasite, cell enlargement and cell lysis, which was only observed to be caused by *Hepatozoon* sp. B that occurred in captive and wild *Di. typus*.

3.5 Conclusion

The low host-specificity, and the possible transmission of *Hepatozoon* between different snake species, suggest possible difficulties for assessing the diversity of these parasites. The elucidation of their true diversity will likely require broad scale sampling of many different snake species from different geographical locations to link distribution to genetic composition. Furthermore, if a faster evolving marker, for use alongside the 18S, is not made available and widely used in the near future, further efforts in determining whether or not the cut-off divergence is in fact 3% is required. Even though it is not encouraged to describe species based on unique morphological characteristics or even differences in gamont size and effects on host cells, it would also be unwise to synonomise species based on a divergence, which in reality does not apply to these parasites. Increased sampling of southern African snake species, as well as prey animals of snakes with different ecological requirements, is encouraged. This will potentially allow better assessment of prey-predator transmission routes of this region. Consequently, it will potentially aid in elucidating the associations between the parasites and their respective hosts, ultimately aiming to clarify their evolutionary histories. It is likely, that the currently represented phylogenetic relationships will change as the genetic identities of already discovered species are fully clarified and as more species are discovered and included in these analyses.

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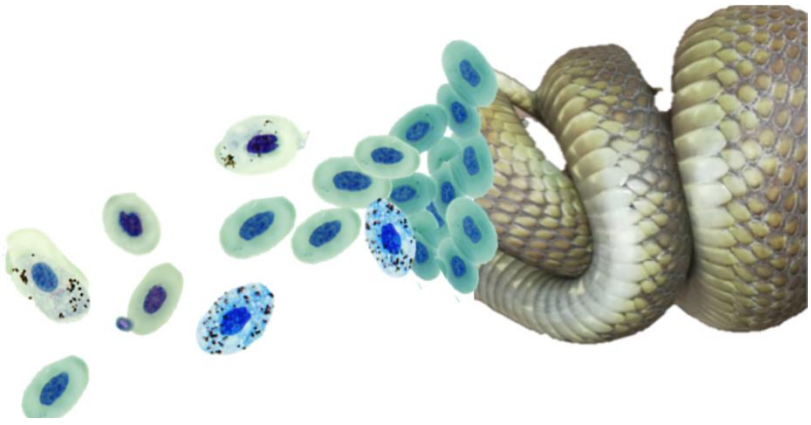
Chapter 4

Haemoproteid diversity in captive and wild cobras in the Mpumalanga province of South Africa

The whole is more than the sum of its parts.

~ Aristotle



Chapter 4: Haemoproteid diversity in captive and wild cobras, *Naja mossambica* and *Naja annulifera* from the Mpumalanga province of South Africa

4.1 Introduction

Members of Haemoproteidae Doflein, 1916 infect both erythrocytes and leucocytes (Cook *et al.,* 2010; Lainson & Naiff, 1998). Studies of haemosporidian parasites have mainly been focused on the genus *Plasmodium* Marchiafava & Celli, 1885, which infects a wide variety of hosts. The majority of interest in this genus is associated with a desire to understand *Plasmodium falciparum* Laveran, 1880, which causes human malaria (Boudenga *et al.,* 2017; Perkins, 2014). However, in order to understand the emergence of disease and parasite life-history traits, expanded sampling, including more host species, is necessary for stabilising phylogenetic relationships (Borner *et al.,* 2016; Boudenga *et al.,* 2017; Lefèvre *et al.,* 2007). Haemosporidian genera infecting chelonians and squamates, such as *Haemocystidium* Castellani & Willey, 1904, have been less studied (Javanbakht *et al.,* 2015; Pineda-Catalan *et al*., 2013) compared to human malaria parasites (*P. falciparum*; *Plasmodium malariae* Golgi, 1886; *Plasmodium ovale* Stephens, 1918; *Plasmodium vivax* Laveran, 1880 and *Plasmodium knowlesi* Garnham, 1957) (Boudenga *et al.,* 2017) and *Haemoproteus* Kruse, 1890 spp. infecting birds (Bennet *et al*., 1965; Levin *et al*., 2011; Levine & Campbell, 1971; Martinsen *et al*., 2008). Unfortunately, there is a lack of information regarding the diversity and distribution of reptile associated haemosporidian parasites thereby making it logistically difficult to study and measure the impacts and effects on hosts (Boudenga *et al.,* 2017; Gonzálelez *et al.,* 2019; Javanbakht *et al.,* 2015; Maia *et al.,* 2016;; Pacheco *et al.,* 2018; Telford, 2008; Valkūnas *et al.,* 2008). Malaria parasites that infect reptiles (lizards, snakes and chelonians) belonging to the order Haemosporidia (Danilewsky 1885) were classified into families; Haemoproteidae (*Haemocystidium*, and *Haemoproteus*), Plasmodiidae Mesnil, 1903 (*Plasmodium*) and Leucocytozoidae (*Saurocystozoon* Lainson & Shaw, 1969) (Lainson *et al.,* 1974; Van As *et al.,* 2016).

The taxonomy of reptile haemoproteid parasites is considered to be more unstable than that of avian haemoproteids (Pineda-Catalan *et al*., 2013). The status of the genus *Haemocystidium* is controversial as it was first considered synonymous to *Plasmodium* and *Haemoproteus* (Castellani & Willey, 1904). There are also limited reports and studies of

Haemocystidium worldwide, which contributes to fragmentary acceptance of this genus in the scientific community (Gonzálelez *et al*., 2019; Javanbakht *et al*., 2015).

As with many other apicomplexan parasites, coalescence regarding the life cycles of reptile associated haemosporidians can perhaps be found by studying the invertebrate vectors and transmission routes more extensively (Javanbakht *et al*., 2015). This led to taxonomical decisions in the past, where it was suggested that avian *Haemoproteus* should be split into two separate genera namely *Haemoproteus*, transmitted by hippoboscid flies and *Parahaemoproteus,* transmitted by culicid midges. However, this was opposed by Levine and Campbell (1971), as the split was considered too extreme and little knowledge of the vectors were known.

To date, in terms of haemosporidian diversity, avian species have generally received more attention than those infecting reptiles (Bennet *et al.,* 1965; Levin *et al.,* 2011; Levine & Campbell 1971; Martinsen *et al.,* 2008). Haemoproteids have close affinities to true *Plasmodium* spp. (or true malarids), which emphasizes the need to conduct research on these organisms in order to better understand their ecology and evolution, especially since 'true malaria' parasites are notorious for causing mortality in a variety of taxonomic groups (Boudenga *et al.,* 2017). Reptilian malaria or malaria-like (haemoproteids) parasites are largely considered non-pathogenic; however, cases of severe anaemia have been reported and some *Plasmodium* spp. are considered to cause blood coagulation in reptile capillary beds (Frye, 1991; Jacobson, 2007). It was also discovered that some *Plasmodium* spp. such as *Plasmodium agamae* Wenyon, 1909 and *Plasmodium giganteum* Theiler, 1930, which infect the common Agama (*Agama agama* (Linnaeus, 1758)), can have physiological effects on hosts, consequently affecting host fitness and behaviour (Jacobson, 2007; Schall, 1990a; 1990b, 1996). This results in lowered reproductive success and ultimately has the potential to lead to lowered survivorship (Jacobson, 2007). Recently, during a revision of the haemoproteid genera, it was suggested that *Haemoproteus* spp. infecting reptiles belong to the genus *Haemocystidium* (Van As *et al.,* 2016; Pineda-Catalan *et al.,* 2013). It was further suggested by Pineda-Catalan *et al.* (2013) that *Haemocystidium* infecting reptiles should undergo subgeneric division, where *Haemocystidium* should be assigned to parasites of squamates and *Simondia* Garnham, 1966 to those infecting chelonians.

Differentiation of the *Haemoproteus* and *Haemocystidium* genera, among others, were previously based only on morphologies of erythrocytic stages and the developmental stages in the tissues of hosts and within the heamatophagous vectors (Davies & Johnston, 2000; Van As *et al*., 2016). Morphological analyses are still considered an indispensable tool, as they allow the identification of different life stages that aid in the elucidation of life cycles, and the

identification of double infections, which are undetectable or otherwise complicated by molecular analyses alone. However, molecular tools alleviated a great deal of the taxonomic uncertainties regarding haemoproteids. Unfortunately, however, many morphologically and formally described *Haemoproteus* and *Haemocystidium* species are not accompanied by molecular information and only morphological characteristics of some of their life stages are provided. This in turn leaves their true taxonomic placements uncertain along with their phylogenetic relationships to one another unknown. It is also possible that the morphology of organisms can change over time (Perkins *et al*., 2011), and that cryptic species may exist that display similar morphological characteristics, but are genetically dissimilar (Perkins, 2000).

The heterogenous life cycle of these parasites in reptiles include the involvement of two hosts, a haematophagous invertebrate (suspected haematophagous dipterans) host and a vertebrate (reptile) host. Sporogony takes place within the invertebrate host, where the infective sporozoite stages are transmitted to reptile hosts during feeding (Jacobson, 2007; Telford, 1984). Asexual life stages such as gametocytes, meronts (in only *Plasmodium* spp.), and trophozoites can be observed in the peripheral blood (Jacobson, 2007).

Haemoproteid species reported to infect African snakes are *Haemocystidium mesnili* (Bouet 1909) Wenyon 1926 (formerly known as *Haemoproteus mesnili*) and *Haemoproteus balli* Telford, 2007. Both of these species were recorded from African cobras (Serpentes: Elapidae). *Haemocystidium mesnili* was first described as *Plasmodium mesnili* bt Bouet (1909) from either a species of *Naja* Laurenti, 1768 or a Rinkhals (*Hemachatus haemachatus* Bonnaterre, 1790) from Odienne, Ivory Coast and Gaoua, Upper Volta, respectively.In the same year, Wenyon (1909) recorded *Haemocystidium najae* from the Black-necked Spitting Cobra (*Naja nigrocollis* Reinhardt, 1843) in Sudan, based on drawings of live specimens in fresh blood. Wenyon (1926) gave priority to Bouet's (1909) description over his own that year and referred to the parasite as *Haemocystidium mesnili.* Additional reports of *H. mesnili* infecting cobra species were made by Macfie (1919); Leger and Leger (1914); Garnham, (1966), Ball (1967) (see Telford, 2007) and Batelli (1949) (see Telford, 2007).

Telford (2007) re-described *H. mesnili* from *N. nigrocollis*, from a location in Tanzania, and as such, not from the type locality in Odienne, Ivory Coast. This author also found that the species recorded by Ball (1967) was not *H. mesnili*, but an additional species that he later described and named *Haemoproteus balli.*

More recently, *H. mesnili* was recorded from a Snouted Cobra (*Naja annulifera* Peters, 1854) from the Kruger National Park located in the Mpumalanga and Limpopo provinces of South Africa. However, this is the first molecular information of *H. mesnili* and there appears to be no morphological information to support its identification. Species distinction between the

above-mentioned haemoproteids infecting African cobras were made based solely on morphological descriptions. The vectors for both these species are unidentified. It is clear that there are still uncertainties regarding the classification of discovered snake haemoproteids and also a major lack of information regarding their diversity, distribution, and vectors.

Although infections of these mentioned haemoproteids in cobras are highly prevalent in several African countries (Telford 2007), little is known about the life cycle and vectors of these parasites. It is reported by Telford (2007) that *H. mesnili* is described only from its erythrocytic stages and that little to nothing is known about its life cycle. The invertebrate hosts for *Haemoproteus* spp. are reported to be hippoboscid, ceratopogonid, and tabanid flies (Davies & Johnston 2000), while invertebrate hosts for *Haemocystidium* spp. are unknown, but it is suspected to also be flying insects (Davies & Johnston, 2000). This was found to be the case for both the above-mentioned snake haemoproteids, where the only concrete available information includes their vertebrate hosts, locality and morphology and morphometrics of their life stages.

The description of haemoproteid species based on morphological observations alone is questionable, especially since there are clear taxonomical instabilities in the available literature. It is crucial that morphological descriptions are accompanied by molecular data, to be able to better approach larger questions such as the biodiversity, ecological roles, pathogenicity, host specificity, and intraspecific variability of these parasites. However, as there are so few records and morphological descriptions available for *Haemocystidium* species infecting snakes, it is also suggested that molecular data should be accompanied by morphological data and supporting information. As these morphological data are available, it is not wise to identify a species based on molecular information alone. There needs to be a combined morphological and molecular approach, with knowledge on the host and type locality taken into consideration. In this chapter the haemoproteid isolates from snakes are referred to as *Haemocystidium.*

The aims of this chapter were to (i) assess the diversity and prevalence of haemosporidian parasites infecting snake species of southern Africa from the Mpumalanga, Limpopo and KwaZulu-Natal provinces in South Africa, based on molecular and microscopic analyses, and (ii) compare the prevalence and diversity of haemospordian parasites between snakes that were recently caught and moved to captivity, captive for a long term, and snakes that were sampled from the wild.

4.2 Methods

4.2.1 General sample information

Samples listed in Chapter 3 (Table 3.1) were utilized for the haemoproteid analysis. The snake husbandry (Chapter 3, section 3.2.1.1) and sampling procedure (Chapter 3, section 3.2.2) are as described in the previous chapter. Wild snakes and BioBank samples, as described in Chapter 3 (section 3.2.1.2), were also utilized during the screening for haemoproteid parasites.

4.2.2 Preparation and screening of blood smears

Samples that were positive for haemoproteids were identified during the collective screening for apicomplexan parasites as described in the previous chapter. Of all the sampled snake species, only the cobras, namely *Naja mossambica* Peters, 1854 and *N. annulifera*, were infected with haemoproteids. The parasitaemia was determined by selecting random counting sites in the slides and determining the number of haemoproteids per ~10^4 erythrocytes, expressed as percentage (Van As *et al*., 2016). The blood smears were examined with the 60× magnification lens, equipped with immersion oil on a calibrated Nikon Eclipse N*i* (Nikon, Amsterdam, Netherlands) compound microscope for the detection of parasites. Photos of the parasites were taken with 100× oil immersion lens at 1000× magnification using the accompanying digital camera and NIS-Elements BR Ver. 4.60 camera analysis software (Nikon, Tokyo, Japan). Parasite morphometrics were measured with the aid of ImageJ software (https://imagej.nih.gov/ij/) and all the measurements were recorded in µm unless stated otherwise. Measurements included total length (TL); total width at the widest part (TW); circumference (CIR), and surface area (SA) recorded in μm^2.

4.2.3 Molecular analysis

DNA was isolated from whole blood samples and from blood spots on FTA® cards (stored at room temperature) with the use of the Machery-Nagel mini kit for DNA from cells and tissue following the standard protocol. Isolated DNA was stored at -20 °C until further processing. The DNA isolates were used as template for PCR, which was performed by amplifying two separate fragments of the cytochrome-*b* (cytb) gene. The primer set HAEMNF (5′-CAT ATA TTA AGA GAA TTA TGG AG-3′) and HAEMNR2 (5′-AGA GGT GTA GCA TAT CTA TCT AC-3′) (Waldenström *et al.,* 2004) was used for the first fragment (~ 500 bp) under the following

PCR conditions: initial denaturation at 94 °C for 3 min, followed by 20 cycles, entailing a 94 °C denaturation for 30 s, annealing at 50 °C for 30 s with an end extension at 72 °C for 45 s, a final extension of 72 °C for 10 min. The second fragment (~ 500 bp) was amplified using the primer set HAEMF (5′-ATG GTG CTT TCG ATA TAT GCA TG-3′) and HAEMR (5′- GCA TTA TCT GGA TGT GAT AAT GGT-3′) (Waldenström *et al.,* 2004). The PCR conditions for the second fragment were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles, entailing a 94 °C denaturation for 30 s, annealing at 50 °C for 30 s with an end extension at 72 °C for 45 s, and a final extension of 72 °C for 10 min. All PCR reactions were performed using the SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Singapore) and reaction volumes were set at 25 µl where the reagents and their respective volumes were as follows: 12.5 µl Thermo Scientific DreamTaq PCR master mix (2×) (2× DreamTaq buffer, dATP, dCTP, dGTP and dTTP, 0.4 mM each and 4 mM $MgCl_2$), 1.25 µl of forward and reverse primer (10 µM) and 25 ng of DNA (1 µl). PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania) was used to make up the reaction volume (9 µl). The PCR products were loaded in 1% agarose gel aligned to a 1kb DNA ladder The gel was run at 90 V for 30 min and amplicons were viewed under UV light. The amplicons that were identified were sent to Inqaba Biotec Company (Pretoria, South Africa) for purification and they were sequenced in both directions.

4.2.4 Phylogenetic analysis

Reverse and forward primer nucleotide sequences were assembled with the use of Geneious ver. 11.1.4 (http://www.geneious.com, Kearse *et al.,* 2012). The Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1990) was then used on NCBI GenBank to compare and determine the closest matches of the sequences to those deposited in GenBank. All of the sequences that were obtained during this study matched closest with *H. mesnili* (Accession number: KF049514), formerly known as *Haemoproteus mesnili*. A total of nine haemoproteid sequences were obtained; one from a captive and six from wild *N. mossambica* individuals and one from a captive and one from a wild *N. annulifera* individual.

These sequences were used for the construction of the phylogenetic tree along with 30 sequences that were obtained from GenBank (Table B.1). *Leucocytozoon gentili* (DQ451435) and *Leucocytozoon majoris* (DQ451439) were used as outgroup following Van As *et al.* (2016). The sequences were aligned using the MUSCLE tool using MEGA X (https://www.megasoftware.net/).

Phylogenetic analysis was performed by implementing Bayesian Inference using the CIPRES (https://www.phylo.org/) platform where the Mr Bayes on XSEDE (MrBayes 3.2.2

Huelsenbeck & Ronquist, 2001) tool was used. The data block was enabled, and runtime was set as eight hours. The MrBayes tool entailed that the analysis was run twice over 10 million generations for the Markov Chains Monte Carlo (MCMC) algorithm. The Markov chain was sampled every 100 cycles, and the MCMC variant contained 4 chains.

In order to determine the most suitable substitution model, the Akaike information criterion (AIC) tests were performed with the use of jModelTest 2.1.10 (Guindon & Gascuel, 2003; Darriba*et al.,* 2012). The best model for both was the General Time Reversible model with estimates of invariable sites and gamma distributed among site-rate variations (GTR+I+G). The maximum likelihood (ML) analysis was performed using the PhyML 3.0 platform (http://www.atgc-montpellier.fr/phyml/) and the substitution model was set as GTR with 1000 bootstrap inferences (Guindon *et al.,* 2010; Guindon & Gascuel, 2010).

Both ML and BI outputs were exported, the trees were viewed using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/) and re-rooted according to the chosen outgroup. The topographies for the ML and BI trees were highly similar, however, the ML values for some branches were low compared to the BI values. The ML and BI values were combined, using the topology produced by the BI tree (Fig. 4.3). The respective values are indicated at the nodes as (ML/BI), where ML values of less than 50 are not indicated.

Estimates of evolutionary divergence between sequences were determined using MEGA X (Kumar *et al.,* 2018). The number of base differences per site from between sequences are shown. This analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 536 positions in the final dataset. The results of the analysis can be observed in Table 4.3.

4.3 Results

4. 3.1 General observations of *Haemocystidium* spp. in captive and wild snakes

A total of 154 snakes (82 wild, 62 captive) of five genera and seven species were sampled and screened for the presence of species of *Haemocystidium*. The overall prevalence of *Haemocystidium* in all snake species was 10/154 (6.5%). Of all the snake species that were screened, only individuals of the two sampled cobra species, *N. mossambica* and *N. annulifera* were infected with *Haemocystidium* (Table 4.1). The overall prevalence in *N. mossambica* was 8/28 (28.6%) and for *N. annulifera* the overall prevalence was 2/30 (6.7%). There were no clear differences between the prevalence of wild and captive snakes, with a prevalence of 6/20 (30%) for wild and 2/8 (25%) for captive *N. mossambica* and 1/20 (5%) for wild and 1/10 (10%) for captive *N. annulifera.* For both these snake species, the sampling pool for captive and wild individuals were different, preventing clear comparison of prevalence between captive and wild individuals. The captive and wild *N. annulifera* individuals that were positive with *Haemocystidium* were both from White River, in the Mpumalanga province, with parasitaemias of 1.3% and 2% respectively. The two captive *N. mossambica* individuals were from Nelspruit (0.2%) and White River (1.75%) respectively, where both locations are situated in the Mpumalanga province. Of the positive wild *N. mossambica*, two were from Hazyview (0.4 and 2.7%), one from Nelspruit (1.6%), two from White River (14% and 19.5 %) and one from Marloth Park (5.5%); all of these locations are also in the Mpumalanga province.

Table 4.1: Prevalence of *Haemocystidium* spp. in wild and captive snakes respectively, and their collective prevalence within each species. MP=Mpumalanga, LP=Limpopo and KZN=KwaZulu-Natal. The estimated time in captivity for captive snakes is also indicated.

Host species	Sampling site	Wild	Captive	Per species
Dendroaspis polylepis	Nelspruit (MP)[2] White River (MP)[2] Hazyview (MP)[2] Marloth Park (MP)[2]Hoedspruit (Lim)[2] Jejane, KNP(MP)[3]	0/11 (0%)	0/12 (0%)	0/23 (0%)
Dendroaspis angusticeps	KZN (Unspecified)[2]	0/1 (0%)	0/10 (0%)	0/11 (0%)
Dispholidus typus	Nelspruit (MP)[2] White River (MP)[3] Malelane (MP)[3] Berberton (MP)[3] Hoedspruit (Lim)[2]	0/9 (0%)	0/13 (0%)	0/22 (0%)
Bitis arietans	Nelspruit (MP)[2] White River (MP)[2] Sterkspruit Nature Reserve (MP)[1] KZN (Unspecified)[1] Marloth Park (MP)[3] Malelane (MP)[3] Sabie (MP)[3]	0/10 (0%)	0/8 (0%)	0/18 (0%)
Naja mossambica	Nelspruit (MP)[2] Whiter River (MP)[1] Hazyview (MP)[2] Marloth Park (MP)[1] Malelane (MP)[3] Hoedspruit (Lim)[1]	6/20 (30%)	2/8 (25%)	8/28 (28.6%)
Naja annulifera	Nelspruit (MP)[2] White River (MP)[2] Hazyview (MP)[2] Malelane (MP)[3] Kanyamazane (MP)[1]	1/20 (5%)	1/10 (10%)	2/30 (7%)
Python natalensis	Nelspruit (MP)[1] White River (MP)[1] Hazyview (MP)[1] Marloth Park (MP)[1] Schoemanskloof (MP)[1] Hoedspruit (Lim)[1] Undetermined site[3]	0/16 (0%)	0/1 (0%)	0/17 (0%)

Only Wild caught[1]; Captive and Wild caught[2]; Only Captive[3]

4.3.2 Phylogenetic tree

Molecular analyses of the haemosporids observed infecting both cobra species in this study were identified as *Haemocystidium mesnili*.Sequences obtained during this study from both *N. annulifera* and *N. mossambica*, were 99.71–99.91% similar, with a divergence of 0%. Collectively, in comparison to *H. mesnili* (KF049514) on GenBank, they were 99.6–99.91% similar, with a divergence of 0%. As such, the phylogenetic results will be discussed first. The sequences that were obtained from *N. mossambica* and *N. annulifera* are observed clustering with *H. mesnili* (KF049514) in the phylogenetic tree (Fig. 4.3), basal to a larger clade containing other species of *Haemocystidium*, *Haemoproteus* and *Plasmodium*, with high nodal support. They grouped separately from *Haemocystidium* species (*H. ptyodactyli* and *H. kopki*) that were isolated from lizards, such as the Fan-footed Gecko (*Ptyodactylus hasselquistii* Donndorff, 1798), and the Frog-eyed Gecko (*Teratoscincus scincus* Schlegel 1858) respectively.

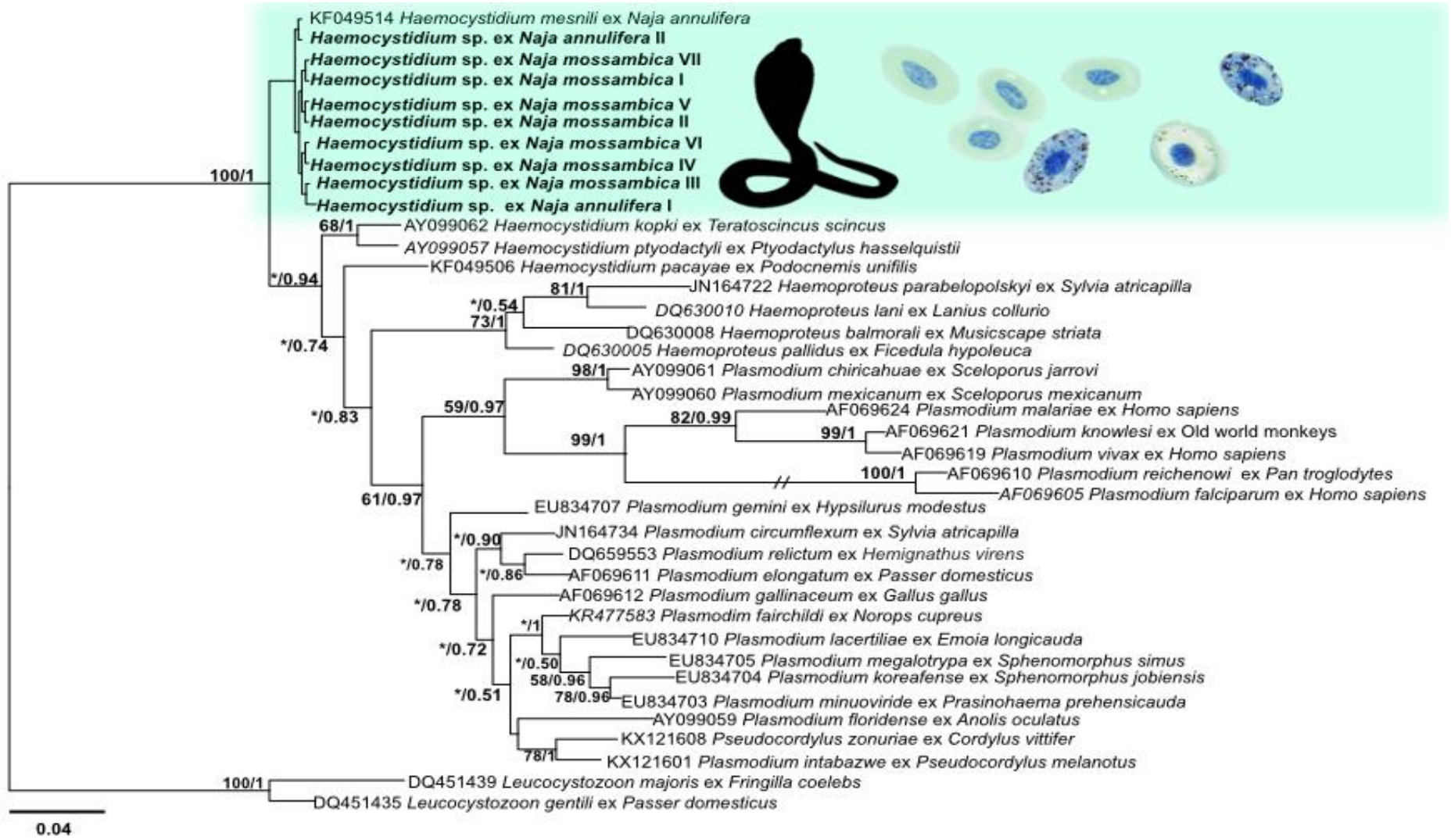


Figure 4.1: Maximum Likelihood (ML) and Bayesian Inference (BI) analysis of *Haemocystidium* species extracted from cobras in the Mpumalanga province of South Africa and their relationships with other haemoproteids based on the mitochondrial cytochrome *b* gene region (cyt *b*). The tree topologies for the ML and BL trees were highly similar. The nodal support values are indicated as bootstrap percentages for the ML tree and posterior probability for the BI tree (ML/BI). ML values below 50 are indicated as a '*' on the tree. Scale = 0.04 nucleotide substitutions per site. Hatchmark on branches represents shortening of the branch by 0.04 nucleotide substitutions per site.

Table 4.2: Evolutionary divergence between *Haemocystidium* sequences isolated from the cytochrome *b* (cyt *b*) gene region of *Naja mossambica* and *Naja annulifera* from the Mpumalanga province of South Africa, expressed as percent similarity (%) (bottom, left) and uncorrected pair-wise distance (p-distance) (top-right).

	Species	1	2	3	4	5	6	7	8	9	10
1	*Haemocystidium* sp. ex *Naja annulifera* I		0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	*Haemocystidium* sp. ex *Naja mossambica* I	99.71		0.00	0.00	0.00	0.00	0.00	0.00	0.0	0.00
3	*Haemocystidium* sp. ex *Naja mossambica* II	99.91	99.71		0.00	0.00	0.00	0.00	0.00	0.00	0.00
4	*Haemocystidium* sp. ex *Naja annulifera* II	99.91	99.71	99.91		0.00	0.00	0.00	0.00	0.00	0.00
5	*Haemocystidium* sp. ex *Naja mossambica* III	99.91	99.71	99.91	99.91		0.00	0.00	0.00	0.00	0.00
6	*Haemocystidium* sp. ex *Naja mossambica* IV	99.91	99.71	99.91	99.91	99.90		0.00	0.00	0.00	0.00
7	*Haemocystidium* sp. ex *Naja mossambica* V	99.91	99.71	99.91	99.91	99.90	99.90		0.00	0.00	0.00
8	*Haemocystidium* sp. ex *Naja mossambica* VI	99.91	99.71	99.91	99.91	99.90	99.90	99.90		0.00	0.00
9	*Haemocystidium* sp. ex *Naja mossambica* VII	99.91	99.71	99.91	99.91	99.90	99.90	99.90	99.90		0.00
10	KF049514 *Haemocystidium mesnili* ex *Naja annulifera*	99.91	99.60	99.91	99.90	99.90	99.90	99.90	99.90	99.90	

4.3.3 Description of stages within the peripheral blood

Class: Aconoidasida Mehlhorn, Peters & Haberkorn, 1980
Order: Chromatorida Euzéby 1988
Family: Haemoproteidae Doflein, 1916
Species: *Haemocystidium meslini* (Bouet 1909) Wenyon 1926

Type host: *Naja nigricollis nigricollis* Reinhardt, 1843 (Serpentes: Elapidae).
Other hosts: *Haemachatus haemachatus* Bonnaterre, 1790 (syn. *Sepedon haemachatus* Merrem, 1820), *Naja annulifera* Peters, 1854, *Naja haje* (L.)*, Naja mossambica* Peters, 1854.
Type locality: Odienne, Ivory Coast (Bouet, 1909).
Other localities: North slope of Uluguru Mountains at edge of Sokoine University campus, Morogoro, Tanzania (06°51'S, 37°38'E) (Telford, 2007); Gaoua, Upper Volta (Bouet, 1909); Bamako, Mali (Leger and Leger, 1914) (see Telford, 2009); Accra, Ghana (Macfie, 1919) (see Telford, 2009); Deleib Hill and Nasser on River Sobat, Sudan (Wenyon, 1909) (see Telford, 2009); Kruger National Park, Limpopo and Mpumalanga, South Africa (Pineda-Catalan *et al*., 2013); Hazyview, Marloth Park, Nelspruit, White River, Mpumalanga, South Africa (this study).

4.3.3.1 Material observed in *Naja annulifera* (morphotype A)

***Locality:** White River (-25.329929; 31.016308), Mpumalanga, South Africa.

Site in host: Peripheral blood.
Prevalence: 7% (2/30)
Parasitaemia: 1.3% for captive individual caught in White River in 2018 and 2% for wild individual from White River.
Vector: Unknown.
Representative DNA sequence(s): Two partial sequences of the 18S rRNA gene, 573 bp and 571 bp in length respectively.

Description: Smaller immature gametocyte stages: Rounded to circular or amoeboid in shape, n=21 (Fig.4.1 B–F). Cytoplasm staining white to light pink with yellow brown granules, or staining deep purple with larger dark brown granules and white vacuoles in the cytoplasm. Granules either dispersed in the cytoplasm or more often concentrated at one of the ends. Rounded stages most prevalent (Fig. 4.1 B–D), circular stages less often observed (Fig. 4.1 E). Amoeboid stages also observed (Fig. 4.1 F–G). White vacuoles occasionally observed

(Fig. 4.1 C). Nucleus not visible in immature stages. Erythrocyte with double infection, containing deep purple and light-stained amoeboid stages (Fig. 4.1 F) Immature stages measured 16.14 ± 4.93 (8.89–27.217) long and 6.54 ± 2.47 (1.90–12.64) wide with a circumference of 38.50 ± 25.81 (20.21–56.43) and surface area of 84.77 ± 8.84 (20.21–130.82) μm^2. White to light pink immature stages usually develop into microgametocytes while those staining dark purple develop into macrogametocytes.

Immature microgametocytes: Rounded in shape, n=29 (Fig. 4.1 H–I), poles slightly bent around nucleus (Fig. 4.1 H). Cytoplasm staining white to light pink, containing dark brown or yellow brown granules. Bright pink granules occasionally visible (Fig. 4.1 H: arrow). Erythrocyte nucleus displaced off-centre by the parasite and slightly pushed to the side. Measuring 2399 ± 9.71 (18.51–72.67) long and 5.60 ± 0.90 (3.29–7.12) wide with a circumference of 49.61 ± 4.41 (42.76–57.30) and a surface area of 124.96 ± 21.40 (89.87–173.14) μm^2.

Immature rounded macrogametocytes: Rounded in shape, n=10 (Fig. 4.1 K–L), erythrocyte nucleus pushed against the side by parasite. Staining deep blue-purple, containing white vacuoles and yellow brown to dark brown granules within cytoplasm. Parasite nucleus occasionally visible as a foamy pale purple discolouration in the parasite cytoplasm. Measuring 23.21 ± 4.38 (15.16–31.57) long and 8.72 ± 1.67 (6.80–12.77) wide with a circumference of 51.51 ± 5.16 (43.13–61.03) and a surface area of 158.97 ± 22.75 (136.76–213.34) μm^2. Occasionally slight elongation of erythrocytes is visible.

Mature microgametocytes: Circumnuclear, n=7 (Fig. 4.1 J), staining white to light pink, measuring 28.76 ± 4.31 (23.04–32.47) long and 3.75 ± 0.68 (2.78–4.42) wide. Dark brown granules visible within cytoplasm, parasite usually not filling the cytoplasm of the erythrocyte completely. Circumference measuring 60.01 ± 8.07 (47.81–66.60) and the surface area 108.42 ± 8.52 (96.77–120.08) μm^2.

Mature macrogametocytes: Circumnuclear, n=28 (Fig. 4.2 M), staining deep purple, measuring 34.80 ± 3.09 (26.93–40.75) long and 4.29 ± 1.53 (2.16–8.68) wide. Containing dark brown granules and white vacuoles within the cytoplasm. Nucleus occasionally visible as a pale purple discolouration in the cytoplasm with a foamy appearance. The circumference measuring 123.90 ± 38.89 (26.93–40.75) and the surface area 90.91 ± 33.80 (49.50–162.06) μm^2.

Remarks: Telford (2007) recorded trophozoites to measure 1 × 1 to 5 × 3 µm (Table 4.2). The cytoplasm and pigment were recorded to be either visible or absent in the smallest stages,

and vacuoles were also occasionally observed in trophozoites. No trophozoite developmental stages of *Haemocystidium* morphotype A were observed in *N. annulifera.* The smaller immature stages infecting *N. annulifera* were larger than those measured by Telford (2007), measuring 16.1 × 6.5 μm as compared to 8–9.5 × 3–5.5 μm respectively. However, the dispersal of granules observed in immature stages infecting *N. annulifera* was consistent with the description provided by Telford (2007), with the granules either dispersed throughout the cytoplasm or concentrated at one of the poles as stages reached sizes of 8–9.5 × 3–5.5 μm and larger in his description. Telford (2007) also described that the vacuoles disappeared as the young gametocytes increased in size. As smaller developmental stages were not observed infecting *N. annulifera,* it may explain why few vacuoles were observed in immature gametocytes in this study. Both immature (23.2 × 8.7 μm) and mature (34.80 × 4.29 μm) macrogametocytes of *Haemocystidium* morphotype A infecting *N. annulifera* were larger as compared to those described by Telford (2007) (18.3 × 7.6 μm). Nuclei were observed in very few smaller stages (stages larger than trophozoites); however, only one was observed within a parasite, compared to Telford's description where there were sometimes three nuclei present. No description was provided by Telford (2007) regarding the shape or form of immature stages, except that as trophozoites grew into immature gametocytes, they become more elongate. No additional measurements such as circumference or surface area were provided. The dispersal of black pigment granules in *Haemocystidium* morphotype A infecting *N. annulifera* is similar to the description provided by Telford (2007), where black pigment granules were dispersed throughout the cytoplasm or concentrated at one of the ends for both pre-mature and mature microgametocytes and macrogametocytes. However, an abundance of yellow-brown granules was observed in premature and mature stages of both sexes of *Haemocystidium* morphotype A, which was not recorded from the specimens described by Telford (2007). Occasionally, bright pink granules were observed in premature and mature microgametocytes of *Haemocystidium* morphotype A. Whether these represent something similar to the multiple nuclei reported in smaller parasites by Telford (2007) is uncertain, but does not seem likely. Premature macrogametocytes of *Haemocystidium* morphotype A contained vacuoles, a finding not recorded by Telford (2007). Different shape classes of premature and mature gametocytes were identified by Telford (2007), describing it as filling the host cell, encircling the nucleus or being halteridial. However, Telford (2007) did not record measurements according to the differentiated shape classes and appeared to have measured all collectively. Only rounded and circumnuclear shape classes of premature and mature stages were observed in *N. annulifera,* respectively. Telford (2007) recorded that few halteridial forms were observed, no such forms were observed in *N. annulifera*. Gametocyte

nuclei of *Haemocystidium* morphotype A were more frequently observed in macrogametocytes than it was observed in microgametocytes.

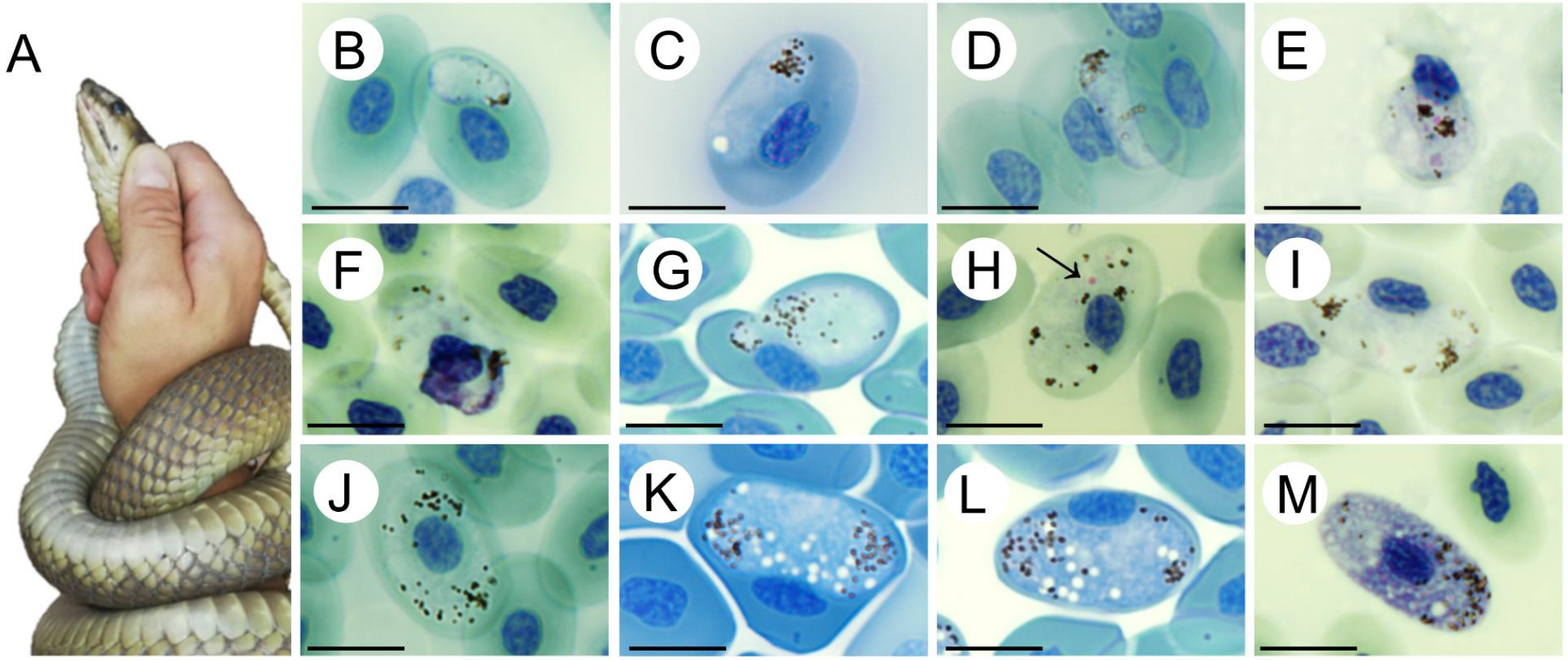


Figure 4.2: *Haemocystidium* sp. observed in snake host, *Naja annulifera.* Host, *N. annulifera* (A); immature rounded stages (B–E) and double infection of erythrocyte with immature amoeboid stages (F); larger immature amoeboid stage (G); rounded immature microgametocyte (H-I); mature circumnuclear microgametocyte (J); immature rounded microgametocyte (K–L) and mature circumnuclear macrogametocyte (M). **Scale bar = 10 µm**.

4.3.3.2 Material observed in *Naja mossambica* (morphotype B)

Locality: Hazyview (-25.033; 31.117); Nelspruit (-25.47448; 30.97033); White River (-25.329929; 31.016308); Marloth Park (-25.367967; 31.776531), Mpumalanga, South Africa.
Site in host: Peripheral blood**.**
Prevalence: 29% (8/28)
Parasitaemia: Hazyview (0.4 and 2.7%), Nelspruit (1.6%), White River (14%–19.5%); Marloth Park (5.5%).
Representative DNA sequence(s): Six partial sequences of the 18S rRNA gene, ranging from 571–574 bp.

Description: Trophozoite stages, n=3 (Fig. 4.2 B), are observed. Non-staining stages measuring 5.35 ± 1.33 (4.45–6.87) long and 3.28 ± 0.77 (2.67–4.15) wide. The surface area measuring 15.03 ± 5.08 (9.61–19.67) μm^2.

Immature smaller stages: Rounded to amoeboid in shape, n=30 (Fig. 4.2 C–H), measuring 10.32 ± 1.96 (6.89–15.59) long and 5.14 ±1.47 (3.03–9.82) wide. Staining white to light pink or deep blue-purple. The surface area measuring 44.63 ± 16.07 (17.49–84.96) μm^2. White to light pink individuals usually developing into microgametocytes while those staining deep blue-purple developing into macrogametocytes.

Immature extra-erythrocytic amoeboid stages: Staining white or deep blue-purple, n=12 (Fig. 4.2 I–J), measuring 14.54 ± 4.32 (7.16–20.97) long and 7.21 ± 2.47 (4.34–12.49) wide with a circumference of 35.01 ± 8.67 (18.01–44.71) and a surface area of 82.04 ± 32.82 (29.19–124.59) μm^2.

Mature amoeboid stages are observed within erythrocytes: Staining deep blue-purple with dark brown granules within the cytoplasm, n=2 (Fig. 4.2 K). Measuring 35.73 ± 1.36 (34.77–36.69) long and 6.28 ± 1.28 (5.38–7.18) wide. The circumference measuring 58.70 ± 23.91 (41.79–75.61) and the surface area 122.23 ± 35.20 (97.35–147.12) μm^2. Causing dehaemoglobinization and lysis of erythrocyte (Fig. 4.2 K).

Immature halteridial microgametocytes: Staining deep blue-purple, n=1 (Fig. 4.2 L), measuring 25.88 long, 4.69 wide with a circumference of 56.01 and surface area of 134.56 μm^2.

Immature halteridial macrogametocytes: Staining white with yellow brown granules, n=1 (Fig. 4.2 M), and measuring 32.01 long, 8.53 wide with a circumference of 66.66 and surface area of 222.10 μm^2.

Immature rounded microgametocytes: Staining deep blue-purple, n=40 (Fig. 4.2 N), and measuring 28.65 ± 3.12 (24.18–32.80) long and 7.55 ± 0.91 (6.02–8.51) wide with a circumference of 59.78 ± 4.60 (53.92–65.92) and surface area of 153.63 ± 16.30 (115.92–174.31) μm^2.

Immature rounded macrogametocytes: Staining white with dark brown granules, n=40 (Fig. 4.2 O). Measuring 19.30 ± 2.35 (14.43–24.30) long and 7.75 ± 2.26 (5.33–19.22) wide with a circumference of 44.96 ± 4.19 (35.99–53.51) and a surface area of 121. 60 ± 25.47 (19.43–198.322) μm^2.

Mature microgametocytes: Circumnuclear in shape, n=4 (Fig. 4.2 P), staining white and containing yellow-brown granules. Measuring 35.13 ± 4.33 (31.82–41.42) long and 5.58 ± 1.24 (4.11–7.11) wide with a circumference of 75.95 ± 15.80 (64.78–98.70) and a surface area of 192.18 ± 69.20 (121.75–284.13) μm^2.

Mature macrogametocytes: Circumnuclear in shape, n=10 (Fig. 4.2 Q), staining deep blue-purple containing dark brown granules. Measuring 34.15 ± 3.51 (30.14–42.53) long and 4.17 ± 0.73 (3.36–5.84) wide with a circumference of 72.68 ± 5.03 (61.49–79.14) and surface area of 148.01 ± 21.60 (109.48–170.471) μm^2.

Remarks: Trophozoites observed in *Haemocystidium* morphotype B were similar in size (5.4 × 3.3 μm) to the larger trophozoite forms described by Telford (2007) (5 × 3 μm) (Table 4.2). In trophozoites of *Haemocystidium* morphotype B, the pigment was absent, as described by Telford (2007). The nuclei of some smaller stages (stages larger than trophozoites) were easily observable; however, no more than one was observed within a parasite, compared to Telford's description where there were sometimes three nuclei present. The dispersal of granules in *Haemocystidium* morphotype B were similar to that described by Telford, where granules were dispersed throughout the cytoplasm or condensed at one of the ends; however, more often it appeared to be dispersed within the cytoplasm in the present study. In *Haemocystidium* morphotype B, large white vacuoles were observed in macrogametocytes of all stages, which was not recorded by Telford (2007). Extracellular and intracellular amoeboid stages were observed in *Haemocystidium* morphotype B, a finding not recorded from the specimens examined by Telford (2007). Large amoeboid intracellular stages appeared to cause dehaemoglobinization of the host erythrocyte. Halteridial forms of both microgametocytes and macrogametocytes were observed of *Haemocystidium* morphotype B, which was also recorded by Telford (2007). The nuclei of macrogametocytes of *Haemocystidium* morphotype B were frequently observable, while those of microgametocytes

were not observed. In Telford's (2007) description nuclei of both micro- and macrogametocytes were visible.

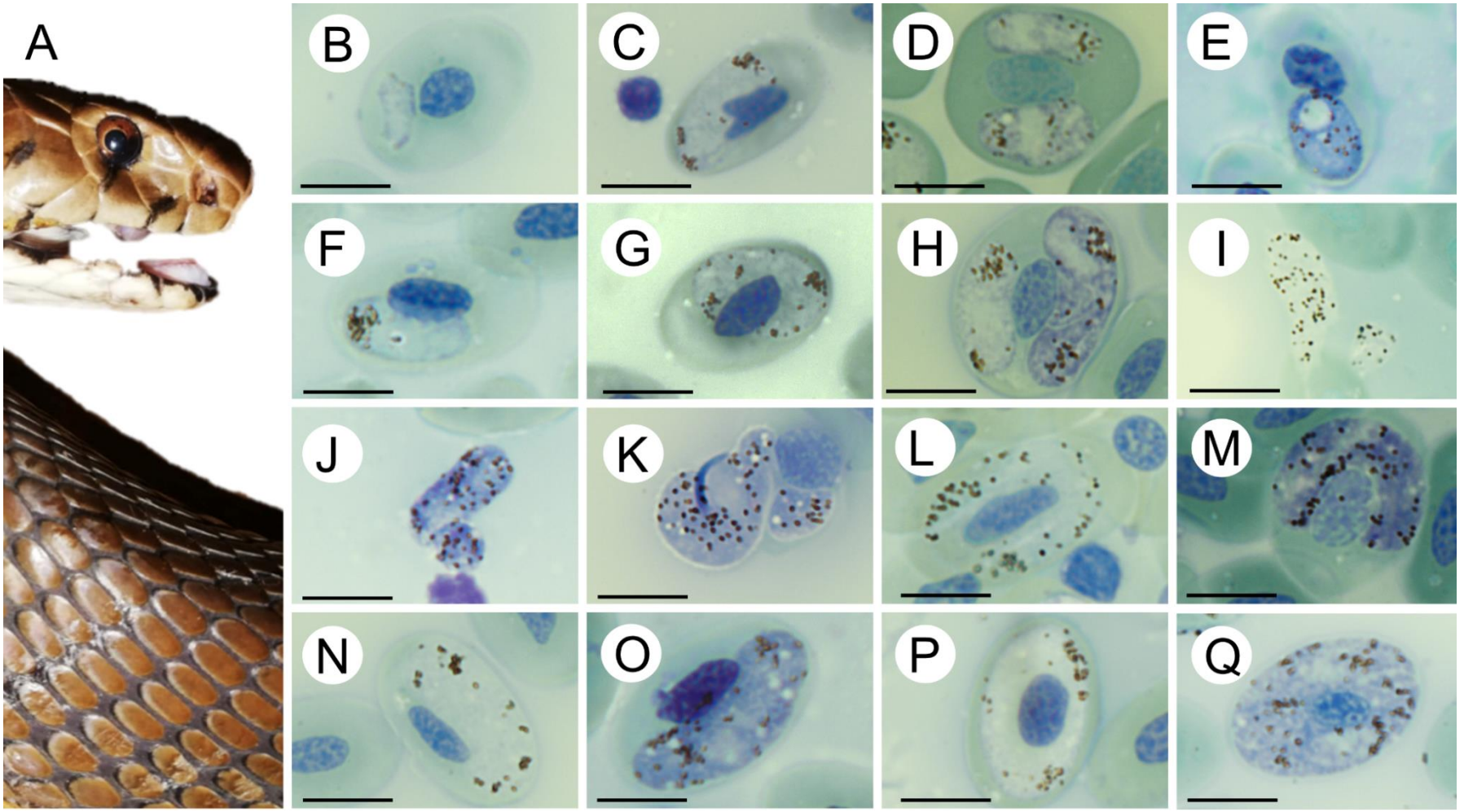


Figure 4.3: *Haemocystidium* sp. observed in snake host, *Naja mossambica.* Host, *N. mossambica* (A); trophozoite (B); immature rounded stages (C–H); double infection of erythrocyte with immature rounded stages (D); erythrocyte with double infection with larger rounded immature stages (H); immature extra-erythrocytic amoeboid stages (I–J); mature amoeboid macrogametocyte, causing dehaemoglobinization and lysis of erythrocyte (K); halteridial immature microgametocyte (L) and immature halteridial macrogametocyte (M); rounded immature microgametocyte (N) and rounded immature macrogametocyte (O); mature circumnuclear microgametocyte (P) and mature circumnuclear macrogametocyte (Q). **Scale bar = 10 µm.**

4.3.4 Remarks on *Haemocystidium* morphotype A and *Haemocystidium* morphotype B

When comparing morphometrics of stages seen in both *Haemocystidium* morphotype A and B, smaller immature stages of morphotype A (16.1 µm x 6.5 µm, circumference 38.5 µm^2, 84.8 µm^2) were larger than those observed for morphotype B (10.3 µm x 5.1 µm, circimference 44.6 µm^2). Regarding shape of these stages morphotype A showed a higher prevalence for rounded, as compared to morphotype B, which showed more amoeboid stages. Throughout development, morphotype B, displayed a wide range of shapes from rounded, to halteridial and amoeboid, whilst only rounded and sometimes amoeboid were seen for morphotype A. In both morphotypes, mature gametocytes were circumnuclear. However, microgametocytes for morphotype B (35.1 µm x 5.6 µm, circumference 76 µm^2, 192 µm^2) were larger than those for morphotype A (28.8 µm x 3.8 µm, circumference 60 µm^2, 108.4 µm^2). Even though macrogametocytes compared closely in length and width for morphotype A (34.8 x 4.3 µm) and B (34.2 x 4.2 µm), overall morphotype B (148 µm^2) appeared to be larger than morphotype A (90.9 µm^2).

Table 4.3: Morphometrics of haemoproteids measured from *Naja annulifera and Naja mossambica* examined as well as measurements provided by Telford (2007) for *Haemocystidium mesnili* (Bouet 1909) Wenyon 1926 observed in *Naja nigricollis nigricollis* and two other cobra host species. Measurements have been rounded to two decimals and were provided as: AVE ± SD (LOW–HIGH).

Species and Study		Measurements of developmental stages					
		Trophozoite	Immature stages	Premature stages		Mature stages	
				Macrogametocytes	Microgametocytes	Macrogametocytes	Microgametocytes
H. mesnili Telford (2007)	**LW**	1 × 1 – 5 × 3	**Smaller stages:** 8–9.5 × 3–5.5 **Larger stages:** 17.7 ± 3.7 (11–29) × 7.3 ± 1.4 (5–10)	18.3 ± 4.0 (11–29) × 7.6 ± 1.4 (5–10)	17.0 ± 3.3 (12–23) × 7.0 ± 1.4 (5–10)	18.1 ± 2.1 (12–21) × 9.2 ± 1.3 (7–12)	15.0 ± 2.6 (10–20) × 8.8 ± 1.5 (6–12)
	L:W	**NG**	2.52 ± 0.70 (1.1–4.4)	2.51 ± 0.70 (1.1–4.20)	2.53 ± 0.71 (1.2–4.4)	2.0 ± 0.36 (1.2–2.6)	.76 ± 0.45 (1.0–2.7)
	CIR	**NG**					
	SA	**NG**					
H. mesnili **(morphotype A)** Present study	**LW**	**NO**	16.14 ± 4.93 (8.89–27.217) × 6.54 ± 2.47 (1.90–12.64)	23.21 ± 4.38 (15.16–31.57) × 8.72 ± 1.67 (6.80–12.77)	23.99 ± 9.71 (18.51–72.67) × 5.60 ± 0.90 (3.29–7.12)	34.80 ± 3.09 (26.93–40.75) × 4.29 ± 1.53 (2.16–8.68)	28.76 ± 4.31 (23.04–32.47) × 3.75 ± 0.68 (2.78–4.42)
	L:W	**ND**					
	CIR	**NO**	38.50 ± 25.81 (20.21–56.43)	51.51 ± 5.16 (43.13–61.03)	49.61 ± 4.41 (42.76–57.30)	123.90 ± 38.89 (26.93–40.75)	60.01 ± 8.07 (47.81–66.60)
	SA	**NO**	84.77 ± 8.84 (20.21–130.82)	158.97 ± 22.75 (136.76–213.34)	124.96 ± 21.40 (89.87–173.14	90.91 ± 33.80 (49.50–162.06)	108.42 ± 8.52 (96.77–120.08)
H. mesnili **(morphotype B)** Present study	**LW**	5.35 ± 1.32 (4.45–6.87) × 3.28 ± 0.77 (2.66–4.15)	10.32 ± 1.96 (6.89–15.59) × 5.14 ± 1.47 (3.03–9.82)	23.17 ± 3.86 (16.98–32.80) × 8.41 ±1.58 (5.61–12.61)	20.98 ± 4.39 (14.43–32.79) × 7.72 ± 2.08 (5.33–19.22)	34.15 ± 3.51 (30.14–42.58) × 4.17 ± 0.72 (3.36–5.84)	35.13 ± 4.33 (31.82–41.42) × 5.57 ± 1.24 (4.11–7.11)
	L:W	**ND**					
	CIR	13.20 ± 3..25 (10.34–16.74)	24.91 ± 4.56 (16.21–36.94)	52 ± 5.32 (40.93–65.92)	47.62 ± 7.13 (65.92–65.92)	72.68 ± 5.03 (61.49–79.14)	75.95 ± 15.80 (64.79–78.70)
	SA	15.03 ± 5.07 (9.61–19.67)	44.63 ± 16.07 (17.49–84.96)	155.55 ± 22.64 (40.93–65.92)	127.37 ± 26.97 (84.95–198.32)	148.00 ± 21.60 (109.48–170.47)	192.18 ± 69.20 (121.74–284.13)

LW, Length × Width; L:W, Length to Width Ratio; CIR, Circumference (μm²); SA, Surface Area, NO, Not Observed; NG, Not Given; ND, Not Determined; AVE, Average; SD, Standard Deviation; LOW, Lowest; HIGH, Highest

4.4 Discussion

Molecular and phylogenetic analyses identify and places the haemosporidian found infecting both *N. annulifera* and *N. mossambica* in this study with *H. mesnili* (KF049514), this latter sequence amplified from an *N. annulifera* individual collected from Kruger National Park, South Africa (Pineda-Catalan, 2013). The latter sample was purely molecular and no morphological descriptions accompanied it. To date, this sequence represents the only currently available molecular data of snake *Haemocystidium* spp. It is unfortunate that this sequence was not accompanied by morphological data, which would have assisted with species confirmation, as there is currently little information available about these parasites. Although the precise locality where the sample was obtained is not stated, it is believed to be from the same larger geographical location to where the samples from which sequences were obtained during this study were collected in the Mpumalanga province, contributing another nine sequences of this parasite species, of which seven are from an additional snake host (*N. mossambica*). Additionally, if the species found infecting *N. annulifera* from the Kruger National Park, and *N. annulifera* and *N. mossambica*, from this study, is indeed *H. mesnili*, the present study increases this specie' distribution range with another four potential localities.

It would appear, as such, that this parasite has a wide distribution throughout Africa (Telford, 2007, 2009; Pineda-Catalan *et al*., 2013; this study), and may, depending on the host species present with somewhat different blood stage morphology. Even though there were morphological similarities between the *Haemocystidium* spp. isolated from *N. mossambica* and *N. annulifera* in this study, they did differ in size and shape. Similar findings could be seen when comparing both *H. mesnili* from the two species of snakes in this study and *H. mesnili* described by Telford (2007). In the latter case, however, different types of measurements were taken as compared to this study. Furthermore, Telford (2007) identified shape classes, but did not measure these separately. Therefore, a clear morphological comparison based on measurements was not completely possible. There are currently no standardised measurements established for measuring haemoproteid parasites, which is problematic as different types of measurements are taken that prevents clear comparisons between specimens based on morphological data.

There is currently very little information available about haemoproteids infecting snakes. The morphological descriptions that are currently available are not accompanied by molecular data. The first genetic information that was made available for *H. mesnili* was done so in the form of a cytochrome *b* sequence deposited in GenBank, without supplementary information regarding its morphology or comparison to existing morphological descriptions to support its

identification. There is another *Haemocystidium* spp. also recorded from African cobras, namely *Haemoproteus balli* Telford, 2007 (potentially *Haemocystidium balli*). However, this species does not compare morphologically, even superficially, as compared to *H. mesnili* or when detailed comparisons are taken into account (see Telford, 2007, 2009). Then again, one cannot account for the potential morphological plasticity that may be occurring in different species and localities of snake hosts.

It was noted by Telford (1996) that *Haemocystidium* spp. from geckos differ from *H. mesnili* in the sense that their meronts develop without the formation of pseudocytomeres (the primary or first generation schizonts), a characteristic he used to uphold the *Haemocystidium* genus (Pineda-Catalan *et al.,* 2013; Telford 1996). In this study, *H. mesnili* was found to group separately from *H. ptyodactyli* and *H. kopki,* with *H. pacayae* isolated from the Yellow-spotted River Turtle (*Podocnemis unifilis* Schweigger, 1812) also grouping separately from *Haemocystidium* spp. isolated from snakes and geckos respectively. It appears as if the inclusion of more representative sequences of a species can affect phylogenetic placements, since *H. mesnili* was found to group with *H. ptyodactyli* and *H. kopki* in the ML phylogenetic tree constructed by Pineda-Catalan *et al*. (2013), contrary to what was observed in this study. It is, thus, clear that there is a need for more molecular data in order to help stabilise phylogenies and accurately represent taxon relatedness.

Studies stated that the host-specificity of chelonian associated haemoproteid parasites is not restricted to the vertebrate hosts and that the parasites demonstrate a stronger host specificity towards the definitive vector (Cook *et al*., 2010; Javanbakht *et al.,*2015; Gupta *et al.,* 2011; Pineda-Catalan *et al*., 2013). However, the snake sampling pool of this study comprised seven different snake species of the same larger geographical location, where only the cobra species (*N. annulifera* and *N. mossambica)* had positive infections with *H. mesnili*. Considering that the available records of haemoproteids in snakes are also from African cobras, this suggests two possibilities; that the vector or parasite has strong host-specificity towards African cobras or that these cobras have a weak immune defence towards this parasite compared to other snake species from the same geographical locations. Furthermore, the prevalence of this parasite was higher in *N. mossambica* as compared to *N. annulifera*, possibly suggesting that the parasite or vector displays a stronger host-specificity towards spitting cobras or that there is a strong association between host ecology and/or these parasites.

It is proposed that the distribution of chelonian associated *Haemocystidium* spp. is associated with ecological and climatic factors that allows for the co-distribution of vectors and vertebrate hosts (Javanbakht *et al.,* 2015). The occurrence and abundance of vertebrate hosts, vectors and parasites are predicted to be strongly influenced by the characteristics of the habitat

(Javanbakht *et al.,* 2015; Gupta *et al.,* 2011). Furthermore, the ability of a parasite to successfully infect a host might be the result of the host's susceptibility towards the parasite, the relative abundance of vertebrate hosts and vectors in the environment or that the vector has a strong preference towards the vertebrate host (Javanbakht *et al.,* 2015; Gupta *et al.,* 2011).

There is no available data on the definitive vectors of snake *Haemocystidium* spp. (Davies & Johnston, 2000; Telford, 2007), as is the case with tortoise associated *Haemocystidium* spp. (Cook *et al.,* 2010; Lainson & Naiff, 1998; Orkun & Güven, 2013; Pineda-Catalan *et al.,* 2013), and the suspected vectors are usually generalized as haematophagous dipterans. The transmission of these parasites in captivity are predicted to be limited if snakes do not come into contact with vectors (Telford, 2009). It is suspected that the parasites are capable of persisting in the circulatory system of cobras for a long duration of time, as the parasite species infecting wild and captive cobras were highly similar morphologically and genetically. In fact, both the captive *N. mossambica* and *N. annulifera* individuals that were in captivity for up to two years and were housed in isolation from possible vector organisms had positive infections with fairly low parasitaemias compared to some of the parasitaemias that were observed in wild cobras. These snakes were housed indoor in temperature-controlled rooms and they were quarantined and screened for ectoparasites before they were moved to their enclosures, which were basic bin setups. This serves as an indication that the snakes became infected prior to being placed in captivity, especially as the same pattern is repeated in captivity than what was observed in wild snakes, that being that only the cobra species were infected from all the snake species in the sampling pool and that the haemoproteids that were observed to infect both wild and captive cobras were similar morphologically and identical genetically.

None of the snakes exhibited any visible symptoms of illness or negative health effects resulting from these infections. It was observed that a few mature macrogametocytes caused cell lysis and completely destroyed host erythrocytes. However, immature rounded stages of micro- and macrogametocytes were the most prevalent stages observed within erythrocytes and caused no visible host erythrocyte deformations other than dislocating the erythrocyte nucleus by pushing it against the inner cytoplasmic membrane of the erythrocyte. *Haemocyctidium* spp. have close affinities to malaria parasites, and reptile associated *Plasmodium* spp. have been reported to negatively affect reptile health (Jacobson, 2007; Schall, 1990a; 1990b, 1996).

4.5 Conclusion

It is recommended that more research be conducted to determine if there are health-risks associated with *Haemocystidium* spp. in reptiles, especially as there is currently little information available about these parasites infecting reptiles. This is particularly so given the close association of this genus to true malaria parasites (*Plasmodium* spp.) that are known to cause mortalities in some vertebrate groups (Javanbakht *et al.,* 2015) and to severely impact reptile survivorship (Jacobson, 2007). However, in order to conduct effective future research in this parasite group, it will be necessary to standardize methods such as measurements for morphological descriptions and to prevent that the first genetic information that is made available for future use by researchers is not accompanied by supportive information justifying the genetic identification of a species. Considering the abundance and diversity of apicomplexan parasites in environments, it is crucial to discover and identify these organisms, especially from an epidemiological perspective and species conservation.

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Chapter 5

Distribution prediction modelling of *Haemogregarina* spp. infecting terrapins and their definitive and intermediate hosts

In all things of nature, there is something of the marvellous.

~ Aristotle

Chapter 5: Distribution prediction modelling of *Haemogregarina* spp. and their definitive and intermediate hosts

5.1 Introduction

Parasites are extremely diverse and abundant, altogether performing fundamental ecological functions. Unfortunately, parasites are also the most threatened and under-protected forms of metazoan life (Carlson *et al.*, 2020; Lafferty & Kuris, 2002; Larsen *et al.*, 2017; Poulin & Morand, 2000; Windsor, 1998). Due to data gaps in their distributions and true population sizes, it is difficult to plan for their conservation (Carlson *et al.*, 2020). Another factor having potential impact on the sampling coverage for parasites and their hosts is biased sampling. In the case of parasites, they are frequently overlooked (Carlson *et al.*, 2020), while more attractive host species are more likely to be prioritized for research (Piccolo *et al.*, 2020).

Species belonging to the *Haemogregarina* Danilewsky, 1885 genus are parasites of ectothermic vertebrates, mostly of aquatic origin, such as chelonians and fish and are globally distributed (Siddall & Desser, 1992). However, one of these species from terrestrial tortoises has since been transferred to the haemogregarine genus *Hepatozoon* (Cook et *al.,* 2014) and another species of *Hepatozoon* has recently been described from the Colombian Wood Turtle *Rhinoclemmys melanosterna* Gray, 1861 (Gutiérrez-Liberato *et al.,* 2021). Those of crocodilians, even though once considered species of *Haemogregarina*, were also transferred to the genus *Hepatozoon* (Siddall, 1995). The classification and species descriptions of *Haemogregarina* were mainly based on the morphological characteristics of the sporogonic stages in pheripheral circulation of the intermediate vertebrate hosts (Dvořáková *et al.*, 2014; Siddall & Desser, 1992) where the mature intraerythrocytic gamonts are almost indistinguishable based on their morphological characteristics alone (Telford, 2009). Species diagnosis of *Haemogregarina* is further complicated by the uniform morphology of gamonts and meronts and the lack of sporogonic information about their life cycles within vectors (Dvořáková *et al.*, 2015). The presence of different developmental stages within the peripheral blood is also dependent on the duration of the infection. Infections are considered to be long-term if there is a high number of gametogonic and merogonic forms with a low number of trophozoites, while old infections are identified by dominant gamonts in the peripheral blood (Dvořáková *et al.*, 2014).

Initial *Haemogegarina* descriptions were influenced by the assumption that they are extremely host-specific, which led to the origin of many synonymous species as each new haemogregarine found in a new host species led to the description of a new parasite species (Dvořáková *et al.*, 2014). However, since apicomplexans emerged in the Precambrian Era and all the species that are currently known are obligatory intracellular parasites (Levine, 1988), it is believed that they co-evolved with their definitive invertebrate hosts. It was suggested that existing species with heterogenous life cycles evolved from monoxenous ancestors that parasitized invertebrates (Barta, 1989; Kopečná *et al.*, 2006). The sexual developmental stages of apicomplexans that are transmitted by vectors are reserved within the invertebrate host, which further disputes that these parasites would display a high-specificity towards vertebrate hosts and that the occurrence of these parasites are more likely linked to the distribution of the definitive vector host rather than the intermediate vertebrate hosts (Dvořáková *et al.*, 2014). Yet the definitive vector is dependent on the host in cases where they parasitize exclusively on that host.

The vectors and routes of transmission of *Haemogregarina* remain unclear (Dvořáková *et al.*, 2014). The only fully described life cycles for *Haemogregarina* species that are available for chelonians, are that of *Haemogregarina stepanowi* Danilewsky 1885 in the European pond turtle *Emys orbicularis* Linnaeus, 1758 (Danilewsky, 1884; Reichenow, 1910) and *Haemogragarina balli* Paterson and Desser, 1976 in Nearctic snapping turtles, *Chelydra serpentine* Linnaeus, 1758 (Dvořáková *et al.*, 2014; Paterson & Desser, 1976; Siddall & Desser, 1990; Siddall & Desser, 1992). For both of these haemogregarine species, the sexual developmental stages occur in leech vectors where merozoites that occur in the proboscis of the leech are inoculated into the hosts during feeding (Dvořáková *et al.*, 2014). Two species, namely *H. stepanowi* and *Haemogregarina bagensis* Ducloux, 1904 have been recorded from terrapins with overlapping distributions (Dvořáková *et al.*, 2014; Fritz & Havaš, 2007; Iverson, 1992). Furthermore, the developmental stages of *H. bagensis* was also recorded in the leech *Placobdella costata* Müller, 1846 which is also the definitive host for *H. stepanowi,* presenting the argument that one leech vector can transmit more than one haemogregarine species and the possibility that a single turtle can serve as intermediate host to more than one *Haemogregarina* species (Bielecki *et al.*, 2012; Dvořáková *et al.*, 2014).

Leeches belonging to the genus *Placobdella* Blanchard, 1893 are known to be implicated in the transmission of haemogregarine parasites to terrapin hosts across the globe (Paperna, 1989; Siddall & Desser, 1991; Siddall & Desser, 2001). A leech from this genus, *Placobdella multistriata,* syn. *Placobdella multistrigata* Johansson, 1909 has been confirmed to be the vector for *Haemogregarina pelusiensi* Pienaar, 1962 from *Pelusios sinuatus* Loveridge, 1941

in Mozambique (Paperna, 1989) and for this blood parasite described from *P. sinuatus* in Limpopo South Africa (Pienaar, 1962). The distribution of *Pl. multistriata* has been recorded from Sudan, Egypt, South West Africa, Zaire, Liberia, Tanzania, Zambia, Uganda and the Republic of South Africa (Oosthuizen, 1979).

The leech, *Pl. multistriata* is a very successful species as they are abundant and reproduce throughout the year. This was evident from samples that were collected throughout the year where the leeches had spermatophores, eggs or young within them year-round. The number of offspring that can be produced, is determined by the size of the parent leech (Oosthuizen, 1979). This leech species seems to solely parasitize ectothermic vertebrates. It has been recorded to parasitize crocodiles (*Crocodylus cataphractus* Cuvier, 1825; *Crocodylus niloticus* Laurenti, 1768 and *Osteolaemus tetraspis* Cope, 1861), and terrapins; *P. sinuatus*, *Pelomedusa subrufa* Bonnaterre, 1789 and *Trionyx triunguis* Forskål, 1775 (Oosthuizen, 1979). Since aquatic reptiles are mobile animals capable of migrating between different waterbodies, it is reasonable to believe that, as hosts, they contribute to the distribution of leeches. Terrapins in particular are known to migrate during the rainy season, inevitably contributing to the distribution of the leeches (Oosthuizen, 1979).

It was also reported that these leeches attach themselves to waterscorpions (Nepidae Linnaeus, 1758) and waterbugs (Belostomatidae Leach, 1815), where many leeches were found on a single insect. It is, however, not believed that the insects serve as hosts for the leeches. The majority of the leeches that were found on insects were well-fed, with their crop contents consisting of vertebrate blood (Oosthuizen, 1979). It appears to be instinctive for the leeches to attach themselves to these insects when they have no need to feed, but the exact reason for this behaviour is not yet confirmed. Furthermore, those insects that fly at night can inadvertently aid in the transport of the leeches to some degree. This can in part explain why these leeches are so widely distributed (Oosthuizen, 1979).

Haemogregarines are known for their ability to cross-infect a wide range of hosts (Siddall & Desser, 2001). Two *Haemogregarina* species, *H. stepanowi* and *Haemogregarina psuedemydis* Acholonu, 1974 for example, have been recorded from almost every known chelonian species in Europe and North-America respectively (Acholonu, 1974; Levine, 1988, Siddall & Desser 2001). It was also discovered that *H. balli* can be transmitted from painted turtles *Chrysemys picta marginata* Schneider, 1783 to snapping turtles *C. serpentine* through the leech *Placobdella ornata* Verrill, 1872 during laboratory experiments (Siddall & Desser, 2001). For this reason, it is necessary to consider the likelihood that cross-infection can occur wherever the distributions of terrapins with positive infections with *Haemogregarina* spp. and the vector leeches overlap.

Species of *Haemogragarina* have been recorded to infect *P. sinuatus*, *Pelusios castanoides* Hewitt, 1931; *Pelusios subniger* (Bonnaterre, 1789); *Pelomedusa subrufa* (Bonnaterre, 1789) and *Pelomedusa. galeat*a (Schoepff, 1792) (Meyer, 2014). This genus is likely to infect more species however, the lack of sampling influences these records. At this time, there are only two described species of the genus *Haemogregarina* that were recorded from *P. sinuatus* in southern Africa, namely *H. pelusiensi* (Paperna, 1989) and *Haemogregarina maputensis* Dias, 1950 (Dias & De Sousa, 1950).

The aims of this chapter were to (i) identify the presence of species of *Haemogregarina* in terrapins from sites in the North-West and KwaZulu-Natal provinces in South Africa through microscopic and molecular analysis (ii) to display the distribution of terrapin species of southern Africa with the use of ArcMap as well as the leech vector and (iii) to visually present the likelihood of occurrence of both *Haemogregarina* sp. and *Pl. multistriata* respectively through Maxent modelling by using currently available distribution data.

5.1.2 Genus: *Pelomedusa* (Wagler, 1830)

It was long thought that the *Pelomedusa* genus was monotypic, but it was recently discovered that there are ten distinct genetic lineages and an additional five distinct lineages based on molecular studies of mitochondrial DNA (Petzold *et al.*, 2014). Some of the species can be distinguished by morphological characteristics alone, while others require molecular confirmation where distributions of more than one species overlap or are sympatric.

Southern Africa is home to two *Pelomedusa* species, namely *Pe. subrufa* and *Pe. galeata*. Based on molecular evidence, it is confirmed that *Pe. subrufa* occurs in countries north of South Africa, including the neighbouring countries of Namibia, Botswana, Zimbabwe, Mozambique, and the most northern provinces of South Africa namely Limpopo (Petzold *et al.*, 2014) and Mpumalanga (Fritz *et al.*, 2015; Petzold *et al.*, 2014). It is, however, uncertain how far south its molecularly-confirmed distribution range extends.

Pelomedusa galeata on the other hand, occurs in all nine provinces of South Africa, but is not molecularly confirmed in the most northern regions of the northern provinces (Petzold *et al.*, 2014). It is also uncertain if and to what extent its distribution overlaps with *Pe. subrufa* in Limpopo and Mpumalanga. The distributions as illustrated in this study were based on molecularly confirmed occurrences for the *Pelomedusa* species (Petzold *et al.*, 2014).

Despite being two different species, *Pe. subrufa* and *Pe. galeata* are difficult to tell apart by eye, as there are only subtle morphological differences. This is confounded by their ecological

requirements being similar. Both species are terrestrial and freshwater that inhabit both fresh and stagnant waterbodies, pans, quarries and farm dams. They are mainly carnivorous, gulping insects from the water surface, and feed on amphibians, fish, crabs, ticks, molluscs, birds, and small mammals, but have also been reported to eat waterweeds and roots (Boycott & Bourquin, 2008). They prefer to travel along easily accessible terrain between different water bodies, especially during the rainy seasons, allowing them to inhabit quite inauspicious terrains (Boycott & Bourquin, 2008). There are still uncertainties regarding their populations, but both *Pelomedusa* species that occur in southern Africa are locally abundant (Boycott & Bourquin, 2008; Fritz *et al.*, 2014).

5.1.2.1 ***Pelomedusa galeata*** (Schoepff, 1792)

Pelomedusa galeata (Fig. 2.4 A) is commonly known as the South African Helmeted Terrapin. It occurs in Eswatini (formerly known as Swaziland), Lesotho and in all nine provinces of South Africa, namely, Eastern Cape, Free State, Gauteng, KwaZulu-Natal, Limpopo, Mpumalanga, North-West, Northern-Cape and Western-Cape. It also occurs in extreme southern Mozambique, but it is uncertain how far north it occurs and whether it also occurs in southern Namibia or southern Botswana (Fritz *et al.*, 2014; Uetz *et al.*, 2020). The true distribution of this species in Limpopo is unknown and also to what extent its distribution overlaps with that of *Pe. subrufa* (Hofmeyr & Fritz, 2018; Petzold *et al.*, 2014). It has a dark coloured carapace and plastron and is a large terrapin species, which can reach a straight carapacial length (SCL) of maximum 325 mm (Fritz *et al.*, 2014). They normally only reach up to 260 mm (Fritz *et al.*, 2014). It has two, small mental barbels, and the legs and head are slightly darker dorsally than they are ventrally (Fritz *et al.*, 2014). This terrapin species is commonly found throughout South Africa and has a conservation status of Least Concern (Hofmeyr & Fritz, 2018).).

5.1.2.2 ***Pelomedusa subrufa*** (Bonnaterre, 1789)

Pelomedusa subrufa (Fig. 2.4 B), known as the Common African Helmeted Terrapin (Boycott & Bourquin, 2008), occurs in South Africa, Namibia, Botswana, Zimbabwe, and Mozambique. In South Africa, it occurs in the Limpopo and Mpumalanga provinces (Fritz *et al.*, 2015; Uetz *et al.*, 2020), but it is uncertain how far south its distribution range in these provinces extends. Adults can reach 197 mm in SCL, although most adults are below 140 mm (Fritz *et al.*, 2014). The carapace is dark to light brown, where older individuals tend to have lighter plastrons and younger individuals have darker plastrons that contain markings (Fritz *et al.*, 2014). There is one large undivided temporal scale at each side of the head and the

pectoral scutes are in broad contact at the plastral midseam, sometimes the pectoral scutes are triangular without midseam contact (Fritz *et al.*, 2014). These terrapins prefer to inhabit ecological niches where *Pelusios*, *Cycloderma,* and *Trionyx* are absent in order to avoid competition, but it was found that they do sometimes share waterbodies with *Pelusios* species (Boycott & Bourquin, 2008). Hinged and softshell terrapins are more selective in occupying permanent waterbodies (Boycott & Bourquin, 2008).

5.1.3 ***Pelusios sinuatus*** (Smith 1838)

Pelusios sinuatus (Fig. 2.4 C), is commonly known as the East African Serrated Hinged Terrapin. It occurs in South Africa, Eswatini, Botswana, Zimbabwe and Mozambique. In South Africa, it occurs in the North-West, KwaZulu-Natal, Limpopo, Gauteng and Mpumalanga (Boycott & Bourquin, 2000; Broadley & Boycott, 2009; Uetz *et al.*, 2020; Vamberger *et al.*, 2019). This is the largest species of the hinged terrapins, with a SCL that can reach up to 550 mm (Boycott & Bourquin, 2000). Its ecological requirements are unique compared to that of other terrapins of the same genus, since they prefer to inhabit deeper perennial rivers, lakes and larger man-made waterbodies in savannah regions (Boycott & Bourquin, 2000; Vamberger *et al.*, 2019). During the rainy season, they migrate in order to colonize smaller waterbodies like pans and waterholes. The carapace and bridge are uniform dark grey or black in colour (Broadley, 1981). The plastron has a prominent hinge and tends to be dark brown, yellow centred with a black angular pattern around the edge (Boycott & Bourquin, 2000; Broadley, 1981). Sometimes black blotches are scattered around the central region of the strongly hinged plastron (Broadley, 1981). Its carapace is high domed and elongated, where it is wider in the back than in the front (Boycott & Bourquin, 2000).

5.1.4 ***Pelusios subniger subniger*** (Bonnaterre, 1789)

Pelusios subniger (Fig. 2.4 D), is commonly known as the Pan-hinged Terrapin, the East African Mud Turtle or the Black Bellied Hinged Terrapin. It occurs in South Africa, tropical southern Africa, Namibia, Botswana, Zimbabwe and Mozambique. In South Africa, it occurs in a small part of the northern KwaZulu-Natal and northern Limpopo provinces (Boycott & Bourquin, 2000; Fritz *et al.*, 2013; Uetz *et al.*, 2020).

They inhabit temporary pans, and permanent swamps, and other waterbodies (Boycott & Bourquin, 2000). Individuals belonging to this species are small to moderate sized terrapins, reaching a maximum SCL of 200 mm (Boycott & Bourquin, 2000; Broadley, 1981). It has a

large head and there are two barbels present on its chin (Boycott & Bourquin, 2000; Broadley, 1981). The snout is blunt and the beak unicuspid (Boycott & Bourquin, 2000). The plastron has a prominent hinge and the auxiliary shield is absent (Boycott & Bourquin, 2000). The carapace is dark brown and the bridge of the carapace is a combination of yellow and brown (Boycott & Bourquin, 2000; Broadley, 1981). The shields on the plastron are yellow in the middle and dark brown towards the outer parts. The vertebrals are not keeled and the third vertebral usually has a greater width than length. The skin is grey to black in colour (Broadley, 1981). The carapace is oval for males and subcircular for females. Females reach a greater mass and larger size than males when fully grown (Boycott & Bourquin, 2000).

5.1.5 ***Pelusios rhodesianus*** Hewitt, 1927

Pelusios rhodesianus (Fig. 2.4 E), is commonly known as the Variable Mud Turtle, Variable Hinged Terrapin, Rhodesian Mud Turtle or the Mashona Hinged Terrapin. This species occurs in South Africa, Botswana, Zimbabwe and Mozambique (Uetz *et al.*, 2020). In South Africa relic populations occur in KwaZulu-Natal (Broadley & Boycott, 2008). It occurs in a variety of habitats like temporary pans and swamps, man-made dams and larger water bodies. They prefer to inhabit water bodies with a decent amount of weedy vegetation (Boycott & Bourquin, 2000; Broadley & Boycott, 2008), and they inhabit the same habitats as *Pelusios castanoides* (Boycott & Bourquin, 2000). During the dry season these terrapins aestivate in mud (Broadley & Boycott, 2008). This is a medium sized terrapin that reaches a SCL of about 220 mm and weight of 700–900 g (Boycott & Bourquin, 2000). The carapace and bridge are uniform black (Boycott & Bourquin, 2000; Broadley, 1981). The carapace is domed, ellipsoid, and wider posteriorly than anteriorly (Boycott & Bourquin, 2000; Broadley, 1981). The vertebral keels are not well developed and the posterior margin of the carapace is rounded (Broadley & Boycott, 2008). The growth rings on the epidermal shields are normally well-defined and the plastral hinge is prominent (Broadley, 1981). The beak is bicuspid and it has two barbels on its chin (Boycott & Bourquin, 2000; Broadley & Boycott, 2008). The plastron has a prominent hinge, is black, and occasionally has irregular yellow patches in the middle (Boycott & Bourquin, 2000; Broadley & Boycott, 2008). The head is brown dorsally with yellow curvy patterns, while the sides of the neck, skin, head, and limbs are yellowish. The outer sides of limbs are grey-brown (Boycott & Bourquin, 2000; Broadley & Boycott, 2008).

The northern populations of the species in southern Africa seem to be stable, but the relict populations in KwaZulu-Natal, Lesotho, Mpumalanga and southern Mozambique are Vulnerable, Threatened or Extinct (Boycott & Bourquin, 2000; Broadley & Boycott, 2008). Veld fires, pollution and habitat destruction pose threats to these terrapins. There is also evidence

that the populations in South Africa are under threat since they are deliberately killed by local people (Broadley & Boycott, 2008). This species is listed as IUCN category 'Least Concern', but was last assessed in 1996 and is in need of updating.

5.1.6 *Pelusios castanoides castanoides* Hewitt, 1931

Pelusios castanoides (Fig. 2.4 F) is commonly known as the East African Yellow-bellied Mud Turtle. It occurs in Mozambique and in the KwaZulu-Natal province in South Africa (Boycott & Bourquin, 2000; Fritz *et al.*, 2013; Uetz *et al.*, 2020). Their distribution is restricted to the Mozambique plain and reaches its southern limit at Mtunzini in northern KwaZulu-Natal (Boycott & Bourquin, 2000).

They inhabit grassy pans, water-filled depressions and swamps. They occur in the same habitat as *P. rhodesianus*. The diet and other ecological requirements of these terrapins may be more specialized than is generally accepted. Detailed studies are needed to explain the occurrence and co-existence in the same habitat of these two similar species.

They are medium sized terrapins that can reach a maximum SCL of 220 mm and a weight of 700–900 g (Boycott & Bourquin, 2000). They have a small, flattened head and moderately pointed snout (Boycott & Bourquin, 2000). The beak is bicuspid and there are two barbels present on the chin. The carapace is domed and elongated, where it is wider posteriorly than it is anteriorly (Boycott & Bourquin, 2000). The plastron has a prominent hinge and the anterior edge is rounded. The head is blackish brown with curvy yellow lines and the skin of the neck and higher portions of the legs are yellow. The carapace is yellow-brown to dark brown, which is usually obscured by algal growth (Broadley, 1981). The bridge is yellow and black where the yellow tends to be dominant (Boycott & Bourquin, 2000; Broadley, 1981). In males, the anal notch is sharply angular while in females it is rounded (Boycott & Bourquin, 2000). There is also slight plastral concavity in males. The auxiliary shield is absent in this species (Boycott & Bourquin, 2000).

They are considered to be carnivorous as they feed on large pulmonate snails. However, a large part of diet consists of plant material, in particular the floating water lettuce (Boycott & Bourquin, 2000). Breeding and nesting likely occurs soon after the first rains of the season and probably continues throughout the summer. Nesting takes place in September and 25 eggs are laid per clutch. The eggs are elliptical and measure 30–33 mm in length and 21.5–23 mm in width (Boycott & Bourquin, 2000). This species is IUCN category 'Least Concern' but was last assessed in 1996 and is in need of updating.

5.1.7 *Pelusios bechuanicus* Fitzsimons, 1932

Pelusios bechuanicus (Fig. 2.4 G), is commonly known as the Okovongo Mud Turtle. It occurs in Namibia, Botswana and Zimbabwe where they are restricted to the Okavango basin (Broadley, 1981; Uetz *et al.*, 2020*).* They tend to prefer lakes, swamps and rivers that are deep, clear, quiet, and well-vegetated. They are mostly recorded from the waterways in the Okavango and Linyanti swamps and in the Zambezi River they are sometimes found in isolated temporary pools (Boycott & Bourquin, 2000; Broadley, 1981). The skin of the neck and dorsal parts of the limbs is grey with a tinge of yellow (Boycott & Bourquin, 2000). This is a large species that can reach a maximum SCL of 330 mm (Boycott & Bourquin, 2000; Broadley, 1981). The hinge of the plastron is strongly developed (Boycott & Bourquin, 2000; Broadley, 1981). The carapace is black, which occasionally has orange-yellow stains along the sides (Boycott & Bourquin, 2000; Broadley, 1981). The carapace is also domed and elongate, where the posterior side is wider than the anterior (Boycott & Bourquin, 2000). The plastron is usually entirely or partially black, and sometimes has a yellowish centre (Boycott & Bourquin, 2000).

5.1.8 *Cycloderma frenatum* Peters, 1854

Cycloderma frenatum (Fig. 2.4 H) is commonly known as the Zambezi Soft-Shelled Turtle. It occurs in tropical eastern and southern Africa. In southern Africa, it occurs in Mozambique and south-eastern Zimbabwe (Broadley & Sachsse, 2011; Uetz *et al.*, 2020). It is a fairly large terrapin that reaches a curvilinear length (CL) of 560 mm and weighs 13–14 kg. The plastron can measure 390 × 310 mm (Boycott & Bourquin, 2000). This is a completely aquatic terrapin that inhabits freshwater waterbodies like rivers and lakes (Boycott & Bourquin, 2000; Broadley & Sachsse, 2011). They are good swimmers and astonishingly good runners. When they feel threatened, they can use their forefeet and snout to bury into soft mud in order to escape danger (Boycott & Bourquin, 2000). The colour of their carapace ranges from dark green to pale grey to olive-grey, and it usually has uniform traces of blotching (Broadley & Sachsse, 2011). It has reduced plastral callosities (Broadley & Sachsse, 2011). The proboscis projects to the front and the nostrils have papilla-like structures that project upwards (Broadley & Sachsse, 2011). The head and neck can be shot forward rapidly to reach almost the length of the carapace (Broadley & Sachsse, 2011).

5.1.9 *Trionyx truiguis* (Forskål, 1775)

Trionyx truiguis (Fig. 2.4 i), is commonly known as the Nile Soft-shelled Turtle. In southern Africa, it only occurs in northern Namibia on the Angola-Namibia border (Uetz *et al.*, 2020; van Dijk *et al.*, 2017), where they may also occur in the sea off the coast of Namibia (Boycott & Bourquin, 2000). They prefer brackish waters and also occur in freshwater and saline habitats such as rivers, ponds, lakes and estuaries (Boycott & Bourquin, 2000; van Dijk *et al.*, 2017). This species is incredibly large and can reach about 900 mm in SCL and can weigh up to 45 kg (Boycott & Bourquin, 2000). These terrapins are soft shelled due to the absence of carapacial and plastral shields (Boycott & Bourquin, 2000). The head is flattened and elongated with protruding eyes that are located on top of the head and a pointed pig-like snout (Boycott & Bourquin, 2000).

5.2 Materials and Methods

5.2.1 Terrapin collection

Permission for sampling of terrapins in the North West province was acquired under permit number: NW 2650/02/2019. Terrapins were captured in shallow muddy pans with the use of baited funnel traps in the North-West Province. Chicken liver was used as bait and placed in perforated containers inside the traps (Fig. 2.1 A–C). The traps were then positioned in shallow water and secured to a sturdy pole in order to prevent it from being fully submerged in the water (Fig. 2.1 E), allowing the terrapins to surface for air whilst they were caught in the trap (Fig. 2.1 F). Captured terrapins were removed from the traps (Fig. 2.1 F–H) and temporarily placed in large bins containing shallow water. They were then moved to a shaded area for processing (Fig. 2.1 H).

5.2.2 Blood collection and screening for *Haemogregarina*

Ethical approval for this study was obtained from the North-West University, Potchefstroom under ethics number: NWU-00062-19-A5. Blood was drawn from the femoral artery with a sterile 1 ml fixed-needle insulin syringe (Fig. 2.1 K). Blood smears were prepared in duplicate by placing a small drop of blood on a microscope slide and carefully dragging another slide over it to create a thin smear. The rest of the blood was preserved in 96% pure ethanol for molecular analysis. The smears were allowed to air dry and were then fixed with absolute methanol for 10 min. They were stained with a 10% solution of Giemsa-stain (FLUKA, Sigma-Aldrich, Germany) for 15 min (Fig. 2.2 B). After staining, the slides were rinsed under tap water to remove excess stain. After the terrapins were sampled, they were marked with a unique notching pattern to one of the marginal scutes to prevent resampling of the same individuals (Plummer and Ferner, 2012), (Fig. 2.1 I). Additionally, archived samples from Ndumo Game Reserve (NGR) (collected in a previous ethically approved study in 2016 and 2017) were utilized.

The blood smears were examined under 60× magnification objective lens, equipped with immersion oil on a calibrated Nicon Eclipse N*i* (Nikon, Amsterdam, Netherlands) compound microscope for the detection of parasites. Photos of the parasites were taken with 100× oil immersion lens at 1000× magnification using the accompanying digital camera and NIS-Elements BR Ver. 4.60 camera analysis software (Nikon, Tokyo, Japan). Parasite

morphometrics were measured with the aid of ImageJ software (https://imagej.nih.gov/ij/) and all of the measurements were recorded in µm. Measurements included total length (TL); total width (TW); nuclear length (NL); nuclear width (NW); assumed anterior to mid-nucleus (AN) and assumed posterior to mid-nucleus (PN).

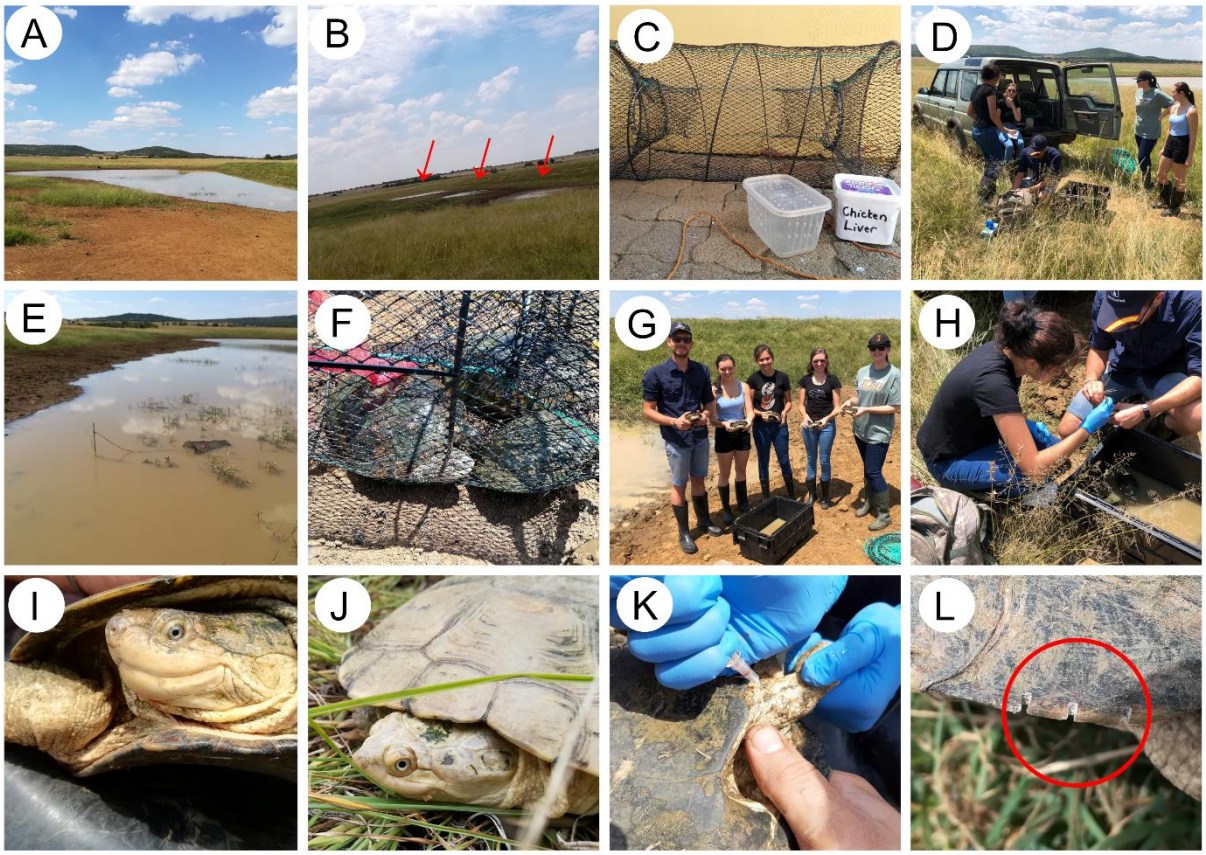

Figure 5.1: Examples of shallow muddy pans that are suitable terrapin habitats, within 10 km from Potchefstroom (A–B: red arrows); funnel trap, perforated container and chicken liver used as bait (C); preparation of equipment to process animals (D); typical example of traps set above water level (E); terrapins caught and removed from the trap (F–G); removal of leeches if found (H); examples of animals that were caught, in this case *Pelomedusa galeata* (I–J); blood collection from the femoral artery (K) and shell notching to mark individuals before release (L: red circle).

5.2.3 Molecular analysis

Genomic DNA was extracted using the 18S rRNA gene from four of the terrapins that were identified as positive for *Haemgregarina* infections through microscopic screening. The KAPA Express Extraction Kit (Kapa Biosystems, Cape Town, South Africa) was used for the extractions by following the manufacturer's instructions for animal blood. The supernatant DNA product was stored at -20 °C and was used as the template for the PCR. The amplification of the 18S rRNA gene was performed using two different primer sets for two different amplification processes. This, to demonstrate the influence that the use of different primer sets for the amplification process has on phylogenetic placements of terrapin associated *Haemogregarina* sequences. All amplification processes were executed using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, Singapore).

For the first PCR process using the 18S rRNA gene, apicomplexan specific forward primer EF (5'-GAAACTGCGAATGGCTCATT-3') and ER (5'-CTTGCGCCTACTAGGCATTC-3') was used as the reverse primer. The PCR conditions were: initial denaturation at 95 °C for 5 min, followed by 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s end extension at 72 °C for 60 s, and final extension at 72 °C for 10 min.

A second PCR process was performed using the 18S rRNA gene, separately from the abovementioned amplification process. For the second amplification process, 4558 (5'-GCTAATACATGAGCAAAATCTCAA-3') was used as forward primer and HepR900 (5'-CAAATCTAAGAATTTCACCTCTGAC-3') as reverse primer. The PCR conditions were as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 95 °C for 30 s, annealing at 55.3 °C for 30 s, 72 °C for 1 min and final extension at 72 °C for 10 min.

All PCR reaction volumes were set at 25 µl where the reagents and their respective volumes were as follows: 12.5 µl Thermo Scientific DreamTaq PCR master mix (2x) (2x DreamTaq buffer, dATP, dCTP, dGTP and dTTP, 0.4mM each and 4mM MgCl2), 1.25 µl of forward and reverse primer (10 µM) and 25 ng of DNA (1 µl). PCR grade nuclease free water (Thermo Scientific, Vilnius, Lithuania) was used to make up the reaction volume (9 µl). The PCR products were loaded in the wells of a 1% agarose gel aligned to a 1kb DNA ladder. The gel was run at 90 V for 30 min where after it was viewed under UV light. The fragment sizes were compared to the ladder with fragments of about 1,150 kb sent to Inqaba Biotec Company (Pretoria, South Africa) for purification and sequencing in both directions with the same primers.

5.2.4 Phylogenetic analysis

The reverse and forward nucleotide sequences were assembled with the use of Geneious ver. 11.1.4 (http://www.geneious.com, Kearse et al. 2012) for the respective primer sets. The Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) was then used on NCBI GenBank to compare and determine the closest matches of the sequences to those deposited in GenBank. All of the sequences that were obtained during this study matched closest with *Haemogregarina* spp. in GenBank. A total of five sequences that were identified as *Haemogregarina* spp. were obtained during this study; two from four *Pe. galeata* individuals, two being from the North-West Province and two from KwaZulu-Natal. For one of the KZN samples, two sequences were obtained. No sequences were successfully obtained from infected *P. sinuatus* individuals.

These sequences were used for the construction of the phylogenetic tree, along with 24 sequences that were obtained from GenBank. *Adelina dimidiata* (DQ096835) was used as outgroup following Cook *et al.* (2015). The sequences were aligned using the MUSCLE tool using MEGA X (https://www.megasoftware.net/). The alignment consisted out of 28 sequences and was 672 bp long. The alignment was exported as NEXUS and PHYLIP format where after the NEXUS file was manually edited and fixed with a command line. The file format was tested with the MrBayes executable (https://nbisweden.github.io/MrBayes/download.html) to test its executability before submitting it to CIPRES.

Phylogenetic analysis was performed by implementing Bayesian analysis using the CIPRES (https://www.phylo.org/) platform where the Mr Bayes on XSEDE (MrBayes 3.2.2 Huelsenbeck and Ronquist 2001) tool was used. The data block was enabled and runtime was set as eight hours. The MrBayes tool entailed that the analysis was run twice over 10 million generations for the Markov Chains Monte Carlo (MCMC) algorithm. The Markov chain was sampled every 100 cycles, and the MCMC variant contained 4 chains.

In order to determine the most suitable substitution model, the Akaike information criterion (AIC) tests were performed with the PHYLIP file by using jModelTest 2.1.10 (Darriba *et al.,* 2012; Guindon & Gascuel 2003). The best model was the General Time Reversible model with estimates of invariable sites and a gamma distribution of rates across sites (GTR+G+I). The ML analysis was performed using the PhyML 3.0 platform (http://www.atgc-montpellier.fr/phyml/) and the substitution model was set as GTR with 1000 bootstrap replicates (Guindon *et al.,* 2010; Guindon & Gascuel, 2010).

Both ML and BI outputs were exported, the trees were viewed using FigTree software (http://tree.bio.ed.ac.uk/software/figtree/) and re-rooted according to the chosen outgroup. The topologies for the ML and BI trees were highly similar. The ML and BI values were combined, using the topography topology produced by the BI tree (Fig. 4.3). The respective values are indicated at the nodes as (ML/BI), where ML values of less than 50 are not indicated.

5.2.5 Distribution maps

The distribution maps for the different species of terrapins were created with the use of ArcMap by using available distribution data obtained from various sources. The distribution maps were compiled using distribution data obtained for *Pe. galeata* and *Pe. subrufa* (Fritz *et al.*, 2015; Petzold *et al.*, 2014); *P. sinuatus* (Broadley & Boycott, 2009); *P. subniger* (Fritz *et al.*, 2013); *P. castanoides* (Fritz *et al.*, 2013) *P. rhodesianus* (Broadley & Boycott, 2008); *P. bechuanicus* (Boycott & Bourquin, 2000); *C. frenatum* (Broadley & Sachsse, 2011); and *T. triunguis* (van Dijk *et al.*, 2017). Polygons were constructed and clipped to the southern African countries' borders where needed. The same procedure was followed for the distribution map for the leech *Pl. multistriata*. The distribution for this species was obtained from Oosthuizen (1979), which was spatially fitted to the country borders of southern Africa and georeferenced in ArcMap in order to obtain the coordinate data from a PDF fie and convert it to point data to use for the prediction maps.

5.2.6 Prediction maps

Haemogregarina distributions were confirmed either by molecular analysis or observation under microscope and the model was collectively run for different terrapin species. The positive records were molecularly confirmed and obtained from a PhD thesis (Meyer, 2014). Additionally raw field data that were microscopically confirmed as positive were obtained (Dr Edward Netherlands, North-West University Potchefstroom). Additionally, the analysis of samples used in this chapter were also added as positive results that were molecularly and microscopically confirmed. MaxEnt (ver. 3.4.4) (https://biodiversityinformatics.amnh.org/open_source/maxent/) was used for the maximum entropy modelling. These models were obtained by running two separate models using the distribution of different terrapin species that were confirmed to be positive for *Haemogregarina* parasites and the leech distribution respectively. The models were run by using the respective distributions along with the 20

environmental variables obtained from https://www.worldclim.org/data/bioclim.html. The environmental variables were imported into ArcMap as raster data and clipped to the boundaries of southern Africa, where after they were converted to ASCII files for Maxent. The number of replications in MaxEnt was set at 1000. The Maxent ASCII output files were then imported back into ArcMap where the leech and *Haemogregarina* distributions were added as point data. The distribution data was obtained from the following terrapin species and was used collectively: *P. sinuatus*; *P. castanoides*; *P. subniger*; *Pe. subrufa;* and *Pe. galeat*a.

5.3. Results

5.3.1 Screening for species of *Haemogregarina*

5.3.1.1 General observations in *Pelomedusa galeata*

In North-West, six *P. galeata* individuals were collected and sampled from the Brits district (Ukutula Lodge -25.518702, 27.661230), and 14 from the Potchefstroom district (two from Atlas Clay -26.866127, 27.089252 and 12 from Lekwena -26.640, 27,179). Since the samples that were collected from North-West had a very low parasitaemia, a larger number of erythrocytes had to be screened to establish infection status with *Haemogregarina*. The number of parasites per 10 000 erythrocytes were counted. Overall, 95 % (19/20) of the North-West individuals were positive for haemogregarines. Parasitaemias ranged between 0.01% and 0.96%, and were determined as number of parasites per 10 000 erythrocytes.

In KwaZulu-Natal, six individuals were sampled from a roadside pool just outside NGR (-26.914327, 32.233462). The prevalence for *Haemogregarina* infection was 100% for *Pe. galeata* from this area with parasitaemia ranging from 0.10% to 0.70% (number of parasites per 1000 erythrocytes).

5.3.1.2 General observations in *Pelusios sinuatus*

In NGR (-26.9120873, 32.2634768), 14 *P. sinuatus* individuals were collected, with 13/14 (92.86%) infected with parasitaemias ranging from 0.10 to 3.10%, which was determined by examining the number of parasites per 1000 erythrocytes. Overall, 38/40 (95%) terrapins were positive for *Haemogregarina*.

5.3.2 Measurements of stages of *Haemogregarina* spp.

5.3.2.1 *Haemogregarin*a sp. isolated from *Pelomedusa galeata*

Trophozoites: the smallest stages, curvilinear in shape, measuring 9.70 ± 0.97 (8.58–12.05) long and 4.047 ± 0.65 (3.28–5.49) wide (n=24). Nucleus staining dark purple comprising of loosely arranged chromatin that is foamy in appearance. Nucleus measuring 5.48 ± 1.15 (4.30–7.68) long and 3.08 ± 0.72 (2.35–4.17) wide (n=19). Small vacuoles occasionally visible at one of the poles (Fig 2.3 A: arrow).

Pre-meronts: bean shaped, measuring 13.54 ± 1.15 (12.18–15.15) long and 5.56 ± 0.81 (3.65–7.14) wide (n=33). Nucleus staining dark purple to lilac, located almost centrally, measuring 4.23 ± 0.67 (3.39–5.62) long and 4.98 ± 0.87 (3.49–7.70) wide (n=33). Cytoplasm between the mid-nucleus and assumed anterior pole has a clear light purple appearance and measures 8.09 ± 1.17 (6.99–10.38), (n=33). Cytoplasm between the assumed posterior pole and mid-nucleus staining light purple with a speckled appearance, measuring 5.32 ± 0.89 (3.86–7.24), n=33 (Fig 2.3 B).

Immature gamonts: Gamonts that did not appear folded and of which the bend was not visible, were identified as immature gamonts and measured accordingly. These are bean shaped, the cytoplasm staining light blue or light purple, measuring 14.31 ± 1.0 (12.46–16.504) long and 4.52 ± 0.96 (2.81–6.50) wide (n=30). Nucleus staining dark purple, located against the assumed posterior pole, measuring 5.83 ± 1.52 (3.44–10.75) long and 3.53 ± 0.52 (2.70–4.53) wide (n=30). Cytoplasm between the mid-nucleus and assumed anterior pole staining light blue or light purple, measuring 10.16 ± 0.84 (8.41–11.80) long, while the cytoplasm between the mid-nucleus and assumed posterior pole measures 4.27 ± 1.3 (2.04–8.08) long, n=30 (Fig 2.3 C).

Mature gamonts: vermiclular in shape, staining light blue or purple, measuring 20.48 ± 1.7 (17.60–23.69) long and 4.36 ± 0.67 (3.28–5.67) wide (n=21). Nucleus staining dark purple, appearing slightly granular, located at the bend between the anterior part of the parasite and the long recurved ‘tail’ (Fig 2.3 d: arrow), measuring 5.44 ± 0.93 (3.52–6.74) long and 3.52 ± 0.65 (2.67–5.23) wide (n=21). Cytoplasm between the mid-nucleus and assumed anterior pole staining dark blue or purple, measuring 8.85 ± 2.1 (2.46–11.68) long; between the mid-nucleus and assumed posterior pole, measuring 11.26 ± 1.59 (8.34–14.24) long, n=21 (Fig. 2.3 D).

5.3.2.2 *Haemogregarina* sp. isolated from *Pelusios sinuatus*

Trophozoites: the smallest and rarest stages. Staining light purple and measuring 10.75 ± 1.08 (9.17–12.83) long and 4.56 ± 0.96 (2.75–5.92) wide (n=11). Nucleus staining dark purple with loosely arranged chromatin and a foamy appearance, positioned against one pole, measuring 5.08 ± 1.3 (3.68–6.93) long and 3.96 ± 1.0 (2.24–5.1) wide (n=7); the opposite pole containing clearly visible vacuoles (Fig 2.3 E: vacuoles indicated by arrow).

Pre-meronts: lentiform to bean shaped, staining light purple to lilac, measuring 12.69 ± 1.57 (10.66–17.7) long and 5.21 ± 0.73 (4.19–6.96) wide (n=29). Nucleus staining light purple, located approximately in the middle of the parasite, spanning the width of the parasite, measuring 4.11 ± 0.84 (1.67-–6.5) long and 4.96 ± 0.81 (3.85–6.92) wide (n=29). Cytoplasm between mid-nucleus and assumed anterior pole appearing clear, measuring 6.92 ± 1.22 (4.93–9.81); while cytoplasm between the mid-nucleus and assumed posterior staining light purple with a speckled appearance, measuring 5.78 ± 1.3 (3.38–10.87), n=29 (Fig 2.3 F).

Immature gamonts: gamonts where the bends were not visible, were identified as immature gamonts and measured accordingly. Bean shaped, staining light purple, containing few cytoplasmic granules, measuring 13.66 ± 1.63 (11.15–17.24) long and 5.59 ± 0.90 (3.57–7.37) wide (n=27). Nucleus staining dark purple, measuring 5.83 ± 1.26 (3.1-8.6) long and 4.36 ± 0.93 (2.94–6.89) wide (n=27). Cytoplasm between the mid-nucleus and assumed anterior pole measuring 9.63 ± 1.72 (6.36–12.58); and between the assumed posterior pole and middle of the nucleus 4.06 ± 1.2 (2.66–7.85), n=27 (Fig 2.3 G).

Mature gamonts: vermicular with a recurved ‘tail’ (Fig 2.3 h: arrow). Cytoplasm staining light purple, containing pink granules, measuring 25.58 ± 2.02 (22.0–29.36) long and 3.81 ± 0.91 (1.90–5.49) wide (n=32). Nucleus staining dark purple, located just before the bend or spread along the bend, measuring 6.76 ± 1.44 (4.51–13.27) long and 3.39 ± 0.91 (1.89–5.42) wide (n=32). Cytoplasm between the mid-nucleus and the assumed anterior pole measures 10.06 ± 2.35 (4.23–14.47); while the distance between the middle of the nucleus and assumed posterior pole is 15.52 ± 3.32 (10.76–23.85), n=32 (Fig. 2.3 H).

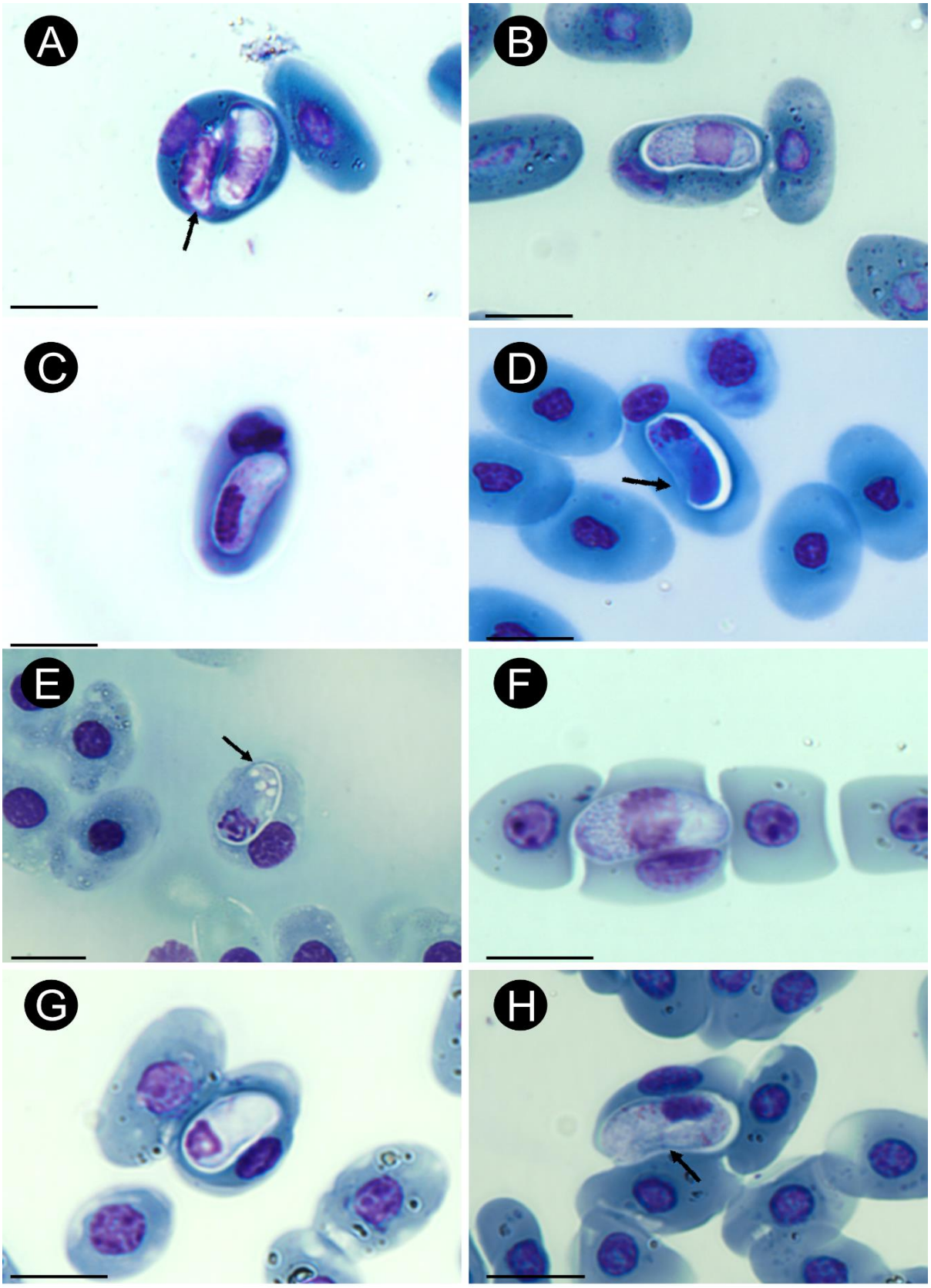

Figure 5.2: Different developmental stages observed in the peripheral blood of *Pelomedusa galeata* (trophozoite (A); pre-meront (B); immature gamont (C); and mature gamont (D)) and *Pelusios sinuatus* (trophozoite (E); pre-meront (F); immature gamont (G) and mature gamont (H)). **Scale bars = 10 µm.**

4.3.3 Phylogenetic tree of *Haemogregarina* spp. infecting *Pelomedusa galeata* and the influence of different primers

Molecular analyses of the *Haemogregarina* spp. observed infecting *Pelomedusa galeata* showed that the sequences that were isolated from *Pe galeata* in this study were 99.91–100% similar, with a divergence of 0.00–0.01%. Phylogenetic relationships, determined through Maximum Likelihood (ML) and Bayesian Inference (BI) analysis of *Haemogregarina* species that were extracted from *Pe. galeata* in the North-West and KwaZulu-Natal provinces in South Africa, revealed that the use of different primers can influence the phylogenetic placements of organisms, consequently affecting how phylogenetic relationships are represented by phylogenetic trees. It was observed that sequences that were obtained with the 4558 and HepR900 primer set group as a sister clade to sequences that were obtained with the use of the EF and ER primer set.

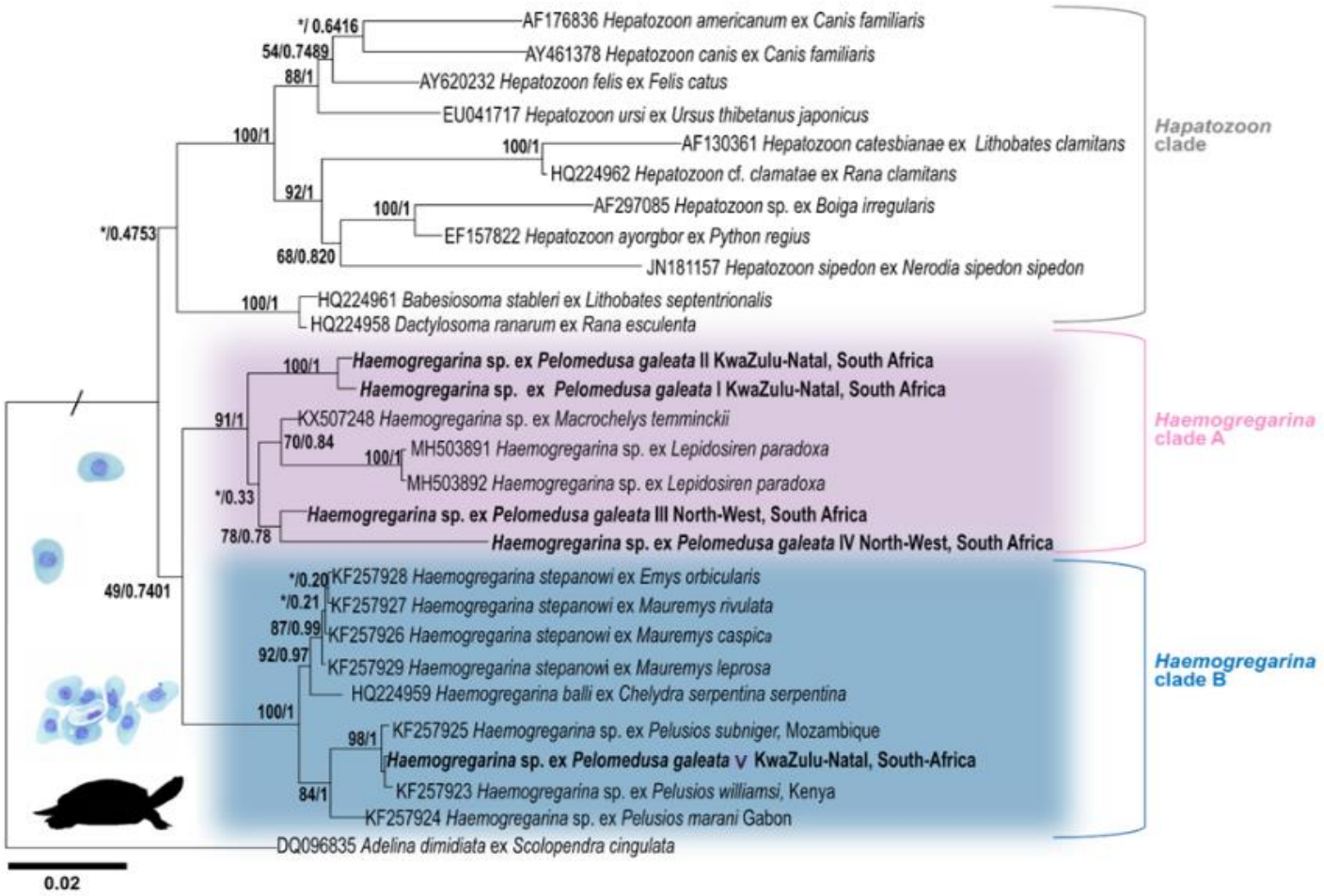

Figure 5.3: Maximum Likelihood (ML) and Byesian Inference (BI) analysis of *Haemogregarina* species extracted from terrapins in the North-West and KwaZulu-Natal provinces in South Africa and their relationships with other haemogregarines based on partial 18S rDNA sequences. The tree topologies for ML and BI are highly similar, the nodal support values, bootstrap for ML and posterior probability for BI tree, are represented as ML/BI on the BI tree. ML values below 50 were indicated as an asterisk '*' in the phylogenetic tree. Obtained sequences are indicated in bold. Scale = 0.02 nucleotide substitutions per site.

Table 5.2: Samples that were used for molecular analyses, the whole blood samples, the names of the sequences and hosts extracted from, as indicated in the phylogenetic tree (Fig. 5.3), as well as the primers that were used during PCR.

Whole Blood Sample number	**Sequences of *Haemogregarina* spp. obtained from terrapins**	**Locality**	**Primer pair used**	
			4558 and HepR900	**EF and ER**
RC160206A1	*Haemogregarina sp.* ex *P. galeata* II	KwaZulu-Natal	×	
RC160206A2	*Haemogregarina sp.* ex *P. galeata* I	KwaZulu-Natal	×	
	Haemogregarina sp. ex *P. galeata* V	KwaZulu-Natal		×
RC190122A3	*Haemogregarina sp.* ex *P. galeata* III	North-West	×	
RC200226A2	*Haemogregarina sp.* ex *P. galeata* IV	North-West	×	

Table 5.3: Evolutionary differences of various 18S rRNA gene sequences of *Haemogregarina* species that were included in the phylogenetic analyses (Fig. 5.3). Evolutionary differences are expressed as uncorrected pair-wise distance (p-distance) (top right).

	Species of *Haemogregarina*	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
1	*Haemogregarina* sp. ex *Pelomedusa galeata* V		0.00	0.01	0.01	0.01	0.00	0.00	0.01	0.00	0.00	0.01	0.00	0.01	0.00
2	*Haemogregarina* sp. ex *Pelomedusa galeata* I			0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.00	0.01	0.00
3	*Haemogregarina* sp. ex *Pelomedusa galeata* II				0.01	0.00	0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01
4	*Haemogregarina* sp. ex *Pelomedusa galeata* IV					0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01	0.01
5	*Haemogregarina* sp. ex *Pelomedusa galeata* III						0.01	0.01	0.01	0.01	0.01	0.00	0.01	0.01	0.01
6	KF257927 *Haemogregarina stepanowi* ex *Mauremys rivulata*							0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00
7	KF257928 *Haemogregarina stepanowi* ex *Emys orbicularis*								0.00	0.00	0.00	0.01	0.00	0.00	0.00
8	KF257929 *Haemogregarina stepanowi* ex *Mauremys leprosa*									0.00	0.00	0.01	0.00	0.00	0.00
9	KF257926 *Haemogregarina stepanowi* ex *Mauremys caspica*										0.00	0.01	0.00	0.00	0.00
10	HQ224959 *Haemogregarina balli* ex *Chelydra serpentina serpentina*											0.01	0.00	0.00	0.00
11	KX507248 *Haemogregarina* sp. ex *Macrochelys temminckii*												0.01	0.01	0.01
12	KF257925 *Haemogregarina* sp. ex *Pelusios subniger*													0.01	0.00
13	KF257924 *Haemogregarina* sp. ex ex *Pelusios marani*														0.01
14	KF257923 *Haemogregarina* sp. ex *Pelusios williamsi*														

4.3.4 Distribution of terrapin species and the leech *Placobdella multistriata* in southern Africa

The reported distributions of some terrapin species in southern Africa, such as *Pelusios rhodesianus*, *Pelusios bechuanicus* and *Trionyx triunguis* are narrow in comparison to species such as *Pelomedusa galeata*, *Pelomedusa subrufa*, *Pelusios sinuatus* and *Pelusios subniger* while the reported distribution of *Pelusios castanoides* and *Cycloderma frenatum* appear to be restricted to the eastern parts of southern Africa (mostly Mozambique). The distribution of terrapins in southern Africa indicate that there is a high degree of overlap among the distribution of terrapin species, particularly in the eastern regions of southern Africa. The reported distribution records of the leech, *Placobdella multistiata* (the definitive host and vector of *Haemogregarina* spp. infecting terrapins) show that the leech occurs in the eastern regions of South Africa and northern Namibia. However, considering the different environmental parameters encompassed within the different regions of the leech's current distribution, it is likely that the leeches occur across a wider range in southern Africa and that the depicted distribution is a reflection of limited sampling and lack of research on leeches as the main focus.

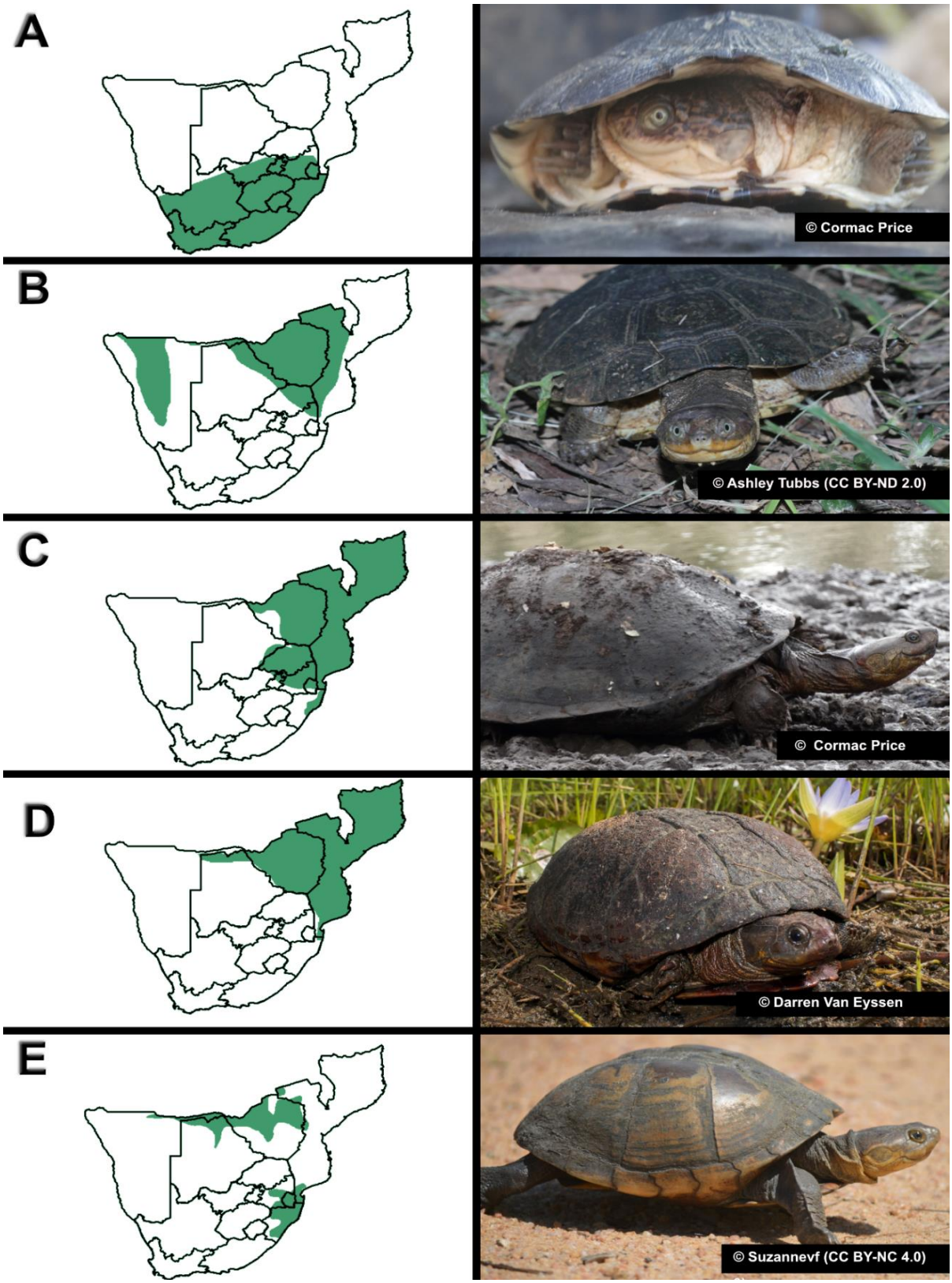

Figure 5.4: Terrapin and vector distribution in southern Africa: *Pelomedusa galeata* (A); *Pelomedus subrufa* (B); *Pelusios sinuatus* (C); *Pelusios subniger* (D); *Pelusios rhodesianus* (E).

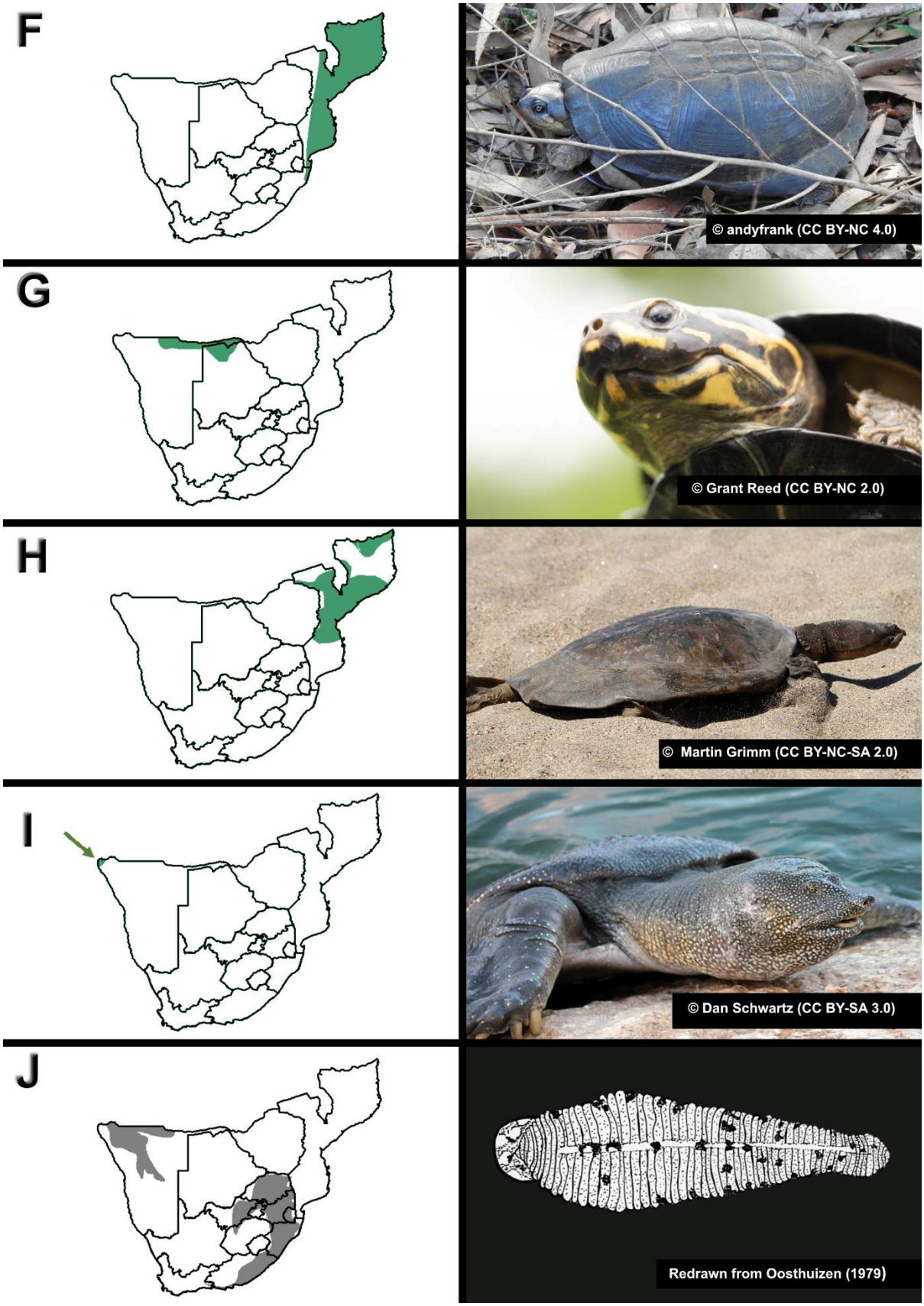

Figure 5.4 continued: *Pelusios castanoides* (F); *Pelusios bechuanicus* (G); *Cycloderma frenatum* (H); *Trionyx triunguis* (I) and *Placobdella multistriata* (J).

4.3.5 Predictive distribution modelling of *Haemogregarina* spp. infecting southern African terrapins as well as their definitive host and vector, *Placobdella multistriata*

The predicted distribution that was determined through MaxEnt modelling indicates that the leech vector and definitive host of *Haemogregarina* spp. infecting terrapins has a relatively wide predicted distribution across a wide array of environmental paramaters, based on available distribution records. The predicted distribution of *Haemogregarina* spp. (based on a limited number of confirmed records) also indicates that *Haemogregarina* spp. occur across a wide range of different environmental paramaters. When taking into consideration that the presence of *Haemogregarina* parasites is highly dependent on the presence of the vertebrate (terrapin) and invertebrate (leech) host, as well as the wide distribution and distribution overlap of these hosts, it is likely that *Haemogregarina* spp. has a wider distribution than what is displayed by the predictive model.

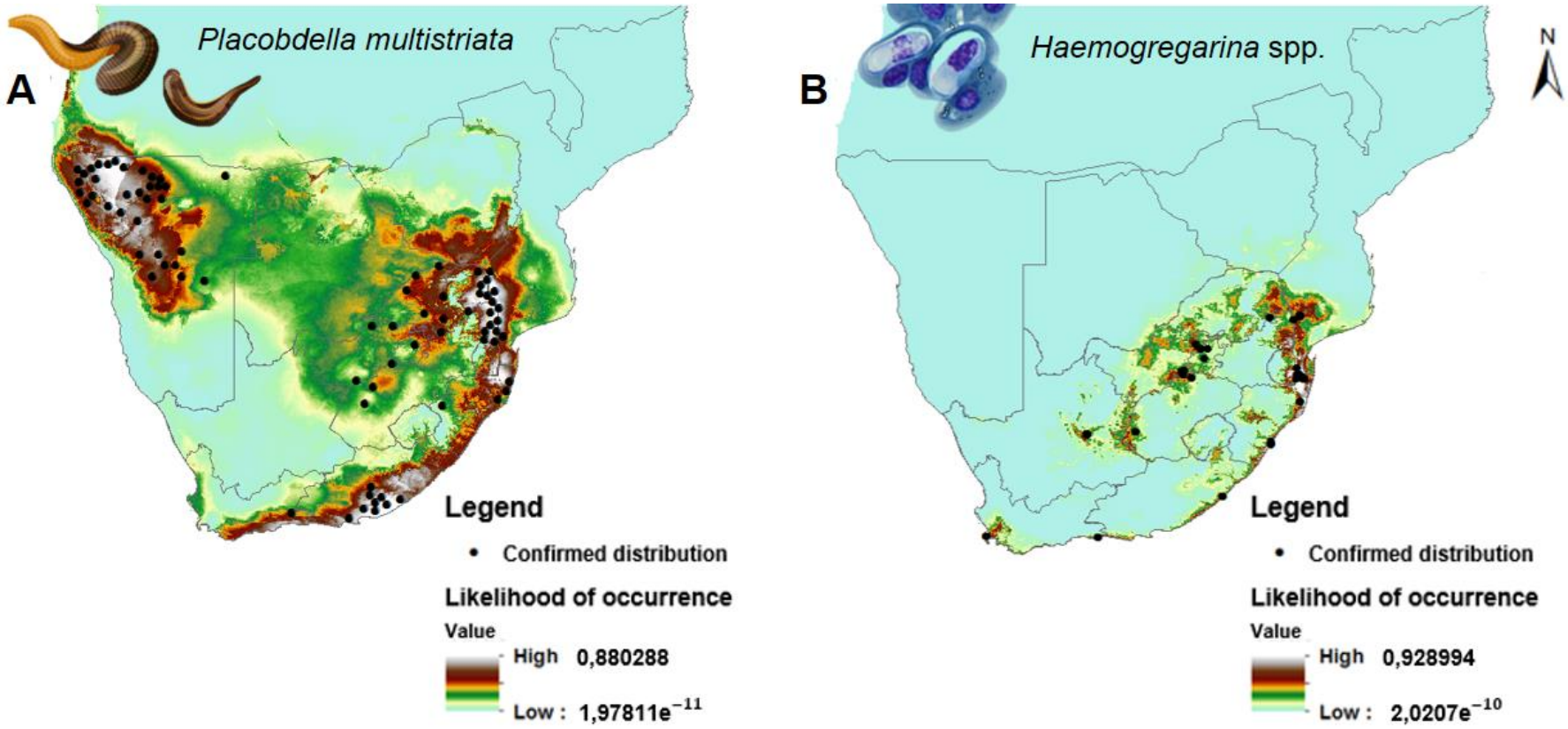

Figure 5.5: Predicted distribution and likelihood of occurrence of *Placobdella multistriata* (A) and *Haemogregarina* spp. (B) based on available data. White indicates a high likelihood of occurrence and light blue indicates a low likelihood of occurrence for *P. multistriata* and *Haemogragarina* spp. respectively.

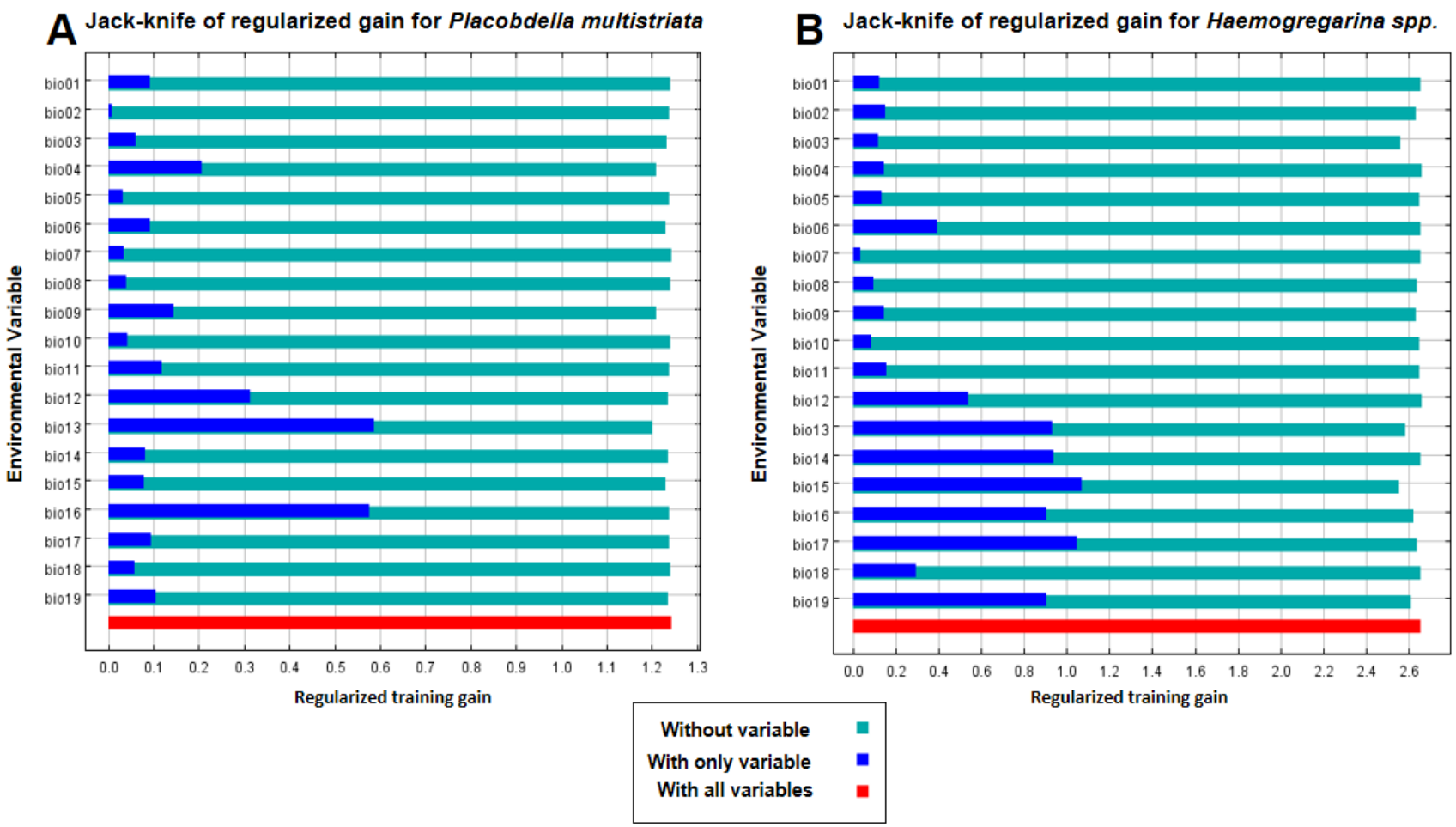

Figure 5.6: Jack-knife test showing the different contributions of the various bioclimatic variables to the regularized training gain of the final predictive model for *Placobdella multistriata* and *Haemogregarina* spp. distribution in southern Africa respectively. 'Only variable' indicates the result of the model with a single variable run in isolation; 'without variable' indicates the effect of removing a single variable from the full Jack-knife model. The values are means from 1000 replicates..

5.4 Discussion

5.4.1 Molecular and phylogenetic analysis

When visualizing the phylogenetic tree, *Haemogregarina* sp. isolates I, II III and IV extracted from *Pe. galeata* are seen falling in *Haemogregarina* clade A, whilst isolate V, also extracted from *Pe. galeata* falls in *Haemogregarina* clade B. The aforementioned four isolates were extracted from terrapins during this study with the use of the 4558 and HepR900 primer set, whilst the latter isolate V was amplified using the EF and ER primer set. Furthermore, isolates I and V were both from the same whole blood sample, where the PCR amplification process was performed using different primer sets that resulted in the respective sequences. This raises questions as to why sequences that are amplified with the use of different primers, in this case the 4558 and HepR900 primer set, and the EF and ER primer set, result in sequences grouping with separate *Haemogregarina* clades.

From the phylogenetic tree, it is apparent that the use of different primer sets for the same purpose of obtaining 18S rRNA sequences for apicomplexan parasites have a major impact on the sequence and subsequently phylogenetic outcome. This demonstrates that in terms of phylogenetic analyses, the primers that are used may greatly affect the placement of an organism in a phylogenetic tree. While this strange outcome can be attributed to many factors, such as the fact that 4558-HepR900 delivers amplicons of shorter length (~ 600 bp) than EF-ER (~ 1700 bp), leaving the phylogenetic analyses with less available genetic information for the shorter sequences, similar outcomes were observed in other studies (Esteves-Silva *et al.,* 2019; Harris *et al.,* 2018; Úngari et al., 2018). Another concern is the possibility of false genetic divergence generated as a result of amplification with different primers, consequently affecting species descriptions and diversity.

In studies where sequences were obtained using HepF300 and HepR900 primers (Esteves-Silva *et al.,* 2019; Harris *et al.,* 2018; Úngari et al., 2018), the obtained sequences are usually found to group as a sister clade to those *Haemogregarina* sequences that were obtained using the EF and ER primer set (Arizza *et al*., 2016; Dvořáková *et al.,* 2014). The HepF300 and HepR900 primer set (Ujvari *et al.,* 2004) is frequently used because of its effectiveness for amplifying apicomplexans of a wide taxonomic range from reptilian, amphibian, and mammalian hosts (Harris *et al.,* 2013; Maia *et al*., 2014).

It was also discovered that a HemoFN and HemoRN primer set was designed for haemogregarines in general to amplify 18S rRNA gene fragments of ~1550 bp

(Alhaboubi *et al.,* 2017). The resulting sequences were found to group with *Haemogregarina* clade A. However, a different outcome was obtained when a primer set 18HemoF1 and 18HemoR1 was designed based on *Haemogregarina* haplotypes that were acquired when using the EF and ER primer set. Additionally, more specific primer sets (18HemoF6/ 18HemoR2, 18HemoF4/ 18HemoR3 and 18HemoF5/ 18HemoR3) were designed in order to distinguish variants of *Haemogregarina* spp. in the same host. The resulting sequences from these newly designed primers grouped with the clade containing the sequences obtained with the EF and ER primer set (El Hili *et al.,* 2020).

It is evident that different primers affect the sequence outcome and that primers that are designed for apicomplexans or haemogregarines in general can aid general identification of the organism but does not necessarily deliver sufficient results for accurate phylogenetic analyses. It is required that more taxa should be sequenced in order to better represent this neglected group in terms of phylogenetic analyses, since sequences of few taxa are presently available to incorporate in phylogenetic analyses for the purpose of elucidating phylogenetic relationships. Further research is necessary to determine why *Haemogregarina* spp. are split into two separate clades when using general apicomplexan primer sets and primer sets that are more specific towards *Haemogregarina* spp. The need for standardised primer sets are therefore emphasized to prevent false representations of the phylogenetic relationships of these taxa.

For the purpose of this study, the aim was only to confirm positive statuses of terrapins for *Haemogregarina,* as the obtained sequences group with *Haemogregarina* clades, the results were interpreted as positive for *Haemogregarina* spp. on molecular grounds. However, it became clear that a diverse group such as *Haemogregarina* spp. should be more closely investigated by researchers, especially in regard to the 18S rRNA gene and the chosen primer sets, since it has such a great influence on phylogenetic placements. The need for additional molecular markers might also be critical for elucidation of *Haemogregarina* species diversity.

5.4.2 Distribution maps

Considering the wide distribution of terrapins in southern Africa and the predicted wide distribution of leech vectors, it can be assumed that the distribution of terrapin-associated haemogregarines are more abundant than what is portrayed by the currently available data in this chapter. This is especially emphasized by the available literature, which suggests that the haemogregarine distribution is more dependent on the distribution of their definitive hosts or vectors, rather than the terrapins themselves that act as intermediate hosts (Dvořáková *et al.*, 2014).

Based on the predicted distribution, resulting from the confirmed positive *Haemogregarina* records, it is clear that species of *Haemogregarina* occur across a wide range of different environmental paramaters ranging across South Africa. Since this was the only available occurrence records for modelling the prediction model, it is possible that their distribution can extend even wider. Another interesting observation was that the predicted distribution of the leeches and the haemogregarines overlap for the most part, supporting the assumption that haemogregarine distribution is dependent on the vector distribution.

A factor that should be taken into account is that both the terrapins and the vector leeches are mobile animals (Boycott & Bourquin, 2000; Oosthuizen, 1979). Their occurrences as predicted by the distributions are not stationary and consequently this has the potential to affect the distribution of the species of *Haemogregarina*. However, for terrapins to become infected, they need to be in contact with the leech vector since members of the genus *Haemogregarina* are considered to be strictly transmitted during a blood meal by the leech (Siddall, 1995). During this, merozoites that are present in the proboscis of the leech are inoculated into the terrapin host (Dvořáková *et al.*, 2014).

It is also assumed that the leech vectors are capable of transmitting *Haemogregarina* spp. from one terrapin to another and between different species of terrapins (Dvořáková *et al.*, 2014; Siddall & Desser, 2001). Visualising the amount of overlap among the distributions of terrapins in southern Africa, especially in eastern-southern Africa, and comparing it to the available *Haemogregarina* distribution data, it is likely that these haemogregarines have a wider distribution and that the few available records are the result of a lack of research focus in this specific field. Studies regarding *Haemogregarina* of terrapins tend to aim to either describe new species and to either elucidate or review current taxonomies, potentially leading to re-description of species or the description of newly found species.

This creates the potential for biased sampling as researchers are likely to selectively sample areas that are easily accessible, have high numbers of terrapin hosts and also to re-sample at areas that are known to be positive for *Haemogregarina*. Similar events have been recorded, showing that researchers tend to identify and choose sampling areas based on the success of previous sampling or areas that have high biodiversity, leading to the potential neglect of other areas (Piccolo *et al.*, 2020). In the case of the *Haemogregarina* prediction map and available distribution data for example, it is clear that the most tropical eastern regions of South Africa are targeted, especially in the area of the Kruger National Park and northern Kwazulu-Natal where game reserves such as Ndumo Game Reserve and Tembe Elephant Park are located, and are frequently more accessible and convenient for sampling purposes. This is another factor that was highlighted by Piccolo *et al.,* (2020) as a potential culprit leading to biased sampling for reptile associated research, where conservation areas become sampling hotspots because of preliminary knowledge that the target species occur there. The distribution maps indicate that there is a likelihood for the vector leeches to occur in Namibia, Botswana, and Zimbabwe. Considering that many southern African terrapin species inhabit these areas, there is potential for the parasites to also occur there. For example, the prediction map shows, especially if you refer back to terrapin distribution for *Pe. subrufa*, that there is a high likelihood for the species to occur in northern Namibia and central Botswana, and these regions should therefore be prioritized for future surveys. There are clearly neglected sampling areas, with very small sampling coverage across southern Africa, according to obtainable distribution data. This is problematic, as the relationships between these parasites cannot be accurately represented if other potential species have not been sampled. Morrison (2009) explains the impediment on phylogenetic representation of apicomplexan parasites resulting from the lack of sampling.

The leeches that were collected and used for the compilation of the leech distribution were not necessarily collected for terrapin-associated research and was collected for the purpose of studying the leech species themselves (Oosthuizen, 1979). They are prone to temporarily attach themselves to insects and vertebrates, although they are known to feed only on ectothermic vertebrates, hence contributing to their wide distribution (Oosthuizen, 1979). A frequent problem with studies regarding parasites is that only positive records tend to be recorded while negative records are rarely formally reported. This can be problematic when interpreting prediction models for current and future research as low probabilities of occurrence can either be a result of lack of sampling or simply because the species in question does not occur there. It became apparent that there is a lack of databases for parasites in general, especially parasites that are not of veterinary or medical importance. Existing databases accommodate parasites such as helminths (https://www.nhm.ac.uk/research-

curation/scientific-resources/taxonomy-systematics/host-parasites/), copepods (https://www.st.nmfs.noaa.gov/copepod /about/copepod.html) and marine parasites (WoRMS) (https://www.nhm.ac.uk/research-curation/scientific-resources/taxonomy-systematics/host-parasites/). There does not appear to be databases that contain general information or complete life cycles of haematozoan parasites. This is problematic, especially when taking into consideration the fundamental roles parasites play in natural ecosystems and that haematozoans form part of apicomplexans, a large component of global biodiversity (Morrison, 2009). It is clear that they are neglected and overlooked compared to many other groups, such as vertebrates or parasites, that are associated with more attractive hosts or that are of either medical or veterinary importance (Morrison, 2009). This can easily be attributed to the fact that parasites like haemotozoans are difficult to study and are perhaps found to be uninteresting by many. However, functional databases that contain available data about these parasites can be extremely useful for current and future research.

Prediction models for species distributions are helpful in illustrating the extent of research priority areas across countries or even more specific areas, allowing researchers to bring into perspective which areas lack attention, which have been potentially oversampled, and in general to see which areas have already been covered (Piccolo *et al.*, 2020). Additionally, these models aid as conservation tools, where they can be used over time to see how distribution expands, shrinks or remains the same. This can then be traced back to identify the attributable factors for these events. This can be a larger set of available data, environmental changes such as anthropogenic disturbances and possible effects of climate change, ultimately aiding in decision-making processes regarding conservation. Unfortunately, prediction models are simultaneously potentially biased as they are dependent on the input data such as the recorded and available occurrence records. This stresses the need for publicly available databases containing reliable occurrence data as well as for more species to be prioritized and recorded in these databases.

5.5 Conclusion

It is clear that many factors contribute to the unstable phylogenetic framework of *Haemogregarina* spp., where the most urgent factors are considered to be the use of different primers and the lack of sampling and prioritization of these parasites. As a result, the lack of research focus on this parasite group acts as an impediment on larger research questions regarding the distribution and diversity of these parasites, and also decelerates the process of establishing standardized analytical methods, particularly molecular and phylogenetic

analyses as there is limited molecular information available. The currently available molecular information is also frequently the result of different methods that were employed, for example the use of different primer sets during amplificaion, hence disrupting the process of making accurate conclusions regarding molecular composition and the phylogenetic placements and relationships of *Haemogregarina* spp.

When considering the predicted distribution for *Pl. multistriata* and *Haemogregarina* spp. respectively, it is clear that conservation research is strongly linked to human perceptions. Species that are less charismatic or difficult to sample seldom receive attention (Piccolo *et al.*, 2020). In this case the reputation of terrapins, leeches and haemogregarines are potentially subconsciously debunked by human perceptions for being reptiles and parasites respectively. Ultimately, the conservation of parasites is problematic in the sense that their survival is most likely dependent on the existence of one or more hosts (depending on the complexity of their life cycles). This means that they face a greater risk for extinction and their conservation directly depends on the conservation of one or more other species (Carlson *et al.,* 2020). It is necessary that sampling and research has to expand in order to assess the diversity and distribution of *Haemogregarina* spp, that including sampling of both the leech vectors and the terrapin hosts.

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Chapter 6

Final Discussion and Conclusion

A little science estranges man from God, but much of it leads them back to Him.
~Louis Pasteur

Chapter 6: Final Discussion and Conclusion

6.1 Introduction

In general, parasites are approached as organisms that cause harm to hosts and are therefore poorly prioritized for research, the only exception to this being those of medical, veterinary and economical importance (Carlson *et al.,* 2020; Gómez & Nichols, 2013). As a vertebrate group that serve as hosts to a variety of parasites, reptiles are at a disadvantage as a study host group due to their apparently lower aesthetic value in comparison to birds and mammals (Alves *et al*., 2008; Alves *et al.,* 2009; Ceríaco, 2012), this despite estimates that diversity of reptile associated haematozoans are higher compared to mammals and birds (Javanbakht *et al*., 2015; Telford, 2007).

The overall aim of this research was to identify the presence and diversity of apicomplexan parasites infecting both snakes and terrapins, accompanying this with estimations on the potential role ecology and diet may play in snake associated apicomplexan infections and diversity, as well as to identify future regions in which further exploratory research may be conducted for haemogregarine parasites of terrapins.

With the aid of morphological (morphometric data through screening of blood smears and measurements of peripheral blood stages, accomplished by microscopy), molecular techniques (such as DNA extraction, PCR amplification, sequencing and phylogenetic analyses) and the use of Maxent modelling (using currently available distribution data), potential diversity of parasites were analysed and potential future areas for exploratory research were identified.

6.2 Findings from the research and recommendations for future research

6.2.1 Aim 1:

Much of the diversity of apicomplexan haematozoans in reptiles is described from peripheral blood stages (Telford, 2009) and the majority of these descriptions are based solely on morphological characteristics of these stages. Additionally, in the past, much of this diversity was differentiated based on new vertebrate host species and new locality, this often warranting

a new species description. Although this may have been common place in the past, present research suggests that many apicomplexan haematozoans display a low host-specificity towards the intermediate vertebrate hosts, with their evolutionary history believed to be more closely associated with the definitive invertebrate hosts (Barta *et al*., 2012; Cook *et al*., 2010; Dvořáková *et al.*, 2014; Gupta *et al.,* 2011; Javanbakht *et al.,* 2015; Pineda-Catalan *et al*., 2013;; Sloboda *et al*., 2007; Tomé *et al*., 2012; Tomé *et al*., 2014; Telford *et al*., 2004). This low host-specificity suggested that many of these past species descriptions may have been erroneous, and that further research is required to comprehend the true diversity of these parasites. As such researchers turned to molecular techniques to resolve questions regarding apicomplexan diversity. However, this too has its downfalls. For instance, at present the most widely available and utilized genetic marker for adeleid apicomplexans is the 18S rRNA gene, a slow evolving gene with limited application for differentiating between species.

During the current study, all the sampled snake spp. (*Dendroaspis angusticeps* (Smith, 1849), *Dendrosaspis polylepis* Günther, 1864, *Dispholidus typus* (Smith, 1829), *Bitis arietans* Merrem, 1820, *Naja mossambica* (Peters, 1854), *Naja annulifera* Peters, 1854, and *Python natalensis natalensis* Smith, 1840) were infected with *Hepatozoon* Miller, 1908 spp (Chapter 3). Morphological analysis identified six morphotypes of *Hepatozoon* spp. infecting snakes of seven species. With the use of molecular analysis, particularly divergence analysis, of the five morphotypes that were sequenced, these represented only two species. This species delineation was based on the commonly accepted 3% cut off divergence for species differentiation. *Hepatozoon* sp. A, comprised eight genotypes according to the haplotype network and divergence analysis. It would thus be expected that these genotypes clade together in the phylogenetic analysis; however, these genotypes claded separately. This would suggest that there are more than only two species of *Hepatozoon* identified in this study. These findings, along with the low divergence (typically 1% and lower) between species and genera seen in other studies of adeleids using the 18S marker (Barta, 1997; Cook *et al*., 2014; Maia *et al*., 2011; Morrisson, 2009; Telford *et al*., 2004;; Tomé *et al*., 2014; Úngari *et al*., 2018), suggest that the 3% cut off divergence for these parasites and the 18S marker need to be reevaluated. However, simultaneously, another faster evolving genetic marker needs to be identified. This would in turn assist with the current difficulty in differentiating species, particularly in situations as was encountered in the current study, where peripheral stages of a single species appeared to have a degree of morphological plasticity, which further confounded species differentiation.

Morphological plasticity was also encountered in the gametocyte stages of *Haemocystdium* observed in snakes *N. annulifera* and *N. mossambica* during the current study (Chapter 4),

making species differentiation difficult. Few species of *Haemocystidium* Castellani & Willey, 1904 have been described from African snakes, with only two recognised species at present *Haemocystidium mesnili* (Bouet 1909) Wenyon 1926 and *Haemocystidium balli* Telford, 2007 (Bouet, 1909; Telford, 2007). Both these descriptions were based solely on morphological descriptions. During this study one species of *Haemocystidium, H. mesnili* was identified, based on molecular data, infecting both cobra species. Morphological analysis of the immature and mature gametocytes identified differences in size and shape of *H. mesnili* that was isolated from the two snake host species, as well as differences between these and the peripheral stages described for *H. mesnili* by Telford (2007). Very little is known about the life cycles of *Haemocystidium* species infecting snakes (Telford, 2007) and as such the vectors are unknown for the above species, as is the case with *Hepatozoon* spp. Parasites belonging to the genus *Haemocystidium* are suspected to have a stronger host-specificity towards the definitive invertebrate hosts than towards the vertebrate snake host (Cook *et al*., 2010; Javanbakht *et al.,* 2015; Gupta *et al.,*2011; Pineda-Catalan *et al*., 2013). This once again suggests that a single species may be able to infect multiple snake spp., as species of this genus do not appear to be vertebrate host-specific. Even though the haemosporid species could be identified as a single species with the use of molecular analyses, it is still not certain as to whether this species is indeed *H. mesnili*. The sequence available in GenBank for this species had no accompanying morphological descriptions and was not collected from the type locality (Pineda-Catalan *et al*., 2013). As such when assigning molecular data, such as a sequence, to a named parasite species, it is important to employ both morphological and molecular methods in conjunction. Therreby allowing visual exemplifications of parasites and the joining of their morphological characteristics with accompanying molecular information.

6.2.2 Aim 2:

As mentioned above, present research suggests that many apicomplexan haematozoans display a low host-specificity towards their vertebrate hosts, suggesting that a single parasite species is capable of infecting multiple orders, genera and species of vertebrate host (Barta *et al*., 2012; Cook *et al*., 2010; Dvořáková *et al.*, 2014; Gupta *et al.,* 2011; Javanbakht *et al.,* 2015; Pineda-Catalan *et al*., 2013; Telford *et al*., 2004; Sloboda *et al*., 2007; Tomé *et al*., 2012; Tomé *et al*., 2014). With regards to *Hepatozoon* of snakes, a three host life cycle is suggested (Smith, 1996), including a definitive vector, a primary intermediate host and a secondary intermediate host, where snakes can fill the role of both primary and secondary host. In order for infection to occur, it is thought that infective stages of *Hepatozoon* need to be ingested, as such snakes may become infected when feeding upon infected prey items. And this, may be

reflected in snake species' ecology and diet. During the current study two potential indications of this could be seen (Chapter 3). The phylogenetic analysis placed *Hepatozoon* sp. B infecting *Di. typus* in a monophyletic clade containing species of *Hepatozoon* from other snake species (*Philothamnus natalensis natalensis* (Smith, 1848)) and anurans. Both *P. n. natalensis* and *Di. typus* can occur sympatrically and both include frogs in their diet (Marais, 2004). Similarly, the phylogenetic analysis and divergence data showed *Hepatozoon domerguei*, infecting both a Malagasy snake species and chameleon genus to be genetically identical to the *Hepatozoon* genotype isolated from the current study's *D. angusticeps*. Even though this was surprising, both the Malagasy snake species and *D. angusticeps* include chameleons in their diets. As such this study identified a potential route whereby a more focused investigation into the influence of diet in snakes on their *Hepatozoon* diversity may be more feasible. That is to screen chameleons and *D. angusticeps* from a more focused study area for species of *Hepatozoon*.

6.2.3 Aim 3:

With the increase in both the legal and illegal trade in reptiles (Halla *et al*., 2014), and the lack of knowledge on reptile associated apicomplexan haematozoan vectors, this study aimed to identify the possible longevity of these parasites in wild-caught snakes in captivity (Chapter 3). As such, snakes for which such data was available formed a focus area. Both wild-caught captive snakes, which had been kept in captivity for an extended period of time, and recently caught wild snakes were compared for prevalence and parasitaemia of apicomplexan haematozoans. The overall prevalence and parasitaemia was higher for wild snakes than for captive snakes, with a prevalence of 42.7% and parasitaemia of 0.01–9.3%, compared to 25.8% and 0.01–7.7% respectively. This likely was as a result of the lack of opportunity for captive snakes to be reinfected through diet or the lack of potential vectors. With this in mind, though, some of the captive snakes that were sampled during this study and were found to be infected with *Hepatozoon* were in captivity for up to three years. Other studies have recorded *Hepatozoon* infections in captive snakes, where snakes have positive infections after eight years in captivity (De Biasi *et al.,* 1989; Úngari *et al.,* 2018), supporting the longevity of these infections in captive snakes.

6.2.4 Aim 4:

Species descriptions of species belonging to the genus *Haemogregarina* Danilewsky, 1885 that infect terrapins were also originally approached as being highly host-specific, thereby resulting in the description of many synonymous species as separate species (Dvořáková *et al.*, 2014). Two *Haemogragarina* spp. infecting southern African terrapin species are currently recognised (*Haemogregarina pelusiensi* Pienaar, 1962 and *Haemogregarina maputensis* Dias, 1950), however, these descriptions too were based on morphology alone (Dias & De Sousa, 1950; Paperna, 1989). The current study aimed to identify the infection status of terrapins in the North-West (NW) and KwaZulu-Natal (KZN) provinces for this genus of adeleid. From these two provinces, two species of terrapin were collected and screened (*Pelomedusa galeata* (Schoepff, 1792) and *Pelusios sinuatus* (Smith 1838)), with *Pe. galeata* collected from both NW and KZN and *P. sinuatus* from only KZN. Both species were found to be infected with species of *Haemogregarina*; overall, 95.7% of *Pe. galeata* and 95% of *P. sinuatus*. As such both species had a highly similar prevalence of infection. Regarding parasitaemias between the two species, *P. sinuatus* had a higher overall parasitaemia at 1.6%, compared to the overall parasitaemia in *Pe. galeata* at 0.49%. The vectors for species of *Haemogregarina* are considered to be leeches (Davies & Johnston, 2000), and this is supported by full life cycle studies into these parasites of terrapins (Dvořáková *et al.*, 2014; Paterson & Desser, 1976; Siddall & Desser, 1990; Siddall & Desser, 1992). Whether parasitaemia in *P. sinuatus* was higher due to KZN being more tropical compared to NW, and thus potentially more likely to provide a better habitat for leech vectors, is uncertain. However, this was further explored using predictive distribution modelling (see Aim 5).

6.2.5 Aim 5:

Although the aim was not to assess the diversity of *Haemgregarina* spp. infecting terrapins, the measurements of the different life stages infecting *Pe. galeata* and *P. sinuatus* were similar, suggesting infection with potentially the same species based on morphometrics. However, molecular data was only successfully obtained from *Pe. galeata,* consequently preventing the assessment of this possibility based on molecular data.

During the assessment of *Haemogregarina* spp. infecting terrapins, it was found that the use of different primer sets for the purpose of sequencing the same apicomplexan parasite delivers different results in terms of phylogenetic placements. This was found to be the case for *Haemogregarina* spp. isolated from *Pe. galeata* where sequences were placed in different *Haemogregarina* sister clades depending on the primers that were used. This demonstrates

that some apicomplexan genera require more specific primer sets and that the use of primers that are suitable for a wide taxonomic range of apicomplexans can affect the sequences and the phylogenetic placements of these parasites. This can potentially lead to inaccurate representations of phylogenetic relationships. Further research entailing experimentation and primer design might alleviate some of the analytical difficulties that are currently experienced for terrapin associated *Haemogregarina* spp. (El Hili *et al.,* 2020).

The distribution of the hosts of *Haemogregarina* spp., the vector leeches (*Placobdella multistriata*) and the terrapin species of southern Africa overlap. This suggests that wherever the leeches or the terrapins occur, there is a possibility that *Haemogregarina* spp. might also be present. The literature revealed that the same leech species can transfer different *Haemogregarina* spp. to terrapin spp. and also that *Haemogregarina* spp. display a low host-specificity towards the vertebrate hosts. It was also experimentally proven that *Haemogregarina* spp. can be transferred between different terrapin species (Bielecki *et al.*, 2012; Dvořáková *et al.*, 2014; Siddall & Desser, 2001). The possibility is considered that wherever the distribution of vertebrate hosts (terrapins) and the definitive host (leeches) overlap in southern Africa, if at least one of these hosts are infected with *Haemogregarina* spp., there exists a possibility that *Haemogregarina* spp. can be transferred from terrapins to leeches, and from leeches to terrapins. This also suggests that *Haemogregarina* spp. are capable of occurring across a wide range of environmental paramaters in South Africa. It is likely that the lack of data and records on *Haemogregarina* spp. infecting South African terrapins are attributed to limited sampling.

Predictive distribution maps, although potentially bias in the sense that the predicted distribution output is dependent on the input data, and that predictive distribution outputs can potentially change as new distribution data is presented, provides valuable insight into the distribution of organisms. In this case, the predictive distribution model for terrapin associated *Haemogregarina* spp. was based on available *Haemogregarina* distribution data, but the predictive model provided valuable insight into factors that influence species conservation. The predicted distribution of *Haemogregrina* spp. indicated that there is a limited sampling range for *Haemogregarina* spp. in South Africa, where sampling possibly targets areas where *Haemogregarina* spp. were previously recorded and where successful sampling is preassured, or where the vertebrate hosts are easily available for sampling. However, when considering the low vertebrate host-specificity displayed by *Haemogregarina* spp., and their potential occurrence over a wide range of environmental parameters, suggest that these parasites are likely much more prevalent across South Africa.

It is recommended that sampling for terrapin associated *Haemogregarina* spp. should be expanded, as the predictive model suggests the occurrence of these parasites in geographical areas where sampling has not taken place yet and that the parasites occur across a wide variety of environmental factors. If a larger geographical sampling scope is not covered in southern Africa, it will be difficult to sufficiently assess the diversity or the distribution of these parasites.

There does not appear to be databases for apicomplexan haematozoans that provide information about their life cycles or distribution, hence the need to obtain raw field data from other researchers for this study so as to depict the predicted distribution of terrapin associated *Haemogregarina* spp. This is not surprising when considering how little information is available about their life cycles and vectors, which makes it difficult to convey information into data platforms. However, if such databases are available, it will aid a great deal in the assessment of the diversity of these parasites as data will be easily available and accessible. The lack of databases further demonstrates how poorly apicomplexan parasites are prioritized in terms of conservation, despite estimates that they are the largest parasite group in abundance and diversity and that only approximately 0.1% of these parasites have been discovered and described.

6.3 Conclusion

The overall aim of this research was to assesses the diversity of apicomplexan haematozoans in snakes and terrapins of southern Africa and to demonstrate the limiting effects that fragmentary research relating to the topic has on progressing research.

In terms of assessing the diversity of *Hepatozoon* spp. infecting snakes, this study contributed important information, demonstrating the low host-specificity that *Hepatozoon* spp. display towards snake hosts and the importance of employing both molecular and microscopic techniques as the genetic diversity of these parasites is not reflected by their morphotype or morphological characteristics. It also provided insights into the dietary infection routes of *Hepatozoon* spp. infecting snakes and the importance of taking ecological factors into account as there appears to be uncertainties concerning the full influence of dietary infection routes in the life cycles of these parasites. Therefore, it is necessary to explore ecological and environmental factors that can contribute in the transmission, distribution and diversity of *Hepatozoon* spp.

Valuable information was obtained during the assessment of haemosporidians infecting snakes, suggesting that *H. mesnili* has a strong host-specificity towards African cobra species, as cobras were the only snakes that were infected from the species of snakes in the sampling pool. In addition, nine sequences of the cytochrome *b* (cyt-*b*) gene for *H. mesnili* were obtained, seven sequences from *N. mossambica* and two sequences from *N. annulifera*, providing more sequences for phylogentic analyses concerning *Haemocystidium* species as well as records of *H. mesnili* occurring in a host (*N. mossambica*) that it was not previously recorded from. However, although the molecular and phylogenetic results suggest that the sequences that were obtained during this study were *H. mesnili*, at the same time we cannot be completely certain that it is *H. mesnili* as the only available sequence for this species in GenBank was not morphologically identified and it was not from the type locality.

During the assessment of the diversity of *Haemogregarina* spp. it was found that the use of different primers can affect sequences to such a degree that it consequently affects phylogenetic placements and interpretations regarding phylogenetic relatedness. It was also observed that terrapin associated *Haemogregarina* spp. and their vector leeches (*P. multistriata*) are likely more widely distributed than what is currently known from their distribution based on available distribution records; a scenario that is likely the case for more groups of reptile associated apicomplexan haematozoans.

Many of the issues that were encountered are considered to be the result of a lack of sampling in this research area and that little progress has been made to date in terms of assessing the diversity of these parasites based on both molecular and morphological analyses. However, as research expands, there is an increased need for a stable phylogenetic framework to make accurate interpretations regarding the diversity of apicomplexan haematozoans infecting reptiles. If no precautions are taken, erroneous research approaches might escalate and further contribute to the unstable phylogenetic framework of apicomplexans. Therefore, it is necessary that future research aims to mitigate factors contributing to uncertain phylogenies and strive towards utilizing analytical tools to their full potential.

It is necessary that the apicomplexan diversity based solely on morphological descriptions need to be reassessed with the use of molecular techniques, while taking morphological characteristics of parasites into consideration to determine the validity of their contributions to the true diversity of apicomplexan haematozoans infecting reptiles. That being said, caution should be exercised when molecular analyses are employed to resolve as many questions as possible following a DNA-based approach and to avoid further contributing to phylogenetic uncertainties. It is also recommended that sampling has to expand, covering more reptile species, and individuals belonging to the same species and to cover more geographical areas as well as to assess the impact of ecological factors on the diversity and distribution of these parasites. In the case of *Hepatozoon* spp., in particular, investigative approaches are necessary to explore the presence of *Hepatozoon* spp. in snake prey animals in order to assess the dietary routes of transmission and its impact on *Hepatozoon* diversity in snakes.

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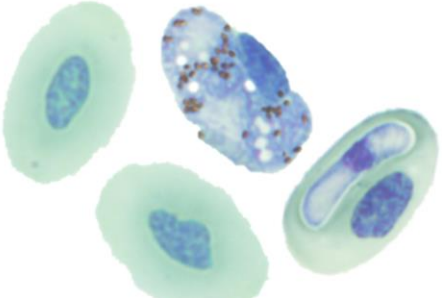
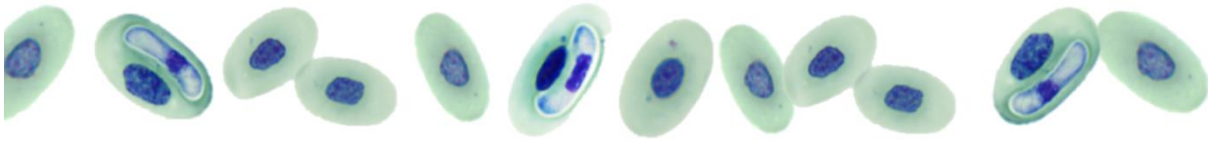
Appendices

The greatest invention of mankind is compound interest.
~Albert Einstein



Appendix A

Chapter 3



Appendix A

A.1 Morphotypes of *Hepatozoon* spp. observed during screening

The screening of the samples that were utilized in Chapter 3 revealed that snakes were infected with several different morphotypes of *Hepatozoon*. Genetic analysis indicated the presence of only two species additionally, some morphotypes were observed but it is suspected that these morphotypes did not amplify during the PCR process. These morphotypes are referred to as morphotype C. All the measurements are recorded in µm.

A.1.1 *Hepatozoon* spp. A

A.1.1.1 Morphotype A1

Mature gamonts, n=121 (Fig A1 A–D). Elongated and ellipsoid in shape, measured 17.84 ± 1.65 (13.40–22.97) long and 4.21 ± 1.32 (1.50–9.15) wide. One pole pointed and slightly hooked (Fig. 3.3 A–B, D: arrowhead), opposite pole usually more pointed than round. For some gamonts both poles appeared more rounded (Fig. A1 C). Gamonts were situated within transparent parasitophorous vacuole (PV) visible as faint gleaming white line around parasite with evident white lines on the outer surface of PV. Nucleus stained dark purple, was densely compacted and measured 6.10 ± 1.81 (3.03–20.28) long and 3.60 ± 1.30 (1.11–6.68) wide n=191. Nucleus appeared to stretch the entire width of parasite or to be situated against the side inconsistently positioned off-centre towards one of the poles. The distance between mid-nucleus and assumed anterior pole was 10.42 ± 1.84 (5.71–16.73) and between mid-nucleus and assumed posterior pole it measured 7.31 ± 1.62 (3.28–12.86) n=191. Occurred within mature erythrocytes, erythrocyte nucleus displaced from centre or pushed to the side by gamont. Morphotype A1 was observed in *Dendroaspis polylepis* and *Naja mossambica.*

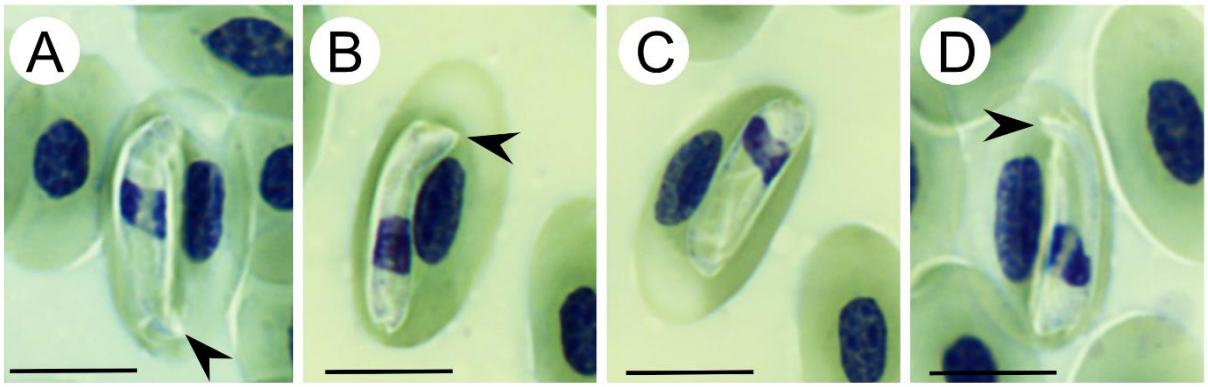


Figure A1: Gamonts of *Hepatozoon* spp. A, morphotype A1 (A–D) observed in *Dendroaspis polylepis* and *Naja mossambic.* **Scale bar = 10**.

A.1.1.2 Morphotype A2

Mature gamonts occured within mature erythrocytes (Fig. A2 A–D). Elongated, slender and slightly crescent-shaped. Measured 15.96 ± 1.29 (14.26–19.82) in length and 2.60 ± 0.65 (1.60–4.20) in width (n=23). Cytoplasm stained light purple. Poles were slightly curved inwards (Fig. A2 B–C: arrow heads). Some gamonts situated loosely within PV (Fig.A2 C), whereas the PV of some were barely visible (Fig. 3.3 F). Nucleus was densely compacted, stained deep dark purple and was situated fairly in the centre of parasite body. The nucleus measured 5.39 ± 1.20 (3.70–7.68) long and 1.94 ± 0.53 (1.22–3.23) wide (n=20). The distance between the assumed anterior pole and mid-nucleus measured 9.27 ± 1.38 (6.36–11.62) long and between mid-nucleus and assumed posterior pole it measured 6.76 ± 1.90 (3.70–10.64), n=20.

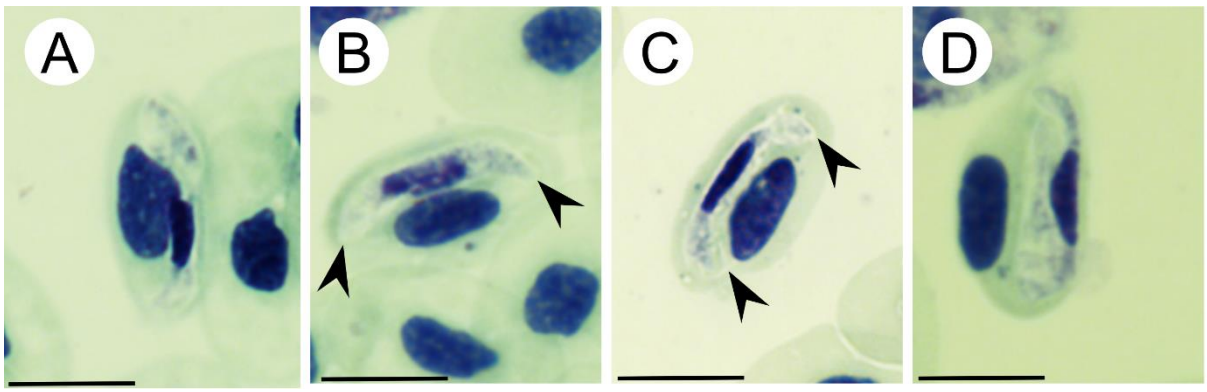


Figure A2: Gamonts of *Hepatozoon* spp. A morphotype A2 (A–D) of observed in *Dendroaspis polylepis* **Scale bar = 10µm**.

A.1.1.3 Morphotype A3

Mature gamonts occurred in mature erythrocytes n=94, (Fig. A3 A–D). Elongated slender parasites with a recurved ‘tail’ (Fig. A3 A–D: arrow heads) with opposite pole pointed and curved slightly inwards. Measured 16.90 ± 2.21 (12.90–22.72) long and 3.15 ± 0.94 (1.64–5.12) wide. Cytoplasm stained dark purple. Nucleus stained dark blue-purple, and was not clearly visible. Measured 5.96 ± 1.07 (3.75–10.30) long and 3.11 ± 1.34 (1.50–10.86) wide. The distance between the assumed anterior pole and mid-nucleus measured 9.82 ± 1.87 (2.21–14.00) and between mid-nucleus and assumed posterior pole it measured 6.90 ± 1.70 (4.26–12.95). Assumed immature gamonts (Fig. A–C stained light opaque purple and the parasite body appeared slightly thicker than mature gamonts (Fig. 3.3 D) which uniformly stained darker.

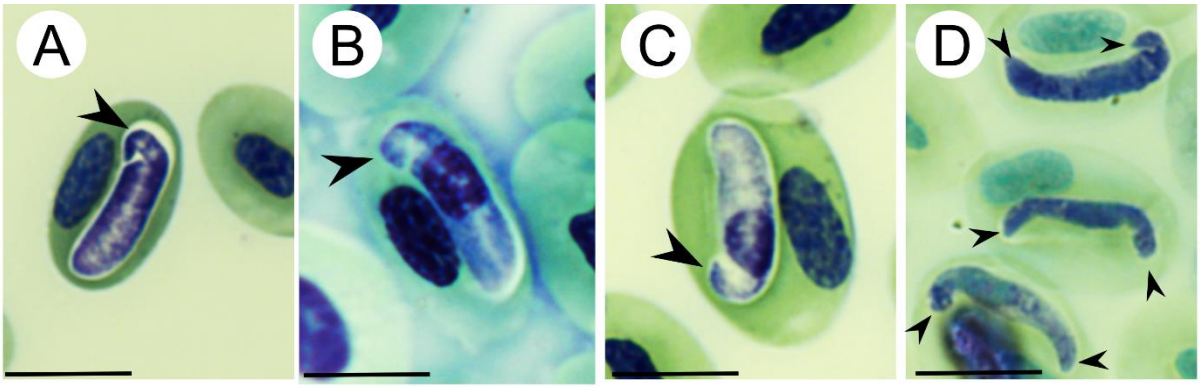


Figure A3: Gamonts of *Hepatozoon* spp. A morphotype A3 (A–D) observed in *Dispholidus typus, Naja mossambica* and *Python natalensis natalenis* **Scale bar = 10µm**.

A.1.1.4 Morphotype A4

Mature gamonts occurred within mature erythrocytes, n=341 (Fig. A4 A–D). Elongated and bean shaped. Measured 15.81 ± 1.65 (11.66–21.10) long and 3.40 ± 0.98 (1.41–5.74) wide. The PV sometimes visible as white ‘halo’ around parasite. The cytoplasm stained light blue to purple. The nucleus was foamy in appearance and stained bright lilac purple. Nucleus positioned slightly off-centre and measured 5.67 ± 1.14 (2.85–10.70) long and 2.77 ± 0.99 (1.01–5.36) wide. The distance between the assumed anterior pole and the mid-nucleus measured 9.20 ± 1.45 (5.43–13.60) and between the assumed posterior pole and mid-nucleus it measured 6.62 ± 1.17 (3.17–13.42). A mature gamont was observed preparing to leave the erythrocyte, with the assumed anterior pole pointed (Fig.A4 E; arrow). Additionally a mature gamont was observed while it was in the process of leaving the PV (Fig.A4 F

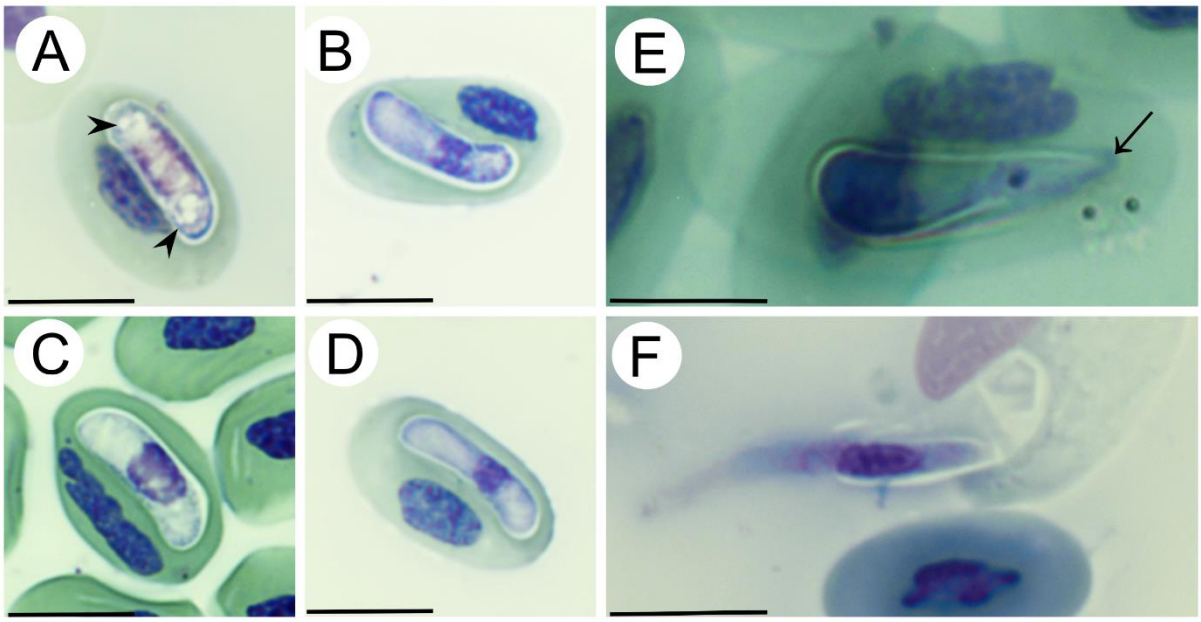


Figure A4: Gamonts of *Hepatozoon* spp. A, morphotype A4 (A–D) observed in *Dendroaspis polylepis*, *Dendroaspis angusticeps*, *Dispholidus typus, Bitis arietans, Naja mossambica* and *Python natalensis natalenis.* A gamont preparing to leave the host erythrocyte (E) and gamont in the process of leaving the parasitophorous vacuole (F) **Scale bar = 10 µm**.

A.1.2 *Hepatozoon* spp. B

A.1.2.1 Morphotype B1

Mature gamonts occurred within mature erythrocytes n=41 (Fig. 3.4 f–h). Gamonts elongated and ellipsoid in shape. One pole slightly pointed (Fig. 3.4 f–g: arrow head) and opposite pole more rounded. Measured 17.60 ± 1.20 (15.23–20.34) long and 4.42 ± 1.03 (2.77–6.63) wide. PV visible as white halo surrounding parasite. Cytoplasm stained light to dark purple. Nucleus stained dark purple, located in middle of parasite and stretched the width of the parasite. Measured 6.44 ± 1.09 (4.34–10.24) long and 4.06 ± 0.94 (2.05–6.00) wide. The distance between the assumed anterior pole and mid-nucleus measured 9.19 ± 1.12 (6.91–11.58) and between the mid-nucleus and assumed posterior pole measured 8.42 ± 1.21 (5.41–10.65).

The effects of the gamonts on host erythrocytes was the most apparent of all the *Hepatozoon* gamonts observed during this study. The cell membrane of the host erythrocyte was extremely thin and cells were stretched in length. Host erythrocytes were dehemoglobinized and the host cell nuclei were situated adherent to the gamonts. In some cases partial cell lysis of the erythrocyte was visible (Fig. A5 A: arrow head). It was frequently observed that the erythrocyte outer membranes and content were not distinguishable and it appeared as if the gamont just

arbitrarily occurred attached to a free cell nucleus. This was the case when cell cytoplasm was completely demolished.

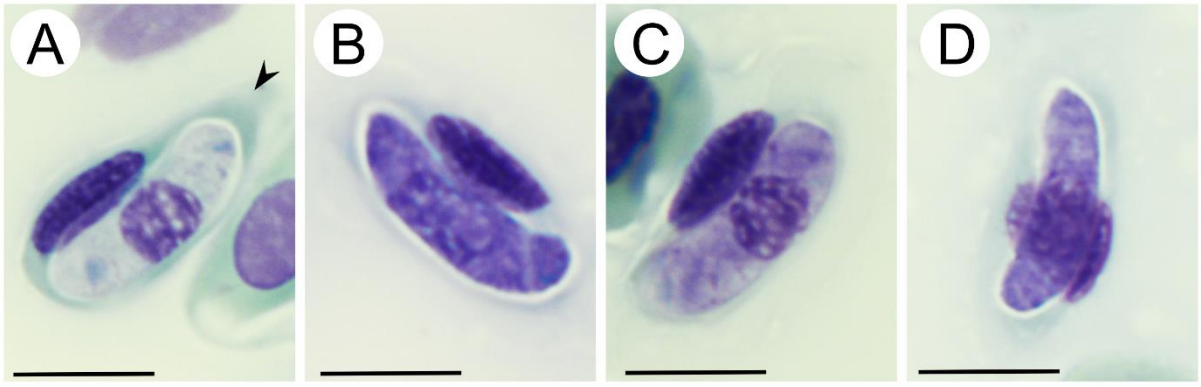


Figure A5: Gamonts of *Hepatozoon* spp B morphotype B1 (A–D) of observed in *Dispholidus typus* **Scale bar = 10 µm**.

A.1.3 Observed *Hepatozoon* spp. without accompanying genetic information

The following gamonts were observed, but not amplified during molecular analyses. Therefore the morphotypes are not accompanied by genotypes as no molecular data was available to classify them genetically.

A.1.3.1 Morphotype C1

One single gamont (Fig.A6 A) was observed in one captive *Naja mossambica* individual. It had the same appearance as morphotype A4 of species A (Fig. A4 A–D), but without accompanying genetic information it is not possible to determine the genotype. Gamont measured 14.58 long and 2.30 wide. The nucleus measured 5.29 long and 1.40 wide. The distance between the assumed anterior pole and mid-nucleus as 9.29 and between the assumed posterior pole and the mid-nucleus it was 5.30. The cytoplasm stained light purple, the nucleus stained dark blue-purple. Elongated bean shaped, poles slightly thicker than parasite mid-body. Since no molecular data was obtained for this individual the genetic identity could not be established.

A.1.3.2 Morphotype C2

Mature gamonts occurred within mature erythrocytes, n=8 (Fig. A6 B–C). Unevenly ovoid shaped. Bright opaque white. Measured 15.14 ± 1.46 (12.08–16.50) long and 6.98 ± 0.52

(6.20–7.49) wide. The genetic material stained dark purple, distorted and pushed tightly against the edges of the parasite. The PV sometimes slightly visible as white lining around parasite (Fig A6 B). Erythrocyte nucleus pushed to the side by gamont.

A.1.3.3 Morphotype C3

Mature gamonts occurred within mature erythrocytes, n=30 (Fig. A6 D–E): Ellipsoid in shape. Measured 16.00 ± 1.28 (13.33–17.59) long and 7.26 ± 1.29 (1.48–9.44) wide. Impure bright crystal appearance, scattered with vibrant white and light pastel purple. Poles are usually whiter than rest of parasite body with larger white specks (Fig. A6 D: black arrow heads). Occasionally clear circular structures are visible at poles with appearance of vacuoles (Fig. A6 E: white arrows). Erythrocyte nucleus displaced or pushed to the side by gamont.

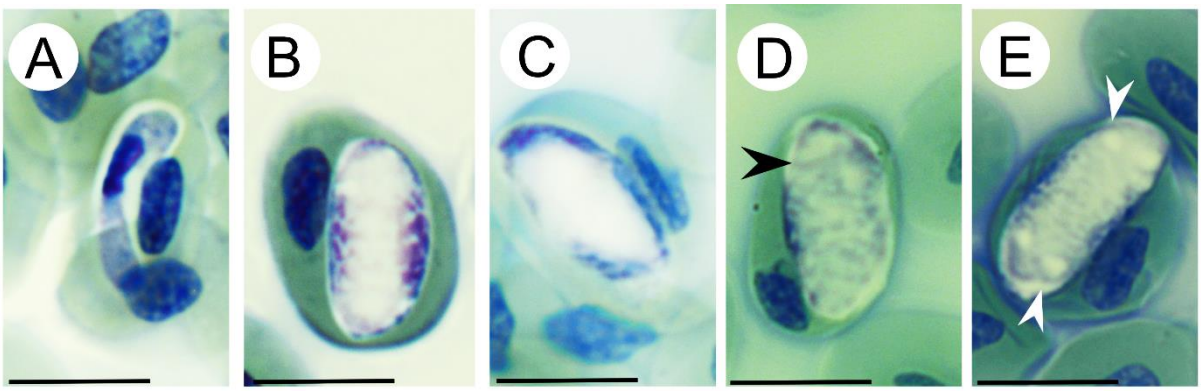


Figure A6: A single gamont of *Hepatozoon* morphotype C1 observed in *Naja annulifera* (A); gamonts of morphoype C2 (B–C) observed in *Dendroaspis polylepis* and gamonts of morphotype C3 (D–E) observed in *Naja mossambica* **Scale bar = 10 µm**.

A.2 Apicomplexan 18S rRNA sequences used for the phologenetic analysis of *Hepatozoon* spp.

Table A1: Accession numbers, species, host, localities and references of sequences obtained from GenBank that were used for the construction of the *Hepatozoon* phylogenetic tree.

Accession number	Organism	Host	Locality	Reference
FJ719817	*Hepatozoon* sp.	*Abrothrix olivaceus*	Chile	Merino *et al*. (2009)
AY600626	*Hepatozoon* sp.	*Clethrionomys glareolus*	Spain	Criado-Fornelio *et al*. (2006)
AY628681	*Hepatozoon felis*	*Felis catus*	Southern Brazil	Criado-Fornelio *et al.* (2006)
KX757032	*Hepatozoon silvestris*	*Felis silvesris silvestris*	Bosnia and Herzegovina	Hodžić *et al.* (2016)
EF222257	*Hepatozoon* sp.	*Martes martes*	Spain	Criado-Fornelio *et al.* (2009)
AY461378	*Hepatozoon canis*	*Canis familiaris*	Spain	Criado-Fornelio *et al.* (2006)
EU041717	*Hepatozoon ursi*	*Ursus thibetanus japonicas*	Japan	Kubo *et al.* (2008)
EF222259	*Hepatozoon* sp.	*Sciurus vulgaris*	Spain	Criado-Fornelio *et al.* (2009)
HQ734789	*Hepatozoon* sp.	*Quedenfeldtia moerens*	Morocco	Maia *et al.* (2011)
HQ734801	*Hepatozoon sp.*	*Timon pater tangitana*	Morocco	Maia *et al.* (2011)
HQ734799	*Hepatozoon sp.*	*Timon pater tangitana*	Morocco	Maia *et al.* (2011)
HQ734798	*Hepatozoon sp.*	*Atlantolacerta andreanskyi*	Morocco	Maia *et al.* (2011)

Table A1 continued: Accession numbers, species, host, localities and references of sequences obtained from GenBank that were used for the construction of the *Hepatozoon* phylogenetic tree.

Accession number	Organism	Host	Locality	Reference
HQ734795	*Hepatozoon sp.*	*Podarcis vaucheri*	Morocco	Maia *et al.* (2011)
HQ734794	*Hepatozoon sp.*	*Podarcis vaucheri*	Morocco	Maia *et al.* (2011)
JX531921	*Hepatozoon sp.*	*Podarcis sp.*	Spain	Maia et al., (2012)
HQ734807	*Hepatozoon sp.*	*Timon pater tangitata*	Morocco	Maia *et al.* (2011)
HQ734787	*Hepatozoon sp.*	*Tarentola mauritanica*	Algeria	Maia *et al.* (2011)
HQ292771	*Hepatozoon sp.*	*Mabuya wrightii*	Seychelles	Harris *et al.* (2011)
HQ734796	*Hepatozoon sp.*	*Eumeces algeriansis*	Morocco	Maia *et al.* (2011)
MN723845	*Hepatozoon ophisauri*	*Pseudopus apodus*	Iran	Zechmeisterova (2019) Unpublished
HQ292773	*Hepatozoon* sp.	*Lycognathophis seychellensis*	Seychelles	Harris *et al.* (2011)
HQ292774	*Hepatozoon* sp.	*Lycognathophis seychellensis*	Seychelles	Harris *et al.* (2011)
KM234646	*Hepatozoon domerguei*	*Madagascarophis colubrinus*	Madagascar	Maia *et al.* (2014)
AF297085	*Hepatozoon* sp.	*Boiga irregularis*	Australia	Jakes *et al.* (2000) Unpublished
KC696568	*Hepatozoon* sp.	*Psammophis elegans*	North Africa	Tomé *et al.* (2013)

Table A1 continued: Accession numbers, species, host, localities and references of sequences obtained from GenBank that were used for the construction of the *Hepatozoon* phylogenetic tree.

Accession number	Organism	Host	Locality	Reference
KF939627	*Hepatozoon* sp.	*Elaphe carinata*	China	Wu *et al*. (2013)
MG519501	*Hepatozoon angeladaviesae*	*Philothamnus natalensis natalensis*	South Africa	Cook *et al.* (2018)
MG519502	*Hepatozoon angeladaviesae*	*Philothamnus natalensis natalensis*	South Africa	Cook *et al.* (2018)
MG519503	*Hepatozoon cecilhoarei*	*Philothamnus natalensis natalensis*	South Africa	Cook *et al*. (2018)
MG519504	*Hepatozoon cecilhoarei*	*Philothamnus natalensis natalensis*	South Africa	Cook *et al.* (2018)
JN181157	*Hepatozoon sipedon*	*Nerodia sipedon sipedon*	Canada	Barta *et al.* (2012)
KR069084	*Hepatozoon fitzimonsi*	*Kinixys zombensis*	South Africa	Cook *et al.* (2015)
KP119773	*Hepatozoon theileri*	*Amietia quecketti*	Africa	Netherlands *et al*. (2014)
KP119772	*Hepatozoon ixoxo*	*Sclerophrys pusilla*	Africa	Netherlands *et al.* (2014)
HQ224962	*Hepatozoon* cf. *clamatae*	*Rana clamitans*	Canada	Barta *et al.* (2012)
HQ224954	*Hepatozoon* cf. *catesbianae*	*Rana catesbeiana*	Canada	Barta *et al.* (2012)

A.3 Evolutionary divergences

Table A2: Evolutionary differences of various 18S rRNA gene sequences of *Hepatozoon* species that were included in the phylogenetic analyses (Fig. 5.3). Evolutionary differences are expressed as uncorrected pair-wise distance (p-distance) (top right).

	***Hepatozoon* species and Hosts extracted from**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	*Hepatozoon* sp. ex *Dendroaspis angusticeps* II		0.004	0.010	0.010	0.010	0.010	0.000	0.004	0.010	0.004	0.010
2	KC696568 *Hepatozoon* sp. *Psammophis elegans*			0.010	0.010	0.010	0.010	0.004	0.000	0.010	0.003	0.010
3	MG519501 *Hepatozoon angeladaviesae* ex *Philothamnus natalensis natalensis*				0,000	0,000	0.003	0.010	0.010	0.002	0.009	0.000
4	MG519502 *Hepatozoon angeladaviesae* ex *Philothamnus natalensis natalensis*					0,000	0.003	0.010	0.010	0.002	0.009	0.000
5	MG519503 *Hepatozoon cecilhoarei* ex *Philothamnus natalensis natalensis*						0.003	0.010	0.010	0.002	0.009	0.000
6	MG519504 *Hepatozoon cecilhoarei* ex *Philothamnus natalensis natalensis*							0.010	0.010	0.004	0.010	0.003
7	KM234646 *Hepatozoon domergue*i ex *Madagascarophis colubrinus*								0.004	0.010	0.004	0.010
8	*Hepatozoon* sp. ex *Bitis arietans* III									0.010	0.003	0.010
9	*Hepatozoon* sp. ex *Dispholidus typus* VII										0.010	0.002
10	*Hepatozoon* sp. ex *Dispholidus typus* V											0.009
11	*Hepatozoon* sp. ex *Dispholidus typus* I											

Table A3: Evolutionary differences of various 18S rRNA gene sequences of *Haemogregarina* species that were included in the phylogenetic analyses (Fig. 5.3). Evolutionary differences are expressed as uncorrected pair-wise distance (p-distance) (top right). **(Table can be made available in excel format as per request**)

	Sequences	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	29	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
1	*Dispholidus typus* VI		0,015	0,007	0,077	0,073	0,077	0,077	0,077	0,077	0,077	0,077	0,077	0,077	0,082	0,082	0,082	0,082	0,082	0,078	0,078	0,078	0,078	0,073	0,070	0,075	0,075	0,062	0,073	0,073	0,073	0,073	0,073	0,073	0,073	0,073
2	*Dispholidus typus* VII			0,002	0,073	0,070	0,073	0,073	0,073	0,073	0,073	0,073	0,073	0,073	0,078	0,078	0,078	0,078	0,078	0,077	0,077	0,077	0,073	0,071	0,068	0,073	0,073	0,060	0,071	0,071	0,071	0,071	0,071	0,071	0,071	0,071
3	*Dispholidus typus* I				0,065	0,061	0,065	0,065	0,065	0,065	0,065	0,065	0,065	0,065	0,067	0,067	0,067	0,067	0,067	0,067	0,067	0,067	0,063	0,059	0,056	0,061	0,061	0,049	0,059	0,059	0,059	0,059	0,059	0,059	0,059	0,059
4	*Dendroaspis polylepis* IV					0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
5	*Dendroaspis polylepis* III						0,002	0,002	0,002	0,002	0,002	0,002	0,002	0,002	0,022	0,022	0,022	0,022	0,022	0,024	0,024	0,024	0,017	0,015	0,013	0,018	0,015	0,009	0,015	0,015	0,015	0,015	0,015	0,015	0,015	0,015
6	*Naja mossambica* VI							0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
7	*Dendroaspis polylepis* X								0,000	0,000	0,000	0,000	0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
8	*Dendroaspis polylepis* IX									0,000	0,000	0,000	0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
9	*Dendroaspis polylepis* VIII										0,000	0,000	0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
10	*Dendroaspis polylepis* V											0,000	0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
11	*Naja mossambica* V												0,000	0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
12	*Dendroaspis polylepis* I													0,000	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
13	*Dendroaspis polylepis* II														0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,027	0,020	0,020	0,018	0,024	0,020	0,013	0,020	0,020	0,020	0,020	0,020	0,020	0,020	0,020
14	*Dispolidus typus* IX															0,000	0,000	0,000	0,000	0,022	0,022	0,022	0,013	0,018	0,007	0,018	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
15	*Dispholidus typus* VIII																0,000	0,000	0,000	0,022	0,022	0,022	0,013	0,018	0,007	0,018	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
16	*Naja mossambica* I																	0,000	0,000	0,022	0,022	0,022	0,013	0,018	0,007	0,018	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
17	*Dispholidus typus* III																		0,000	0,022	0,022	0,022	0,013	0,018	0,007	0,018	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
18	Dispholidus typus II																			0,022	0,022	0,022	0,013	0,018	0,007	0,018	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
19	*Python natalensis* VI																				0,000	0,000	0,015	0,018	0,015	0,015	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
20	*Python natalensis* V																					0,000	0,015	0,018	0,015	0,015	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
21	*Python natalensis* I																						0,015	0,018	0,015	0,015	0,015	0,011	0,018	0,018	0,018	0,018	0,018	0,018	0,018	0,018
22	*Dendroaspis angusticeps* I																							0,015	0,011	0,015	0,011	0,007	0,015	0,015	0,015	0,015	0,015	0,015	0,015	0,015
23	*Dispholidus typus* IV																								0,000	0,007	0,004	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
24	Python natalensis III																									0,007	0,004	0,002	0,000	0,000	0,000	0,000	0,000	0,000	0,000	0,000
25	*Bitis arietans* III																										0,004	0,006	0,007	0,007	0,007	0,007	0,007	0,007	0,007	0,007
26	*Naja mossambica* III																											0,002	0,004	0,004	0,004	0,004	0,004	0,004	0,004	0,004
27	*Python natalensis* IV																												0,002	0,002	0,002	0,002	0,002	0,002	0,002	0,002
28	*Bitis arietans* II																													0,000	0,000	0,000	0,000	0,000	0,000	0,000
29	*Naja mossambica* IV																														0,000	0,000	0,000	0,000	0,000	0,000
30	*Dendroaspis polylepis* VII																															0,000	0,000	0,000	0,000	0,000
31	*Dendroaspis polylepis* VI																																0,000	0,000	0,000	0,000
32	*Python natalensis* II																																	0,000	0,000	0,000
33	*Dispholidus typus* V																																		0,000	0,000
34	*Bitis arietans* I																																			0,000
35	*Naja mossambica* II																																			

Appendix B

Chapter 4

Appendix B

B.1 Apicomplexan cytochrome *b* (cyt *b*) sequences used for the phologenetic analysis of haemoproteid spp.

Table B1: Accession numbers, species, hosts, localities and references of sequences obtained from GenBank that were used for the construction of the phylogenetic tree in Chapter 4.

Accession number	Parasite	Host	Locality	Reference
EU834703	*Plasmodium minuoviride*	*Prasinohaema prehensicauda*	Papua New Guinea	Perkins & Austin (2009)
EU834704	*Plasmodium koreafense*	*Sphenomorphus jobiensis*	Papua New Guinea	Perkins & Austin (2009)
EU834705	*Plasmodium megalotrypa*	*Sphenomorphus jobiensis*	Papua New Guinea	Perkins & Austin (2009)
KR477583	*Plasmodium fairchildi*	*Norops cupreus*	Mexico	Falk *et al*. (2015)
EU834710	*Plasmodium lacertiliae*	*Emoia longicauda*	Papua New Guinea	Perkins & Austin (2009)
KX121608	*Plasmodium zonuriae*	*Cordylus vittifer*	North-West, South Africa	Van As *et al*., (2016)
KX121601	*Plasmodium intabazwe*	*Pseudocordylus melanotus*	Free State, South Africa	Van As *et al*., (2016)
AY099059	*Plasmodium floridense*	*Anolis oculatus*	Dominica	Perkins & Schall (2002)

Table B1 continued: Accession numbers, species, hosts, localities and references of sequences obtained from GenBank that were used for the construction of the phylogenetic tree in Chapter 4.

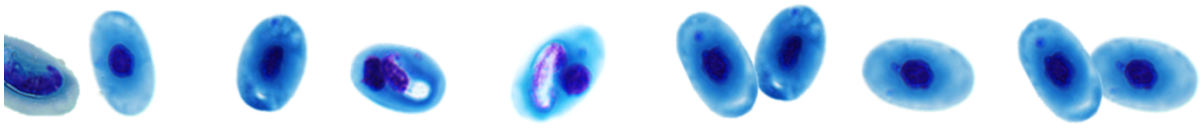
Accession number	Parasite	Host	Locality	Reference
AF069612	*Plasmodium gallinaceum*	*Gallus gallus*	Asia	Escalante *et al.,* (1998)
AF069611	*Plasmodium elongatum*	*Passer domesticus*	North America	Escalante *et al.,* (1998)
DQ659553	*Plasmodium relictum*	*Hemignathus virens*	Hawaii	Beadell *et al.,* (2006)
JN164734	*Plasmodium circumflexum*	*Sylvia atricapilla*	Spain	Perez-Rodriguez & Perez-Tris (2011)
EU834707	*Plasmodium gemini*	*Hypsilurus modestus*	Papua New Guinea	Perkins & Austin (2009)
AY099060	*Plasmodium mexicanum*	*Sceloporus mexicanum*	USA California	Perkins & Schall (2002)
AY099061	*Plasmodium chiricahuae*	*Sceloporus jarrovi*	USA: Arizona	Perkins & Schall (2002)
AF069619	*Plasmodium vivax*	*Homo sapiens*	Brazil	Escalante *et al.,* (1998)
AF069621	*Plasmodium knowlesi*	Old world onkeys	Malaysia	Escalante *et al.,* (1998)
AF069624	*Plasmodium malariae*		Uganda	Escalante *et al.,* (1998)
AF069605	*Plasmodium falciparum*	*Homo sapiens*	Tropical regions	Escalante *et al.,* (1998)

Table B1 continued: Accession numbers, species, hosts, localities and references of sequences obtained from GenBank that were used for the construction of the phylogenetic tree in Chapter 4.

Accession number	Parasite	Host	Locality	Reference
AF069610	*Plasmodium reichenowi*	*Pan troglodytes*	Central Africa	Escalante *et al.,* (1998)
DQ630010	*Haemoproteus lanii*	*Lanius collurio* Linnaeus, 1758	Sweden	Hellgren *et al.,* (2007)
JN164722	*Haemoproteus parabelopolskyi*	*Sylvia atricapilla*	Europe	Perez-Rodriguez & Perez-Tris (2011)
DQ630008	*Haemoproteus balmorali*	*Muscicapa striata*	Sweden	Hellgren *et al.,* (2007)
DQ630005	*Haemoproteus pallidus*	*Ficedula hypoleuca*	Sweden	Hellgren *et al.,* (2006) Unpublished
AY099062	*Haemocystidium kopki*	*Teratoscincus scincus*	Pakistan	Perkins & Schall (2002)
AY099057	*Haemocystidium ptyodactyli*	*Ptyodactylus hasselquistii*	Israel	Perkins & Schall (2002)
KF049506	*Haemocystidium pacayae*	*Podocnemis unifilis*	Israel	Pineda-Catalan *et al*., (2013)
KF049514	*Haemocystidium mesnili*	*Naja annulifera*	South Africa	Pineda-Catalan *et al*., (2013)
DQ451435	*Leucocystozoon gentili*	*Passer domesticus*	Israel	Martinsen *et al.,* (2006)
DQ451439	*Leucocystozoon majoris*	*Fringilla coelebs*	Israel	Martinsen *et al.,* (2006)

Appendix C

Chapter 5



Appendix C

C.1 Apicomplexan 18S rRNA sequences used for the phologenetic analysis of *Haemogregarina* spp.

Table C1: Accession numbers, localities and references of apicomplexan 18S rRNA sequences that were used for the construction of the *Haemogregarina* phylogenetic tree in chapter 5.

Accession number	Organism	Host extracted from	Locality	Reference
AF176836	*Hepatozoon americanum*	*Canis familiaris*	–	Matthew *et al.,* (2000)
AY461378	*Hapatozoon canis*	*Canis familiaris*	Spain	Criado-Fornelio *et al.,* (2006)
AY620232	*Hepatozoon felis*	*Felis catus*	Spain	Criado-Fornelio *et al.,* (2006)
EU041717	*Hepatozoon ursi*	*Ursus thibetanus japonicas*	Japan	Kubo *et al*., (2008)
AF130361	*Hepatozoon catesbianae*	*Lithobates clamitans*	–	Carreno *et al.,* (1999)
HQ224962	*Hepatozoon* cf. *clamatae*	*Rana clamitans*	Canada	Barta *et al*., (2012)
AF297085	*Hepatozoon sp.*	*Boiga irregularis*	Australia	Jakes *et al.,* (2000) Unpublished
EF157822	*Hepatozoon ayorgbor*	*Python regius*	Africa	Sloboda *et al.,* (2007)

Table C1 continued: Accession numbers, localities and references of apicomplexan 18S rRNA sequences that were used for the construction of the *Haemogregarina* phylogenetic tree in chapter 5.

Accession number	Organism	Host extracted from	Locality	Reference
JN181157	*Hepatozoon sipedon*	*Nerodia sipedon sipedon*	Canada	Barta *et al*., (2012)
HQ224961	*Babesiosoma stableri*	*Lithobates septentrionalis*	Canada	Barta *et al*., (2012)
HQ224958	*Dactylosoma ranarum*	*Rana esculenta*	France	Barta *et al*., (2012)
KX507248	*Haemogregarina sp.*	*Macrochelys temminckii*	Texas	Alhaboubi *et al.,* (2017)
MH503891	*Haemogregarina sp.*	*Lepidosiren paradoxa*	Amazon	Esteves-Silva *et al.,* (2019)
MH503892	*Haemogregarina sp.*	*Lepidosiren paradoxa*	Amazon	Esteves-Silva *et al.,* (2019)
KF257928	*Haemogregarina stepanowi*	*Emys orbicularis*	Bulgaria	Dvořáková *et al.,* (2014)
KF257927	*Haemogregarina stepanowi*	*Mauremys rivulata*	Syria	Dvořáková *et al.,* (2014)
KF257926	*Haemogregarina stepanowi*	*Mauremys caspica*	Iran	Dvořáková *et al.,* (2014)
KF257929	*Haemogregarina stepanowi*	*Mauremys leprosa*	Algeria	Dvořáková *et al.,* (2014)
HQ224959	*Haemogregarina balli*	*Chelydra serpentina serpentina*	–	Barta *et al*., (2012)

Table C1 continued: Accession numbers, localities and references of apicomplexan 18S rRNA sequences that were used for the construction of the *Haemogregarina* phylogenetic tree in chapter 5.

Accession number	Organism	Host extracted from	Locality	Reference
KF257925	*Haemogregarina sp.*	*Pelusios subniger*	Mozambique	Dvořáková *et al.,* (2014)
KF257923	*Haemogregarina sp.*	*Pelusios williamsi*	Kenya	Dvořáková *et al.,* (2014)
KF257924	*Haemogregarina sp*	*Pelusios marani*	Gabon	Dvořáková *et al.,* (2014)
DQ096835	*Adelina dimidiata*	*Scolopendra cingulata*	Bulgaria	Kopečná *et al*., (2006)

C.2 Photo credits and CC licences of terrapin photos

C.2.1 *Pelomedusa subrufa*

Photographer: Ashley Wahlberg (Tubbs)

Link downloaded from: https://www.flickr.com/photos/47745688@N05/14991981141/

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C.2.2 *Pelusios rhodesianus*

Photographer: suzannevf

Link downloaded from: https://www.inaturalist.org/taxa/73896-Pelusios rhodesianus/browse_photos

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C.2.3 *Pelusios castanoides*

Photographer: andyfrank

Link downloaded from: https://www.inaturalist.org/taxa/73895-Pelusios-castanoides/browse_photos

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C.2.4 *Pelusios bechuanicus*

Photographer: Grant Reed

Link downloaded from: https://www.inaturalist.org/taxa/109021-Pelusios-bechuanicus/browse_photos

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C.2.5 *Cycloderma frenatum*

Photographer: Martin Grimm

Link downloaded from: https://www.flickr.com/photos/mgrimm82/14677691277/in/photostream/

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C.2.6 *Trionyx triunguis*

Photographer: Dan Schwartz

Link downloaded from: https://en.wikipedia.org/wiki/File:African-Softshell-Turtle-On-Rock---Alexander-river---Israel.jpg

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Appendix D

Permits and Ethics

D.1 North-West province collection permit

MCPO 00664/1/2019 NW 7650/02/2019 Application ID 7650

North West Province

Issued in terms of the provisions of:
The National Environmental Management Biodiversity Act, Act 10 of 2004 as amended

Issued in terms of the provisions of:
(1) Bophuthatswana Nature Conservation Act, Act No.3 of 1973; (2) Transvaal Nature Conservation Ordinance, No.12 of 1983; (3) Cape Nature and Environmental Conservation Ordinance, 19 of 1974.

Biodiversity North West Integrated Permit Ordinary Catch, Release, Research Trap

Conduct Research or Scientific Project

APPROVED SPECIES AND NUMBERS, RESTRICTED ACTIVITIES AND CONDITIONS AS PER ADDENDUM AND PAGES ATTACHED

PERMIT HOLDER

Details		Physical Address		Postal Address	
Surname:	Weldon	Building:		Private Bag	X6001
Full Name:	Che	Street:	6 Meul street	Post Office:	North-West University
Id Number:	7502075129088	Suburb:			
Passport:		Town:	Potchefstroom	Town:	Potchefstroom
Cell Home:	0825689001	Area Code:	2531	Postal Code:	2520
Tel Home:		Division/Region:	Potchefstroom	District/Region:	Potchefstroom
Tel Work:	0182992375	Province/State:	North West Province	Province/State:	North West Province
Fax Home:		Country:	REP OF SOUTH AFRICA	Country:	REP OF SOUTH AFRICA
Email:	che.weldon@nwu.ac.za				

REGION

NorthWest

LOCATION

Facility

Property
Property Name: North West Province excluding provincial protected areas
Building:
Street:
Suburb:
Town:
Area Code:
Division/Region:
Province/State:
Country:

NATURE CONSERVATION PERMIT OFFICE
NORTH WEST PROVINCE
28 Feb 2019, 10:19 AM
Private Bag X2039 Mmabatho 2735
Tel: +27 (0)18 389 5231 Fax: +27(0)18 389 5640

Stamp of Issuing authority

VALIDITY PERIOD
FROM 09/01/2019 TO 31/12/2021

Stamp if applicable

Permit holder / Dealer

Signature of Issuing Authority
(Basie Diole)
28 Feb 2019, 10:19 AM

Signature of Permit Holder
(Che Weldon)
28 Feb 2019, 10:19 AM

orth West Department of Rural, Environment and Agricultural Development. (READ), Cnr. Dr. James Moroka Drive & Stadium Road, Mmabatho
Contact Information: Tel:+27 (0)18 389 5130, Fax:+27 (0)18 389 5130, E-mail:jdenga@nwpg.gov.za
Postal Address: Private Bag X 15, Mmabatho, 2735

ADDENDUM

PROPERTIES

Name	Town	Province	Country
North West Province excluding provincial protected areas			

SPECIES INFORMATION

Scientific Name	Common Name	Number	Gender	Description/Markings
Xenopus laevis	African clawed frog	20	Both (Male and/or Female)	None
Pelomedusa subrufa	Marsh Terrapin	20	Both (Male and/or Female)	None

OTHER PARTIES INVOLVED

Full Name	ID Number	Contact	Involvement	Physical Address
Jean Ruhan Verster Verster	9112045171083	Cell:0836418472	PhD student	
Nicolaas Phillipus du Preez du Preez	9403225066080	Cell:0834437654	PhD student	
Carla Smit Smit	9603220041083	Cell:0815538563	MSc student	
Alicia Fouche Fouche	9603120094083	Cell:0783756920	MSc student	
Celia Cloete Cloete	9602270014081	Cell:0711143911	MSc student	

ACTIVITIES

Activity Name
Catch
Release
Research

METHODS

Method Name
Trap

STANDARD CONDITIONS

GENERAL CONDITIONS - ALL PERMITS

1) The Issuing Authority for this licence is : The Northwest Department of Rural,Environment and Agricultural Development (READ), Chief Directorate Environmental Services, Private Bag X 2039, Mmabatho, 2735, hereafter named "the Issuing Authority". 2) This permit, unless otherwise stated, is only valid within the boundaries of the North West Province (hereafter named "the Province") and then specifically as specified on the permit. 3) This permit is valid only : - a) for the specific species, sex and numbers as specified on this permit. b) for the specific activity / activities authorised. c) for the specified methods or instruments authorised. d) for the specific property / locality as specified. e) for the specific day, time or period stipulated. 4) This permit is only deemed valid : - a) in the original format and with the content as issued by the Issuing Authority. b) once it has been printed and the signature of the permit holder has been endorsed thereon in ink. 5) the Issuing Authority reserves the right to amend, withhold, withdraw or cancel any permit at any time. 6) This permit is not transferable to any individual, natural person, juristic person or any other legal identity. 7) Any alterations or attempt thereto, whether electronically or in any other way, shall immediately render it invalid. 8) This permit shall lapse and be deemed invalid when it is altered, lost or destroyed and no copy thereof shall be issued. 9) This permit shall also become invalid as soon as the permit holder loses possession of any animal, plant or derivative as the case may be, as specified on the permit. 10) This permit does not grant the permit holder automatic access to any Protected Area, National Park, Provincial Nature Reserve or privately owned land and : - a) the permit holder must beforehand obtain all other relevant written permissions, documents, rights and licences. b) the permit holder must comply with any other / further conditions or restrictions that the manager / landowner may stipulate at his / her discretion. 11) The permit holder must at all times while performing any restricted activity authorised by this permit, have the permit and all other relevant documentation in his / her possession and without delay make it available upon request by any authorized person. 12) An authorized person must also be allowed access onto the property at any reasonable time for any inspection needed and can remain on such property as long as it is needed to do the inspection. 13) The permit holder must immediately after completion of any activity authorised by this permit, record the required particulars in the space provided therefore or on the annexure or document attached hereto or in the prescribed register related to the permit. 14) The permit holder must return the original signed permit to the Issuing Authority within (14) fourteen days : - a) after performing or completing the authorised restricted activity, or b) after the date of expiry thereof whichever happens first, and c) if applicable furnish the Issuing Authority with a prescribed written feedback report on the results of every activity conducted. 15) The permit holder must retain a copy of the permit together with all other relevant written permissions, documents, rights and licenses for a period of at least (2) two years from date of issue or for as long as the permit holder is in possession of the animal, plant or derivative, whichever period is the longer. 16) If applicable, the permit holder shall apply for the renewal of the permit to the Issuing Authority, on the appropriate application form, at least (3) three months prior to the expiry date thereof. 17) Applications for renewal of this permit will only be processed after the original signed permit together with the prescribed written feedback report has been returned to the Issuing Authority. 18) This permit, during the period of validity thereof, is also subject to: - a) all applicable norms and standards in existence at the time of issuance. b) the provisions of any law in force, in respect of the specific species, activity, method or instrument to which this permit applies. 19) It is the permit holder's responsibility to obtain the correct information on any other legislation, specification, requirement or changes thereto that may be applicable or are required by any other Issuing Authority / Organization / Institute, relating to this permit. 20) By signing this permit, the permit holder declares that he / she is aware of the fact that : - a) any transgression or failure to return the original permit or failure to render the required reports can lead to criminal prosecution and also jeopardize any future applications by or in the name of the permit holder. b) if the permit holder contravenes or fails to comply with any permit condition or requirement, he / she shall be guilty of an offence. 21) The prescribed fees paid

MCPO 00664/1/2019 NW 7650/02/2019 Application ID 7650

ADDENDUM

to the Issuing Authority for the issue of this permit shall not be refunded.

HARVESTING - COLLECT FOR SCIENTIFIC RESEARCH

1) The permit holder must ensure that : - a) specimens collected in terms of this permit is utilised for scientific purposes and only by the applicable institution or Issuing Authority as specified on this permit. b) specimens collected in term of this permit is not sold, bartered or given away. c) the written permission of the owner or occupier of land is obtained before any plant is harvested / picked. d) quantities of soil, rock or specimens is limited to the absolute minimum necessary for the research project. e) no endangered or specially protected specimens is collected. f) detailed record is kept on the specimens, quantities and localities where specimens are collected.

OCCUPATION - RESEARCH - (Non-Bioprospecting)

1) Should the research be conducted by a group, numbers specified are for the group as a whole and not per individual. 2) Written permission of the landowner or relevant authority must be obtained and must always be carried on such person with the valid permit. 3) Care should be taken not to harm non-target species. 4) An update report must be forwarded to the Issuing Authority which includes the permit holders name and contact details, species scientific and common name, numbers, date and locality (grid reference and description), Latitude and longitude as well as a copy of the written permission of the landowner where the activity had been conducted. 5) If applicable, at least one specimen per species should be lodged / housed at a South African Institute / Herbarium / Museum. 6) A copy of all completed reports, publications, or articles resulting from the project must be submitted free of charge to the Issuing Authority. 7) Should a report, publication, article or thesis arise from this project, an acknowledgement to the Issuing Authority. 8) Unless otherwise specifically indicated in writing, no material or specimens collected with this permit or material or specimens bred or propagated, from material or specimens collected with this permit, may be donated, sold or used for any commercial purpose by any party. 9) Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognized South African institution / museum / herbarium (preferably within the Province), where such material will be available to other researchers. 10) This permit does not authorize the collection within the boundaries of any Protected area, National Park, Provincial Nature Reserve, unless specified in the permit. 11) No Critically Endangered or Endangered species may be collected unless specified on the permit. The permit holder should take photographic records of rare / endangered species (where recognized). 12) No habitat must be disturbed at all. 13) Please ensure the validity of all other certificates and permits issued by other Institutes during the validity of this permit 14) This permit is only valid if a valid ethics letter for the relevant project is obtained and supplied to the Issuing Authority.

OCCUPATION - RESEARCH

1) "Plant and Quality Control" and any other regulations must be adhered to when exporting material out of the country. 2) No live specimens / material may leave the province, unless otherwise specified. 3) No habitat must be disturbed at all.

TRANSPORT - EXPORT - RESEARCH

1) An update report of species translocated to be forwarded to the Issuing Authority with the application for renewal of a research permit.

D.2 Section 20 Permit

agriculture, land reform & rural development

Department:
Agriculture, Land Reform and Rural Development
REPUBLIC OF SOUTH AFRICA

Directorate Animal Health, Department of Agriculture, Land Reform and Rural Development Private Bag X138, Pretoria 0001
Enquiries: Mr Herry Gololo • Tel: +27 12 319 7532 • Fax: +27 12 319 7470 • E-mail: HerryG@dalrrd.gov.za
Reference: 12/11/1/3 (1711AC)

Prof Ché Weldon
Unit for Environmental Sciences and Management
North-West University
Tel: 018 299 2375
Email: Che.Weldon@nwu.ac.za

Dear Prof Weldon,

RE: PERMISSION TO DO RESEARCH IN TERMS OF SECTION 20 OF THE ANIMAL DISEASES ACT, 1984 (ACT NO 35 OF 1984)

Your application received on 21 October 2020 requesting permission under Section 20 of the Animal Disease Act, 1984 (Act No. 35 of 1984) to perform a research project or study, refers. I am pleased to inform you that permission is hereby granted to perform the following study, with the following conditions:

Conditions:

1. This permission does not relieve the researcher of any responsibility which may be placed on him by any other act of the Republic of South Africa;
2. The research project is approved as per the application form received 21 October 2020 and the correspondence thereafter. Written permission from the Director: Animal Health must be obtained prior to any deviation from the conditions approved for this research project under this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za;
3. The study must be conducted in compliance with the Veterinary and Para-Veterinary Professions Act 1982 (Act No. 19 of 82);
4. Ethical approval for all components of the study must be obtained from the relevant authority before the study may start;
5. Only reptile (snake, monitor, tortoise and terrapin) samples may be collected as part of this study. Samples that may be collected include skin moulds, swab samples, scale biopsies, faeces, blood and organ samples. Samples must be fixed in 10% buffered formalin or PrimeStore MTM as indicated;

6. Sampling of reptiles may only be conducted in the specified areas for which a state veterinary letter of no objection has been received:
   a. NWP – Dr Kenneth Kaunda District
   b. MP – Mbombela (Nelspruit), Hazyview areas
   c. LP – Hoedspruit, Soutpansberg areas

   This includes the Hoedspruit Reptile Centre and Lowveld Venom Suppliers (Hazyview);
7. Only reptile samples may be obtained from the Hans Hoheisen Wildlife Research Station (HHWRS) Biobank for which a letter of permission has been received;
8. For transport of any reptile samples out of the Foot and Mouth Disease Controlled Areas and the African Swine Fever Controlled Areas, relevant movement permits from the responsible state veterinarian must be obtained;
9. All samples must be packaged and transported in accordance with the National Road Traffic Act, 1996 (Act No. 93 of 1996);
10. Processing, testing and analyses of the reptile samples must be performed in the Herp Health Laboratory (E6, G03) of the North-West University, Potchefstroom campus. These facilities may be subject to an on-site inspection by the Directorate Animal Heath. If any critical non-conformances are identified, the facility (including all Section 20 approved studies connected there to) will be suspended until these non-conformances are rectified;
11. Sampes may be tested for the following pathogens using the relevant test procedures, which may inlcude histopathology, Transmission Electron Microscopy and molecular diagnostics. No propagation of these agents is allowed:
    a. Mycoplasma agassizii
    b. Ophidiomyces ophiodiicola (Snake Fungal Disease)
    c. Plasmodium
    d. Ranavirus
    e. Salmonella
12. The detection of any controlled or notifiable animals diseases (including Ranavirus) must be reported to the responsible state veterinarian and the Director Animal Health;
13. All potentially infectious material utilised, collected or generated during the study is to be destroyed at the completion of the study using the specified waste contractor.
14. Records must be kept for five years for auditing purposes;
15. Extracted DNA from reptile samples may be stored under access control at -80°C in Laboratory G03, building E6, North-West University Potchefstroom campus;
16. Stored samples may not be outsourced or used for further research without prior written approval from the Director: Animal Health;
17. If required, an application for an extension must be made by the responsible researcher at least one month prior to the expiry of this Section 20 permit. Please apply in writing to HerryG@dalrrd.gov.za.

Title of research/study: *"Initiate and conduct disease surveillance in the North West province, Mpumalanga and Limpopo to primarily assess the prevalence and characterize infectious diseases in reptiles, including zoonosis"*
Researcher: Prof Ché Weldon
Institution: Unit for Environmental Sciences and Management, North-West University
Our ref Number: 12/11/1/3 (1711AC)
Your ref: NWU-00063-19-S5
Expiry date: December 2022

Kind regards,

DR. MPHO MAJA
DIRECTOR: ANIMAL HEALTH
Date: 2021 -02- 16

D.3 Lowveld Venom Suppliers collection permit

Mpumalanga
TOURISM AND PARKS AGENCY

Permit No. MPB/V/ 2003

PERMIT

TO ESTABLISH AND OPERATE A VENOM EXTRACTION FACILITY

(Issued in terms of the provisions of the Nature Conservation Act 10 of 1998)

Name of permit holder: *Christopher Hobkirk*
Residential address: *3 Akwameryn Street*
West Acres
Nelspruit 1200

In terms of and subject to the provisions of the Nature Conservation Act, 1998 (Act No. 10 of 1998) and the regulations framed thereunder, the above-mentioned person is hereby authorised, subject to the conditions and requirements appearing on this permit to establish or carry on the institution referred to hereunder during the period of validity of this permit.

PARTICULARS OF INSTITUTION

Name of institution: *Lowveld Venom Suppliers*

Place where business is carried out: *Cnr Sabie & Main Road - HAZYVIEW*

ANIMAL SPECIES WHICH MAY BE KEPT AND EXHIBITED IN TERMS OF THIS PERMIT

Number	Species	Scientific name
As per attached addendum		

- **Gaboon Adder should Not be sourced from Mpumalanga Province**
- **New Condition: TOPS regulations from DEAT will apply from 1st February 2008(*Gov. Gazette No. 29657 of 23 February 2007*)**

Period of validity of permit: From date of issue to: *30 June 2022*

For Chief Executive Officer

Signature of permit holder

Conditions

1. The permit shall not be transferable
2. Only a person authorised thereto by the C E O may make an alteration on the permit
3. The permit shall be subjected to the provisions of any law in force during the period of validity of the permit in the area to which the permit applies
4. No wild animals may be brought into or removed from the property unless authorised by a permit.
5. All animals received from the public must be kept in separate facilities and must be handed to the MTPA
6. Register must be kept on all animals received
7. The animals may not be handled by the public and the public may not be allowed into the cages.
8. The necessary precautions must be taken to ensure the safety of the public.
9. The permit holder of this permit may not sell, donate, import, export or convey any wild or exotic animal/s referred to on the permit or any progeny of such wild animal/s, unless he is the holder of a permit.

General Information

(a) Keeping of live wild animals or exotic animals in certain conditions(section 30)
(b) Prohibited acts with exotic animals(section 34)
(c) This permit may be cancelled or amended at any time.(Section 86.)
(d) The holder of a permit who contravenes or a fails to comply with any one of the conditions or requirements to which the permit is subject, shall be guilty of an offence.(Section 86.)
(e) The holder of a permit shall, at the request of a person authorised in terms of the provisions of the Act so to demand, forthwith produce such permit to such person.(Section 103.)

As per permit MPB/V/2003 dd. 26/07/2019

Mpumalanga
TOURISM AND PARKS AGENCY

10	Berg Adder	*Bitis atropos*
10	Many Horned Adder	*Bitis cornuta*
10	Horned Adder	*Bitis caudalis*
10	Peringuey's Adder	*Bitis peringueyi*
10	Common Night Adder	*Causus rhombeatus*
10	Snouted Night Adder	*Causus defilippii*
100	Cape Cobra	*Naja nivea*
99	Forest Cobra	*Naja melanoleuca*
99	Rinkhals	*Hemachatus haemachatus*
10	Coral Snake	*Aspidelaps lubricus*
10	Shield Nose Snake	*Aspidelaps scutatus*
10	Zambezi Garter Snake	*Elapsoidea boulengeri*
9	Twig Snake	*Thelotornis capensis*
10	Bibron's Stiletto Snake	*Atracaspis bibronii*
9	Natal Black Snake	*Mecrlaps microlepidotis*
10	Rufous Beaked Snake	*Rhamphiophis rostratus*
10	Olive Grass Snake	*Psammophis mossambicus*
10	Short Snouted Grass Snake	*Psammophis brevirostris*
10	Spotted Skaapsteker	*Psammophylax rhombeatus*
10	Striped Skaapsteker	*Psammophylax tritaeniatus*
10	Eastern Bark Snake	*Hemirhagerrhis nototaenia*
10	Common Tiger Snake	*Telescopus semiannulatus*
9	Herald Snake	*Crotaphopeltis hotamboeia*
10	Natal Purple-Glossed Snake	*Amblyodipsas concolor*
10	Common Purple-Glossed Snake	*Amblyodipsas polylepis*
30	Southern African Python	*Python natalensis*
10	Brown House Snake	*Lamrophis capensis*
10	Olive House Snake	*Lamrophis inornatus*
9	Aurora House Snake	*Lamrophis aurora*
10	Spotted Bush Snake	*Philothamnus semivariegatus*
10	Green Water Snake	*Philothamnus hoplogaster*
10	Natal Green Snake	*Philothamnus natalensis*
10	Cape Wolf Snake	*Lycophidion capense*
10	Cape File Snake	*Mehelya capensis*
10	Black File Snake	*Mehelya nyassae*
10	Rhombic Egg Eater	*Dasypeltis scabra*
100	Black mamba	*Dendroaspis polylepis*
100	Mozambique spitting cobra	*Naja mossambica*
100	Snouted Cobra	*Naja annulifera*
100	Boomslang	*Dispholidus typus*
97	Green mamba	*Dendroaspis angusticeps*
97	Puff adder	*Bitis arietans*
100	Gaboon adder Eastern	*Bitis gabonica*
50	Gaboon adder Western	*Bitis gabonica rhinoscerous*
50	Jamesons mamba	*Dendroaspis jamesoni*
19	Rock monitor	*Varanus albigularis*
19	Water monitor	*Varanus niloticus*
47	Leopard tortoise	*Geochelone pardalis*
20	Spekes hinged tortoise	*kinixys spekii*

Private Bag X11338 Mbombela 1200. N4 National Road, Halls Gateway,
Mataffin, MBOMBELA - MPUMALANGA. www.mpumalanga.com
Tel: +27 (0) 13 759 5300/1 Fax: +27 (0) 13 759 5490

MPUMALANGA
THE PLACE OF THE RISING SUN

26/11/19

D.4 Transport permit for BioBank samples

Permit in	Permit out
	/

DEPARTMENT OF AGRICULTURE

MPUMALANGA

Permit number: 20210329ORK - UKUPI

DEPT. LANDBOU

LITIKO LETEKULIMA

VETERINARY PERMIT TO MOVE ANIMALS / ANIMAL PRODUCTS
VEEARTSENYPERMIT VIR VERVOER VAN DIERE / DIEREPRODUKTE
IMVUMO YEKUHAMBISA TILWANE / TINTFO LETIHABULISAKO TETILWANE

In terms of the Animal Diseases Act No. 35 of 1984 permission is hereby granted to:
Kragtens die Wet op Dieresiektes Nr. 35 van 1984 word toestemming hiermee verleen aan:
Ngeligunya leMtsetfo weTifo teTilwane No. 35 wa 1984:

Name: / Naam: / Libito:	CHÉ WELDON	ID No.:	7502075129088
Address: / Adres: / Likheli:	NORTH WEST UNIVERSITY, POTCHEFSTROOM, 2531		
to move with: / om te beweeg met: / uvunyelwa yekuhambisa:	THREE HUNDRED THIRTY SEVEN	VARIOUS REPTILE SPECIES	LIVE / PRODUCT
identified as follows: / geïdentifiseer as volg: / letikhonjiswe nga:	337 REPTILE SAMPLES (115 BLOODSMEARS, 112 FTA CARDS, 107 SKIN SAMPLES + 3 PARASITES)		
from the farm/diptank area/place: / vanaf die plaas/dipbakgebied/plek: / kusuka epulazini/edibhini/endzaweni:	HANS HOHEISEN WRS (STOCK CARD NUMBER)	in the district of / in die distrik / esigodzini sase	BOHLABELA
to the farm/diptank area/abattoir/place: / na die plaas/dipbakgebied/abattoir/plek: / kuya epulazini/edibhini/emadzeleni/endzaweni:	POTCHEFSTROOM	in the district of / in die distrik / esigodzini sase	DR KENNETH KAUNDA

On the following conditions:
1. This permit:
(a) is valid for 10 days from date of issue and for one consignment only
(b) must accompany the animals / products mentioned above;
(c) must be produced for inspection on demand by any land or animal owner or police or veterinary official;
(d) must be kept at a destination until collected by a veterinary official.
2. For game / game products a nature conservation permit is also needed.
3. Animals and products must be visibly free from external parasites.
4. Animals are to be transported in accordance with animal welfare guidelines.
5. Other conditions:

Op die volgende voorwaardes:
1. Hierdie permit:
(a) is geldig vir 10 dae vanaf datum van uitreiking en slegs vir een beweging;
(b) moet die diere / produkte hierbo vermeld vergesel;
(c) moet vir inspeksie getoon word op aanvraag van enige grond- of dieresienaar of polisie- of veeartsenybeampte;
(d) moet by bestemming gehou word totdat dit deur 'n veeartsenybeampte geneem word.
2. Vir wild / wildprodukte word 'n natuurbewaringspermit ook benodig.
3. Diere en produkte moet sigbaar vry van uitwendige parasiete wees.
4. Diere moet volgens dierewelsynriglyne vervoer word.
5. Ander voorwaardes:

Ngaphansi kwemibandzela lechazelwe ngaphansi:
1. Lemvumo:
(a) [illegible]
(b) [illegible]
(c) [illegible]
(d) [illegible]
2. [illegible]
3. [illegible]
4. [illegible]
5. [illegible]

* SAMPLE LIST TO PLEASE ACCOMPANY PERMIT

6. Retention for / Quarantine for / Direct slaughter within: days after arrival at destination

Seal no/s	/	/	Place / Plek / Indzawo:	HANS HOHEISEN WRS
Vehicle reg. no/s	KKM860 NW	/	Date / Datum / Lusuku:	2021-03-29

I hereby certify that all applicable control measures for issuing of this permit have been complied with.

DR HKM RAMPET
Issuing Veterinary Official:

SIGNATURE
State Veterinarian / Staatsveearts / Dokotela weTilwane

IMPORTANT NOTE / BELANGRIKE NOTA / SATISO LESIBALULEKILE
Permit holder who does not comply with this permit or any condition thereof is guilty of an offence.
Permithouer wat in stryd met hierdie permit of enige voorwaarde daarvan optree, is skuldig aan 'n misdryf.
Lonalemvumo longalandzela lemvumo kumbe muphi umbandzela wayo uphula umtsetfo.

DISTRIBUTION: VETERINARY OFFICIAL AT DESTINATION

REPUBLIC OF SOUTH AFRICA
DEPARTMENT OF AGRICULTURE
OFFICIAL VETERINARIAN
DATE STAMP
2021-03-29
DR H.K.M. RAM
REG. NO D18/159
MPUMALANGA PROVINCE

D.5 Ethics certificate

Private Bag X1290, Potchefstroom
South Africa 2520

Tel: 086 016 9698
Web: http://www.nwu.ac.za/

North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC)

Tel: 018 299-1208
Email: Ethics-AnimCare@nwu.ac.za (for animal studies)

7 March 2021

ETHICS APPROVAL LETTER OF STUDY

Based on approval by the North-West University Animal Care, Health and Safety Research Ethics Committee (NWU-AnimCareREC) on 07/03/2021, the NWU-AnimCareREC hereby approves your study as indicated below. This implies that the NWU-AnimCareREC grants its permission that, provided the general conditions specified below are met and pending any other authorisation that may be necessary, the study may be initiated, using the ethics number below.

Study title: Pathogen survey of aquatic reptiles in the North-West Province of South Africa
Principal Investigator/Study Supervisor/Researcher: Prof C Weldon
Student: C Smit - 25996509

Ethics number:

N	W	U	-	0	0	0	6	2	-	1	9	-	A	5
Institution				Study Number						Year			Status	

Status: S = Submission; R = Re-Submission; P = Provisional Authorisation; A = Authorisation

Application Type: Single study
Commencement date: 07/03/2021
Expiry date: 31/03/2022

Risk: Category 2

Approval of the study is provided for a year, after which continuation of the study is dependent on receipt and review of a yearly monitoring report and the concomitant issuing of a letter of continuation. A monitoring report is required at the end of March annually until completion.

General conditions:

While this ethics approval is subject to all declarations, undertakings and agreements incorporated and signed in the application form, the following general terms and conditions will apply:

- *The principal investigator/study supervisor/researcher must report in the prescribed format to the NWU-AnimCareREC:*
  - *Annually on the monitoring of the study, whereby a letter of continuation will be provided annually, and upon completion of the study; and*
  - *without any delay in case of any adverse event or incident (or any matter that interrupts sound ethical principles) during the course of the study.*
- *The approval applies strictly to the proposal as stipulated in the application form. Should any amendments to the proposal be deemed necessary during the course of the study, the principal investigator/study supervisor/researcher must apply for approval of these amendments at the NWU-AnimCareREC, prior to implementation. Should there be any deviations from the study proposal without the necessary approval of such amendments, the ethics approval is immediately and automatically forfeited.*
- *Annually a number of studies may be randomly selected for active monitoring.*

9.1.5.4.2 Ethics Approval Letter of Study 1

- *The date of approval indicates the first date that the study may be started.*
- *In the interest of ethical responsibility, the NWU-AnimCareREC reserves the right to:*
  - *request access to any information or data at any time during the course or after completion of the study;*
  - *to ask further questions, seek additional information, require further modification or monitor the conduct of your research or the informed consent process;*
  - *withdraw or postpone approval if:*
    - *any unethical principles or practices of the study are revealed or suspected;*
    - *it becomes apparent that any relevant information was withheld from the NWU-AnimCareREC or that information has been false or misrepresented;*
    - *submission of the annual monitoring report, the required amendments, or reporting of adverse events or incidents was not done in a timely manner and accurately; and/or*
    - *new institutional rules, national legislation or international conventions deem it necessary.*
- *NWU-AnimCareREC can be contacted for further information via Ethics-AnimCare@nwu.ac.za or 018 299 1208*

Special conditions of the research approval due to the COVID-19 pandemic:

Please note: Due to the nature of the study i.e. (field work involving the collection of animal samples), this study will be able to proceed during the current alert level, following receipt of the approval letter. No additional COVID-19 restrictions have been placed on the study except that the researcher must ensure that before proceeding with the study that all research team members have reviewed the North-West University COVID-19 Occupational Health and Safety Standard Operating Procedure as well as that of the Unit for Environmental Sciences and Management regarding COVID-19 precautions during field work.

NWU-AnimCareREC would like to remain at your service and wishes you well with your study. Please do not hesitate to contact the NWU-AnimCareREC for any further enquiries or requests for assistance.

Yours sincerely,

Digitally signed by
Christiaan B Brink
(Tiaan)
Date: 2021.03.08
10:54:30 +02'00'

Chairperson: NWU-AnimCareREC

Current details:(22654704) G:\My Drive\9. Research and Postgraduate Education\9.1.5.4 Templates\9.1.5.4.2_NWU-AC_EAL.docm
28 February 2021

File Reference: 9.1.5.4.2