CONSERVATION OF SOUTH AFRICAN TORTOISES WITH EMPHASIS ON THEIR APICOMPLEXAN HAEMATOZOANS, AS WELL AS BIOLOGICAL AND METAL-FINGERPRINTING OF CAPTIVE INDIVIDUALS

By

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“We need another and a wiser and perhaps a more mystical concept of animals. Remote from universal nature, and living by complicated artifice, man in civilization surveys the creature through the glass of his knowledge and sees thereby a feather magnified and the whole image in distortion. We patronize them for their incompleteness, for their tragic fate of having taken form so far below ourselves. And therein we err, and greatly err. For the animal shall not be measured by man. In a world older and more complete than ours they move finished and complete, gifted with extensions of the senses we have lost or never attained, living by voices we shall never hear. They are not brethren, they are not underlings; they are other nations caught with ourselves in the net of life and time, fellow prisoners of the splendour and travail of the earth.”

Henry Beston (1928)

ABSTRACT

South Africa has the highest biodiversity of tortoises in the world with possibly an equivalent diversity of apicomplexan haematozoans, which to date have not been adequately researched. Prior to this study, five apicomplexans had been recorded infecting southern African tortoises, including two haemogregarines, *Haemogregarina fitzsimonsi* and *Haemogregarina parvula*, and three haemoproteids, *Haemoproteus testudinalis*, *Haemoproteus balazuci* and *Haemoproteus* sp. A. The taxonomy of all of these species was questionable, and therefore one goal of this study was to examine at least some in great detail with the view to resolving taxonomic issues. This involved using a number of techniques such as light microscopy and image analysis, transmission electron microscopy, and molecular analysis. Outcomes were the transfer of one *Haemogregarina* species (*Haemogregarina fitzsimonsi*) to the genus *Hepatozoon,* the suggestion that the genus *Hemolivia* might be more appropriate for another haemogregarine (*Haemogregarina parvula*), the synonymisation of two known species of *Haemoproteus* (*Haemoproteus balazuci* with *Haemoproteus testudinalis*), and the naming of a third haemoproteid (*Haemoproteus natalensis* Cook, Smit and Davies, 2010). In addition, a likely new species of haemogregarine (*Haemogregarina* sp. A.) was described. To achieve all this, 367 tortoises were collected representing 62% of the species and all five genera, of South African tortoises. Tortoises were both wild (287) and captive (80), with these being both live (270) and dead (97) when taken. They were located in four different provinces, including Gauteng, KwaZulu-Natal, the Northern and the Western Cape, and in four different biomes (semi-arid grassland, Kalahari desert, subtropical thorn bushveld, and coastal endemic fynbos). Light photomicroscopy examination of Giemsa stained peripheral blood smears prepared from the subcarapacial vessels of live tortoises allowed for descriptions and comparisons of the observed haematozoans. Of the live tortoises, 14.8% had haemogregarines, including 13.3% with *H. fitzsimonsi*, 0.7% with *H. parvula*, and 0.7% with a previously unknown, intraleucocytic, *Haemogregarina* sp. A. A further 1.1% had haemoproteids, including 0.7% with *Hp. testudinalis/Hp. balazuci* and 0.4% with *Haemoproteus* sp. A. The host and locality records of previously described haematozoan species were increased and records for likely new species provided. Subtropical areas (KwaZulu-Natal) compared to arid regions (Northern Cape) presented with a higher diversity of apicomplexans, along with a higher prevalence of ticks, possible vectors of the tortoise blood parasites. Overall, male tortoises had the highest haematozoan and tick prevalences compared to females and juveniles,

whilst juveniles showed the highest parasitaemias. Ticks collected from tortoises included *Amblyomma marmoreum*, *Amblyomma sylvaticum*, and *Ornithodorus compactus*. Tick prevalence was high in areas where wild tortoises showed high prevalence with *H. fitzsimonsi*, while captive tortoises from which ticks had been eradicated, showed low haematozoan prevalence. Infections with *Haemogregarina* and *Haemoproteus* species were not concurrent, and *H. fitzsimonsi* occurred in the absence of all other apicomplexans. However, *H. parvula* and *H. fitzsimonsi* were always found concurrently, and *Haemogregarina* sp. A and *H. fitzsimonsi* co-existed in 50% of the recorded *Haemogregarina* sp. A. infections. Ultrastructural examination of gamonts of *H. fitzsimonsi* and *H. parvula* differentiated between these species, *H. fitzsimonsi* resembling species of *Haemogregarina* and *Hepatozoon* at this level, and the encased gamonts of *H. parvula* closely resembling those of species of *Hemolivia*. Molecular analysis of 18S rDNA sequences extracted from methanol-fixed, Giemsa stained tortoise blood films containing *H. fitzsimonsi* gamonts, and DNA taken from sporogonic stages in formalin and ethanol fixed *H. fitzsimonsi* infected tick impression slides and tick viscera, revealed that *H. fitzsimonsi* likely belonged to the genus *Hepatozoon.* Such analysis also implicated both *Amblyomma* species as vectors for this parasite, as well as providing insights into the phylogeny of the ticks themselves. No DNA sequences were obtained for *Haemogregarina* sp. A., likely due to the low parasitaemias observed in tortoise peripheral blood films. However, based on the apparent lack of dividing stages within blood smears and its intraleucocytic nature, *Haemogregarina* sp. A. was suggested to be a new species of *Hepatozoon*. Morphometrics allowed for the synonymisation of *Hp. balazuci* with the earlier described *Hp. testudinalis*, and *Haemoproteus* sp. A. was studied more extensively than previously and described as a new species, *Haemoproteus natalensis*. Other major goals of the thesis were to assess *H. fitzsimonsi* as a bio-indicator for tortoise body condition, and as a biological tag. However, the apparent relationships among prevalence, parasitaemia and host body condition in two selected tortoise species, *Chersinia angulata* and *Stigmochelys pardalis*, were statistically insignificant. Additionally, prevalence and parasitaemia were apparently higher in tortoises in anthropogenically impacted areas than in those in unimpacted reserve areas, but these differences were again statistically insignificant. *Haemogregarina fitzsimonsi* was thus rejected as a bio-indicator for future tortoise population 'health' assessment. Host-specificity, geographical range, prevalence and parasitaemia of the recorded haematozoans suggested that several might eventually prove useful as biological tags, but with *H. fitzsimonsi* perhaps requiring further genetic analysis to determine whether different strains

of the parasite occur across distinct host species and different tortoise populations. A final goal of the thesis was to attempt to metal-fingerprint tortoises by site. Of all wild tortoise tissues (bone, scute and nail) collected and analysed by inductively coupled plasma spectrometery, nail provided the best resolution among tortoises from different sites. Nail collection, being non-invasive compared to the sampling of other tissue types, supported the proposal of a nail metal-fingerprint database for the future determination of captive tortoise origin, perhaps allowing their return to the wild and thus aiding South African tortoise conservation.

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CHAPTER 1

INTRODUCTION

1.1. Introduction to the field of study

South Africa has the highest biodiversity of tortoises in the world (Branch, 2008) with possibly an equivalent diversity of protistan (protozoan) apicomplexan blood parasites. Until the MSc preceding this research had been undertaken (Cook, 2008), very little research had been done on these parasites infecting southern African tortoises, with only a species described from South Africa by Laveran (1905) and three species reported by Dias (1953) in Mozambique having been recorded from the region. Based on this knowledge, Cook (2008) hypothesised that a greater diversity of haematozoans would be present in South African tortoise species and that the different tortoise genera would be infected by different species of haematozoans. Lastly, Cook (2008) hypothesised that the species of haematozoans described from Mozambique tortoises would infect the same genus of tortoise within South Africa.

Cook (2008) sampled 5/13 tortoise species occurring in four South African provinces, recording three species of apicomplexan blood parasites. Collections undertaken included both wild and captive tortoises, during all four seasons, with the provinces sampled representing different environments, ranging from arid grasslands to subtropical and coastal regions. The three species of parasites found had already been recorded. The first, was a haemoproteid, *Haemoproteus testudinalis* (Laveran 1905) found in two species of tortoises (see below), a parasite possibly originally recorded from the Cape by Laveran (1905) in *Stigmochelys pardalis* (syn. *Testudo pardalis*) (Bell, 1828), and which Dias (1953) unknowingly described as another species, *Haemoproteus balazuci* Dias, 1953 from *Kinixys belliana belliana* (syn. *Kinixys belliana zuluensis*) Gray, 1830 in Mozambique. The second and third parasites found by Cook (2008), originally recorded by Dias (1953) from *Kinixys belliana zuluensis* (syn. *K. b. belliana*) in Mozambique, were the haemogregarine species, *Haemogregarina fitzsimonsi* Dias, 1953 found in five tortoise species (see below) and *Haemogregarina parvula* Dias, 1953 located in two tortoise species (see below).

Cook *et al.* (2009a) redescribed the two haemogregarine species with their new locality and host species records. Furthermore, Cook *et al*. (2010a) compared the haemoproteid recorded by Cook (2008) with those reported by Laveran (1905) and Dias (1953) and found it to be identical to both these known species. Cook's (2008) parasite and *Hp. balazuci* of Dias (1953) were thus synonymised with *Hp. testudinalis* of Laveran (1905) (see Cook *et al*., 2010a) and this action is recorded in this thesis. Recently, a fourth species of haemoproteid, also recorded by Cook *et al.* (2010a), was found infecting *Kinixys natalensis* Hewitt, 1935. It was new to science and named *Haemoproteus natalensis* Cook, Smit and Davies 2010. This species is also recorded in this thesis.

Given the diversity of blood parasites found in five species of tortoise by Cook (2008), it was considered essential that more research be undertaken, sampling as many of the remaining eight species of tortoise found in South Africa as possible, as well as completing the description of any new species of haematozoans that might be found (for example, see above). Cook *et al*. (2009a) recorded *H. fitzsimonsi* in all five of the tortoise species examined at the time, *Chersina angulata* (Schweigger, 1812), *Kinixys belliana belliana*, *Kinixys lobatsiana* (Power, 1927), *K. natalensis* and *S. pardalis*, across four South African provinces, Gauteng, KwaZulu-Natal, North West, and Western Cape. *Haemogregarina parvula*, on the other hand, was recorded from two tortoise species, *K. b. belliana* and *S. pardalis*, in KwaZulu-Natal (see Cook *et al*., 2009a). The haemoproteid, *Hp. testudinalis* (syn. *Hp. balazuci*) was found in two tortoise species, *K. lobatsiana* and *S. pardalis*, in North West. Finally, *Haemoproteus natalensis* was recorded recently from a single individual of *K. natalensis* in KwaZulu-Natal (see Cook *et al*., 2010a and this thesis).

Siddall (1995), placed all chelonian haemogregarines, terrestrial and aquatic, into the genus *Haemogregarina* Danilewsky, 1885 (*sensu stricto*), all transmitted by a leech vector. Landau and Paperna (1997), after reviewing Brumpt's (1938) proposed life cycle of *Haemogregarina mauritanica* (Sergent and Sergent, 1904) infecting the Palearctic tortoise, *Testudo graeca* Linnaeus, 1758, transferred this haemogregarine to the genus *Hemolivia* Petit, Landau, Baccam and Lainson, 1990. The genus *Hemolivia* is defined as having an ectothermic vertebrate host (the tortoise) in which erythrocytic gamogony, erythrocytic merogony, extra-erythrocytic merogony and cystogony occur (Široký *et al*., 2007). Furthermore, *Hemolivia* requires the ingestion of the tick vector by the tortoise host for transmission to occur, with sporogony occurring within the gut epithelial cells of the tick (Široký *et al*., 2007). Landau and Paperna's (1997) findings were therefore in contrast to Siddall's (1995) classification of chelonian haemogregarines. The logic of an aquatic vector (leech) being responsible for transmission of a haemogregarine to a terrestrial tortoise is also questionable.

During the MSc study of Cook (2008), no leeches were recorded parasitizing the tortoises collected. Only ticks were found, suggesting that ticks may be the invertebrate hosts for the haemogregarines *H. fitzsimonsi* and *H. parvula* and that these two species may belong to the genus *Hemolivia*, rather than *Haemogregarina.* However, during the redescriptions of both of the above haemogregarines (Cook *et al*., 2009a), no conclusive erythrocytic merogony and cystogony was observed for *H. fitzsimonsi* and *H. parvula*, suggesting the further possibility that the two parasites might just as well be members of the genus *Hepatozoon* Miller, 1908. *Hepatozoon* is defined by having one or more ectothermic vertebrate hosts (usually involving predator-prey relationships, including a rodent, or

amphibian and a snake species) in which erythrocytic gamogony and extra-erythrocytic merogony occurs (Telford, 2009). Unlike *Hemolivia*, it is rare for erythrocytic merogony or cystogony to occur with *Hepatozoon* (see Smith *et al*., 2000). Like *Hemolivia* though, the *Hepatozoon* life cycle necessitates that the sporocyst-infected invertebrate host (recorded in the past to be a mite or tick) is ingested by the vertebrate for infection to occur (Telford, 2009).

To determine whether *H. fitzsimonsi* belongs within *Hemolivia,* stellate oocysts would need to be identified (Landau and Paperna, 1997; Boulard *et al*., 2001; Paperna, 2006), along with sporokinetes and sporocysts within the intestinal cells of the invertebrate host (Landau and Paperna, 1997). If however, non-stellate oocysts and the sporocysts are found within the haemocoel of the invertebrate, this may suggest that the parasite belongs within *Hepatozoon* (see Telford, 2009). Yet, even if parasite stages are present within the tick host, as well as in tortoise blood films, they cannot be guaranteed to be of the same species. Parasite stages in blood films and infective stages within the invertebrate would need to be linked, either by use of laboratory experiments or by molecular analysis.

The first has been achieved by Široký *et al.* (2007) who fed *Hemolivia mauritanica* infected ticks (see above) to clean examples of the ticks' natural tortoise hosts, resulting in an infection; the recipient tortoise parasite stages were subsequently carefully documented. However, laboratory experiments of this nature can be difficult and expensive to set up, and the possibility of morphologically variable blood parasites exists between host species. Recently, this parasite variability between hosts and within hosts was further illustrated in reptile *Hepatozoon* lineages by molecular means (Maia *et al*., 2011). Also, duplicating the natural course of infection when there is a lack of appropriate natural vectors or vertebrate hosts may be difficult. Interspecific variability of parasite stages may occur, since the capacity of an unnatural host to support a naturally functioning infection may be unreliable (see Wozniak and Telford, 1991; Telford *et al*., 2001).

Molecular methods can be equally valuable, if not more so, and recently there has been great interest in sequencing apicomplexan blood parasites, especially within the herpetological field. Some herpetologists have begun to compare reptile blood parasite phylogenetics to the phylogenetics of the reptiles themselves, aiding inference of cryptic reptile species relationships (see Harris *et al*. 2011; Maia *et al*., 2011). Though little has been done on sequencing of *Haemogregarina* and *Hemolivia*, a large number of *Hepatozoon* sequences have been uploaded onto websites such as the NCBI (National Center for Biotechnology Information). This website with links to GenBank, at least,

may provide for some comparison if *H. fitzsimonsi*, a main focus of the present study, can be sequenced successfully. Recently, molecular techniques, such as those mentioned above, using the 18S ribosomal RNA gene, have been used to identify suspected vectors (Mathew *et al*., 2000; Vilcins *et al*., 2009a). Similar work would be of great value to this study, possibly identifying conclusively, the already suspected, tortoise tick species as the vectors of *H. fitzsimonsi*.

The vector(s) and mode of transmission of the haemoproteid infections recorded by Cook *et al*. (2010a) are uncertain. It is suggested though, that the vectors may include mosquitoes and biting flies such as sand flies, which have been reported to transfer members of the closely related genus *Plasmodium* Marchiafava & Celli, 1885 genus (see Telford, 2009). To date, no such vectors have been collected directly off haemoproteid infected tortoises (Cook, 2008; Cook *et al*., 2010a).

Cook (2008) found *H. fitzsimonsi* to be the most prevalent species of blood parasite recorded, occurring in all five of the species of tortoise examined at the time. As already noted, the finding necessitates a further need to examine as many as possible of the remaining species of tortoises found in South Africa, for the presence of this haemogregarine and other apicomplexan blood parasites. Telford *et al*. (2001) has suggested that reptilian haemogregarines are not particularly host specific. *Haemogregarina fitzsimonsi* seemingly fits this profile, and appears to be in contrast to *H. parvula*, which was found in *K. b. belliana* (1/5 tortoise species examined by Cook (2008)) and what was presumed to be an unnatural infection in a single *S. pardalis* specimen (Cook, 2008).

Haemogregarines and haemoproteids are considered non-pathogenic; possibly only mild cases of anaemia occur in their presence and reflect a well-adapted host-parasite relationship (Jacobson, 2007). Thus, in addition to the above research on *H. fitzsimonsi*, the wide vertebrate host and geographical range of this parasite (see Cook *et al*., 2009a) advocate its possible use as an 'effect' bio-indicator, perhaps demonstrating a correlation between prevalence, parasitaemia and tortoise body condition. Whether such correlation could benefit future studies of host populations, anthropogenic impacts, or stress indicators is debateable, but is worthy of investigation. Furthermore, the effects that variable host immunity, condition and susceptibility (Beldomenico and Begon, 2009) might have on the parasite are unpredictable (Vidal-Martínez *et al*., 2010). It is intended that prevalence and parasitaemia levels of *H. fitzsimonsi* are, for this study, compared against tortoise body condition (body mass divided by shell volume, BM/SV) (see Loehr *et al*., 2006). Apicomplexan haematozoans not only occur in the peripheral blood of the intermediate host (the tortoise), but also occur in other organs such as the liver and spleen (Davies and Johnston, 2000),

allowing persistence of the infection (see Široký *et al*. 2007). The vertebrate host accommodates haematozoan asexual reproduction, the result of which is the sexual stages known as gamonts/gametocytes within the peripheral blood (Telford, 2009). It is the parasitaemia levels of these stages which may increase or decrease in correlation with tortoise body condition.

It is well known that many tortoises are illegally collected by well-meaning citizens as pets (Branch, 2008); however, many of these tortoises are later abandoned or confiscated by Nature Conservation authorities. Many of these tortoises are subsequently placed under the care of zoological institutions and cannot be returned to the wild for concern that they may transmit disease back into the wild population, or, if placed in a wild population from which they did not originate, may contaminate the genetic pool (Wimberger *et al*., 2009). Conservation projects aimed at developing procedures with which tortoises may be returned to the wild have not yet been successful (Wimberger *et al*., 2009). Even though some of these projects released suitable species which had been quarantined for disease and injuries, into pre-approved environments, mortality was high (Wimberger *et al*., 2009). As mentioned above, tortoises also could not be released into environments in which a pre-existing population occurred for fear of pathogen transfer (Armstrong, 2012; Branch pers. comm.), regardless of whether the individual had originated from that specific area (Newton pers. comm.). The approach of placing tortoises into a new area with possibly unrecognisable food sources also may be fatal to more cautious individuals which will not readily try new food sources (personal observation). Furthermore, it is not always the pathogen that may be transmitted to an already existing population or environment that may be problematic, but pathogens within the population or new environment that may cause disease to introduced individuals.

To determine what would be the most suitable environment or area in which to release tortoises, it is important to understand, or have a good knowledge of, where they originally came from. In the same way that it is possible to barcode species of a particular population genetically, it may be possible to provide animals with a metal-fingerprint. Genetic barcoding is an efficient method for species level identification (Hajibabaei *et al.*, 2007; see www.boldsystems.org) based on the use of short, standardised gene regions (Hebert and Gregory, 2005) such as the mitochondrial gene cytochrome oxidase I (*co*I) (Hebert *et al*., 2003) or (*cox*) (Chinnery and Schon, 2003). Metal fingerprinting, however, is based on trace metal analysis, in which the metal concentrations of different localities or populations are comparably different (Nikkarinen and Mertanen, 2004).

In the past, reptiles were excluded from ecotoxicological research, thus metal-fingerprinting had not been undertaken in these vertebrates, and they were the least studied of all vertebrates for environmental contaminants and trace elements (Smith *et al*., 2007). However, more recently, contaminant and trace element accumulation has been documented in reptiles such as crocodilians, lizards and snakes, and mechanisms of contaminant uptake discussed (Smith *et al*., 2007). The two primary routes of contaminant exposure or trace metal accumulation in reptiles include ingestion and dermal contact. Ingestion of contaminated food as well as the indirect consumption of contaminated soil, may lead to an accumulation of these contaminants or trace metals within certain organs (Gardner *et al*., 2006). The current research project does not, however, intend to determine contaminant exposure, but will focus on the end result of metal or trace element uptake, so as to develop a preliminary procedure for the return of tortoises to the wild, based on ratios of metals in tissues compared to those found in soil samples. As the procedure may be used in future for tortoise conservation and the return of these animals to the wild, it is imperative that sampling of tissues is non-invasive. Past research into non-invasive trace element exposure, utilised egg (Guirlet *et al*., 2008), blood and tail clips (Hopkins *et al*., 2001); however, these samples did not provide a life history of trace element or contaminant exposure. Therefore, nail, scute and bone samples (from the carapace and plastron) are used in the current study, which should provide these data. Additionally, research into trace metal accumulation in this study may provide the basis for future research into the utilisation of tortoises as environmental stress-indicators. These animals may prove to be good indicator species because of their remarkable longevity, and feeding habits (Gardner and Oberdörster, 2006).

1.2. Hypotheses and aims of the study

Based on above information the following five hypotheses, and the aims to test each, have been formulated:

1. It is hypothesised that the existing diversity of tortoise apicomplexan parasites includes more species than that already recorded. The aim will be to screen the blood of as many of the 13 species of South African tortoise as possible and describe any new species.
2. It is hypothesised that the ticks collected off the tortoises are vectors of the haemogregarine *H. fitzsimonsi*, and in a similar manner to that illustrated by Široký *et al*. (2007), infected ticks are ingested by tortoises resulting in an infection. The aim will be to elucidate the life cycle of *H. fitzsimonsi*.

3. It is hypothesised that *H. fitzsimonsi* and *H. parvula* belong to the genera *Hemolivia* or *Hepatozoon*, but not *Haemogregarina.* The aims will be to correct the taxonomic placement of these two species based on the elucidation of their life cycles if possible, or through molecular analysis.
4. It is hypothesised that a correlation will exist between the prevalence and parasitaemia levels of *H. fitzsimonsi* and tortoise body condition, and knowledge of the above relationship will aid in the development of a tortoise population stress bio-indicator. The aim will be to determine whether such a correlation exists for *H. fitzsimonsi*, and whether it might be used as a stress indicator.
5. It is hypothesised that through metal-fingerprinting of captive individuals their original place of collection may be determined. The aim will be to assess the possibility of a relationship between trace metal concentrations within wild tortoise tissue and soil from specific localities. If a relationship exists, the aim will be to provide the information to Nature Conservation authorities for their possible use in tortoise population evaluations and tortoise release protocols.

1.3. Objectives of the study

Objectives of the study include:

1. To collect blood through venepuncture, and use it to make thin blood smears for analysis using light microscopy. Haematozoans discovered within the smears will be measured and compared morphologically to known species. Any new species will be described and new locality and host records recorded for described species.
2. To collect and fix tortoise blood samples for molecular analysis. Ticks collected from tortoises will be fixed for both histology and molecular analysis.
3. To find oocyst and sporocyst formation within collected ticks. Ticks found to be infected with these stages will be assessed by molecular means to determine whether they have the same genetic makeup as tortoise peripheral blood stages of the haematozoans. Also, as it may prove difficult to distinguish *H. fitzsimonsi* molecularly from *H. parvula*, since they can occur concurrently in the tortoise species *K. b. belliana* and extremely rarely in *S. pardalis*, the haemogregarines will be differentiated at the ultrastructural level using TEM (transmission electron microscopy).

4. To collect thin blood smears, and calculate the prevalence and parasitaemia of the infections, subsequently comparing them to tortoise body condition (body mass/volume). The relationship between the body condition and correlating prevalence and parasitaemia will be statistically analysed using the appropriate statistical tests, the results of which will determine the feasibility of the above method for future tortoise population studies and subsequently the use of such a method in the conservation of these animals.
5. To collect nail in live tortoise specimens, and either one or all of nail, scute and bone in dead specimens. Analyse subsequently, through ICP (inductively coupled plasma)-MS (mass spectrometry) and ICP-OES (optical emission spectrometry), the trace metal array against that of soil samples collected from the same localities where tortoise samples were taken. The relationship between metal concentrations within tissues and soil samples will be statistically analysed using the appropriate statistical tests. The future use of the above research in tortoise conservation will be determined on the results obtained.

1.4. Outline of the thesis

This brief introduction (Chapter 1), is followed by a detailed literature review (Chapter 2). Chapter 2 will include a review on the biomes of South Africa and the influence these environments may have on tortoise and associated parasite diversity. It will also include a general introduction to tortoises, their classification, South African species, the evolution of these creatures and their capacity to host haematozoan parasites. This will be followed by a similar general introduction, classification and evolution of, specifically, chelonian haematozoans, and the haematophagous ectoparasites of tortoises. Emphasis will be placed on haematozoan and tick species infecting South African tortoises. The use of South African haematozoans, in particular *Haemogregarina fitzsimonsi,* as bio-indicators, will be reviewed. Lastly, Chapter 2 will assess the possible use of metal-fingerprinting as a means of biological tagging in tortoises and thus as a possible aid in the conservation of these animals.

Chapter 3 will record the materials and methods used to capture and collect data from tortoises and their localities, as well as the techniques and statistical tests used to assess these data.

Chapter 4 will detail the tortoise species and number of those species examined for haematozoans, including the province and site from which these species were sampled and the haematozoan infections and tick infestations.

Chapter 5 will deal with *H. fitzsimonsi*, the light microscopy and ultrastructural observations, as well as the molecular findings of peripheral blood stages of this parasite. Further in the chapter, focus will be on tick diversity, especially species collected off South African tortoises and therefore, the suggested vectors of *H. fitzsimonsi*. Histological and molecular findings of possible *H. fitzsimonsi* stages within these ectoparasites will be correlated with peripheral blood infections in an attempt to dismiss or implicate them once and for all as vectors for *H. fitzsimonsi*. Furthermore, the chapter will conclude in a discussion of the phylogenetic findings for *H. fitzsimonsi* in comparison to those for other published species of the same genus, and a comparison of the possible tick vectors of this haemogregarine with other published species of the same tick genus.

Chapter 6 will concentrate on two species of haemogregarine recorded infecting South African tortoise species, describing *H. parvula* using light microscopy and ultrastructural observations, and a new species *Haemogregarina* sp. A discovered during the course of this research, will be described by light microscopy observations. Chapter 7 will focus on two species of South African tortoise haemoproteids (*Hp. testudinalis* and *Hp. natalensis*), describing both species using light microscopy, as published in Cook *et al*. (2010a). Chapters 5, 6 and 7 will also all include taxonomic summaries of the parasites described. Chapter 8 will follow with results obtained for the possible use of *H. fitzsimonsi* as a stress bio-indicator, the capacity for South African tortoise haematozoans as biological-tags, and results of metal-analysis comparisons between tortoise tissue and soil sample collections. All three (bio-indicator, biological-tag and metal-fingerprinting) results will be discussed and their possible future use in tortoise conservation determined.

Chapter 9 will conclude the thesis with overall findings, suggestions for future research and a conclusion. Lastly, a complete list of references, acknowledgements to relevant parties, as well as additional appendices will follow.

Research done during this thesis has been published in an international journal, Folia Parasitologica (Cook *et al*., 2010a). Also, work from this thesis has been presented at a national level at the annual and biennial conferences of the 39th Annual Congress of the Parasitological Society of Southern Africa (Cook *et al*., 2010b) and the 10th Herpetological Association of Africa congress (Cook *et al*., 2011a) respectively; internationally at the 35th international congress for the World Association for

the Advancement of Veterinary Parasitology (Cook *et al*., 2009b), and the 12th International Congress of Parasitology (Cook *et al*., 2010c). The abstract of one of these presentations at a national conference has also been published (Cook *et al*., 2011b).

CHAPTER 2

LITERATURE REVIEW

Aims of Chapter 2

The aims of the following chapter are to review South African tortoise species, the apicomplexan blood parasites that infect them, and the invertebrate vectors which may transmit these blood parasites. It will provide a brief overview of the different South African biomes and their possible influence on the diversity of tortoise species, their blood and ectoparasites. The evolution and classification of tortoises, apicomplexan haematozoans, especially those that infect tortoises, and possible vectors will be also reviewed. This will lead into a discussion on tortoises as hosts, and invertebrate ectoparasites as vectors for haematozoans. Later, attention will be given to the tortoise apicomplexan blood parasites, by evaluating tortoise haematozoan taxonomy, by considering an already described tortoise haematozoan life cycle, and by exploring the use of molecular analysis to determine taxonomic placement of some of these parasites. The possible use of the most common of these South African tortoise blood parasites as a possible host stress bio-indicator, as well as tortoise haematozoans as biological tags will be assessed, and finally the need for, and use of, metal-fingerprinting of tortoise populations and individuals as a conservation tool will be discussed.

2.1. BIOMES OF SOUTH AFRICA

South Africa has six different biomes (vegetation zones). These include the endemic fynbos and renosterveld, the arid karoo (karroid) or semi-desert, the desert, the savannah (grassland) and arid woodland, the montane forest and grassland, as well as the mesic woodland (see DEA (2012)) (Fig. 2.1).

2.1.1. Influence on tortoise and associated parasite diversity

The diversity of these biomes has a direct effect on the tortoise diversity found within the region. It provides for different sets of challenges for these chelonians and likely, has served evolutionarily in South Africa by providing the highest diversity of terrestrial tortoises in the world (Branch, 2008) (see Fig. 2.2).

Tortoise distributions are largely governed by these biomes or vegetation types, many species being specific to certain regions (Branch, 2008). Overlaying tortoise biodiversity (Fig. 2.2) on the biome map (Fig. 2.1), indicates that most species occur within the western Cape region. The region is

predominately fynbos, forming less than 10% of the rest of South Africa's vegetation. Fynbos is an endemic vegetation type, made up of evergreen heathlands and shrublands with grasses, with trees being rare. Fire is a very important component in fynbos renewal and regeneration (van Wilgen, 2009) and thus indirectly important to the tortoises. Fynbos is recognised as having one of the most distinct and diverse floras in the world, possibly resulting in the high diversity of tortoises and possibly their parasites.

2.2. CHARACTERISTICS OF TORTOISES

2.2.1. General introduction

Tortoises (terrestrial chelonians) are endemic to all continents except Australia and Antarctica, inhabiting a wide range of regions from temperate to tropical (Palkovacs *et al*., 2002; Branch, 2008). In the past, giant tortoises were also found populating a number of Indian Ocean islands, including Madagascar, Réunion, Mauritius, Rodrigues, Aldabra and the Seychelles as well as the Mascarene islands and the most popular, the Galápagos (Austin and Arnold, 2001). The last of the giant tortoises still remain in critically small populations on Aldabra and the Galápagos (Austin and Arnold, 2001; Bourn *et al*., 1999).

Tortoises are ectothermic, pre-historic reptiles, the only reptiles known to have formed a shell from lateral expansions of the rib-cage, vertebrae, sternum and clavicles (Boycott and Bourquin, 2000; Branch, 2008). Relying therefore on external environmental temperatures, tortoises tend to be active during the warmer months of the year and to hibernate during the colder months (Boycott and Bourquin, 2000), making examination of these animals in the wild difficult during those periods. Besides the characteristic shell of these reptiles, they may also be recognised from their beak-like, toothless mouths (Boycott and Bourquin, 2000). The skull of the tortoise is unique compared to other extant vertebrate groups, having a solid bony cranium and chelonians are therefore the only anapsids (see section 2.2.2. below) existing today (Boycott and Bourquin, 2000). Also, distinctively, the pelvic and pectoral girdles are situated within the rib-cage. Covering the bones of the shell is a layer of horny, keratin shields called scales or scutes. Fascinatingly, these scutes do not correspond to the size or shape of the shell bones beneath even though they grow in size with these bones (Boycott and Bourquin, 2000).

Tortoises generally move slowly due to their heavy armour. Smaller species cannot swim as they cannot float (Boycott and Bourquin, 2000). Only the giant tortoises possess the morphological features necessary for flotation (Caccone *et al*., 1999), the importance of which will be discussed further below (see section 2.2.4.).

Tortoises are largely herbivorous, but occasionally include snails, insects or the chewing of carcasses in their diet (see below) for extra calcium and phosphate (Boycott and Bourquin, 2000). It is not certain how often tortoises indulge in carnivorous behaviour, but it may be important when considering transmission of haematozoans such as those of the genera *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 and *Hepatozoon* Miller, 1908, both haemogregarines being transmitted through ingestion of an infected arthropod vector (such as those mentioned above) by the vertebrate host (Davies and Johnston, 2000).

Tortoises are oviparous, the eggs being laid in a nest dug in the ground by the female. The ratio of males to females produced during incubation relies on the temperature of the nest environment (Boycott and Bourquin, 2000; Branch, 2008), another important factor in the evolution of these animals (Caccone *et al*., 2004). Tortoises, like other reptiles, grow throughout their lives, and this is influenced greatly by climatic and environmental conditions (Boycott and Bourquin, 2000), making it difficult to estimate the age of an individual. Depending on the species, tortoises have been recorded to live from 20 to over 150 years (Boycott and Bourquin, 2000). The long life-span of these animals may well contribute to the tortoise serving as a reservoir host for haematozoan infections.

2.2.2. Classification

[Boycott and Bourquin (2000); Rhodin *et al*., (2009); Zoological Museum, Hamburg: Reptile Database (2012) and The Taxinomicon (2012)]

Phylum: Chordata Bateson, 1885
Class: Reptilia Laurenti, 1768
Subclass: Anapsida Linnaeus, 1758
Order: Testudines Batsch, 1788
Suborder: Cryptodira Cope, 1868
Superfamily: Testudinoidea Fitzinger, 1826
Family: Testudinidae Batsch, 1788

Worldwide, approximately 16 tortoise genera covering 82 species, including subspecies, are known (Rhodin *et al*., 2009). The genera are listed below with numbers of species/subspecies per genus appearing in square brackets:

Aldabrachelys Loveridge and Williams, 1957 [4],
Astrochelys Gray, 1873 [2],
Chelonoidis Fitzinger, 1835 [15],
Chersina Grey, 1830 [1],
Cylindraspis Fitzinger, 1835 [5],
Geochelone Fitzinger, 1835 [3],
Gopherus Rafinesque, 1832 [4],
Homopus Duméril and Bibron, 1835 [6],
Indotestudo Lindholm, 1929 [3],
Kinixys Bell, 1827 [9],
Malacochersus Lindholm, 1929 [1],
Manouria Gray, 1854 [3],
Psammobates Fitzinger, 1835 [5],
Stigmochelys Gray, 1873 [2],
Pyxis Bell, 1827 [4],
Testudo Linnaeus, 1758 [15].

South Africa has five of these genera and 18 species (including subspecies), 22% of the world's species of terrestrial tortoises.

2.2.3. South African genera

[Compiled from Boycott and Bourquin (2000); Branch (2008); Reptile Database (2012)].

Chersina Gray, 1830
    Chersina angulata (Schweigger, 1812)
Homopus Duméril and Bibron, 1835

Homopus areolatus (Thunberg, 1787)

Homopus boulengeri Duerden, 1906

Homopus femoralis Boulenger, 1888

Homopus signatus (Gmelin, 1789)

H. s. signatus (Gmelin, 1789)

H. s. cafer (Daudin, 1801)

Kinixys Bell, 1827

Kinixys belliana Gray, 1830

K. b. belliana Gray, 1830

K. b. zuluensis Hewitt, 1931 (synonym)

Kinixys lobatsiana (Power, 1927)

Kinixys natalensis Hewitt, 1935

Kinixys spekii Gray, 1863

Psammobates Fitzinger, 1835

Psammobates geometricus (Linnaeus, 1758)

Psammobates oculiferus (Kuhl, 1820)

Psammobates tentorius (Bell, 1828)

P. t. tentorius (Bell, 1828)

P. t. trimeni (Boulenger, 1886)

P. t. verroxii (Smith, 1839)

Stigmochelys Gray, 1873

Stigmochelys pardalis Bell, 1828

S. p. pardalis (Bell, 1828)

S. p. babcocki (Loveridge, 1935)

***Chersina angulata* (Schweigger, 1812) (Figures 2.2a, 2.3a)**

Angulate Tortoise

Size: medium, between 220 – 300mm, weighing 1- 1.5kg. Habitat: varied, including sandy coastal and fynbos in the west to mesic thicket in the east, with inland populations inhabiting moist Karoo. Distribution: wide, from southern most coastal Namibia along the west coast to the east coast of South Africa, in the last century introduced to two offshore islands Dyer's and Dassen Island. Diet: varied, including grasses, annuals and succulents.

***Homopus areolatus* (Thunberg, 1787) (Figures 2.2b, 2.3b)**

Common Padloper

Size: small, between 100 – 160mm, weighing 140 – 300g. Habitat: varied, coastal fynbos, karroid broken veld and open mesic thicket. Distribution: endemic, mostly Cape coastal region. Diet: varied, possibly including grasses, annuals and succulents.

***Homopus boulengeri* Duerden, 1906 (Figures 2.2c, 2.3c)**

Boulenger's Padloper

Size: small, between 100 – 160mm, weighing 100 – 150g. Habitat: karroid, rocky regions especially dolerite ridges. Distribution: endemic, the Great Karoo. Diet: unknown as it is a secretive species; however, the diet is thought to be very specific, as the species does not survive long in captivity.

***Homopus femoralis* Boulenger, 1888 (Figures 2.2d, 2.3d)**

Karoo Padloper

Size: small, between 100 – 168mm, weighing 200 – 300g. Habitat: grasslands of mountain plateaus especially along Karoo escarpment. Distribution: endemic except for small populations in Lesotho, from inland mountains of Eastern Cape to southern and central Free State and southern Northern Cape. Diet: unknown, however as in Boulenger's Padloper, must be specific, as the species does not adjust well to a captive substitute diet.

***Homopus signatus cafer* (Daudin, 1801) (Figures 2.2e, 2.3e)**

Speckled Padloper

Size: the world's smallest tortoise species between 80 – 96mm, weighing 96 – 163g. Habitat: restricted to western succulent Karoo, extending into fynbos in the south. Distribution: endemic, from Western Cape north into southern Northern Cape. Diet: selective, feeding on leaves and flowers of small herbaceous plants, geophytes and sometimes grasses and insects.

***Homopus signatus signatus* (Gmelin, 1789) (Figures 2.2f, 2.3f)**

Namaqualand Speckled Padloper

Size, habitat and diet the same as the Speckled Padloper. Distribution: endemic, Namaqualand of the Northern Cape.

***Kinixys belliana belliana* Gray, 1830 (Figures 2.2g, 2.3g)**

Bell's Hinged Tortoise

Size: medium, 190 – 230mm, weighing 1.5 – 2kg. Habitat: wet savannah, coastal grasslands, dune forest edge and humid forests. Distribution: western Africa to rainforest of the Congo Basin, Somalia south to KwaZulu-Natal in South Africa; introduced to Madagascar. Diet: omnivorous, feeding on fruit, fungi, grasses, sedges and insects.

***Kinixys lobatsiana* (Power, 1927) (Figures 2.2h, 2.3h)**

Lobatse Hinged Tortoise

Size: medium, 170 – 200mm, weighing 820g – 1.5kg. Habitat: rocky hillsides with mixed *Acacia* and *Combretum* woodland to tropical bushveld, thornveld and *Burkea* savannah. Distribution: Northern South Africa and south eastern Botswana. Diet: varied, feeding on annuals, grass, fruit, mushrooms, beetles, snails and millipedes.

***Kinixys natalensis* Hewitt, 1935 (Figures 2.2i, 2.3i)**

Natal Hinged Tortoise

Size: small to medium, 130 – 160mm, weighing 650 – 300g. Habitat: dry rocky thornveld and bushveld at an elevation of 300 – 1000m and mesic thicket. Distribution: endemic from KwaZulu-Natal to Mpumalanga in South Africa, with small populations through the Lebombo Mountains between Swaziland and Mozambique. Diet: omnivorous, feeding on small annual plants to insects.

***Kinixys spekii* Gray, 1863 (Figures 2.2j, 2.3j)**

Speke's Hinged Tortoise

Size: medium, 170 – 200mm, weighing 820g – 1.5kg. Habitat: savannah, thornveld, coastal plain and dune forest. Distribution: western Africa from southern Democratic Republic of Congo (DRC), Zambia and western Angola; central and eastern Africa through Zimbabwe to northern provinces of South Africa, along the coastal plain of Mozambique to Swaziland. Diet: varied omnivorous, feeding on leaves and flowers of small annuals, grass, mushrooms, fruit, millipedes and giant land snails.

***Psammobates geometricus* (Linnaeus, 1758) (Figure 2.2k, 2.3k)**

Geometric Tortoise

Size: small, 106 – 165mm, weighing 207 – 436g. Habitat: west coast renosterveld. Distribution: endemic to the south western Western Cape occurring in small isolated populations. Diet: selective, including mostly geophytes and grasses with snails.

***Psammobates oculiferus* (Kuhl, 1820) (Figures 2.2l, 2.3l)**

Kalahari Tent Tortoise

Size: small, 80 – 147mm, weighing 319 – 489g. Habitat: arid savannah and scrub desert. Distribution: endemic to southern Africa from central western South Africa Northern Cape Province east to Limpopo up north into Namibia, Botswana almost to the border of Zimbabwe. Diet: varied, including succulents, grasses and annuals along with game droppings.

***Psammobates tentorius tentorius* (Bell, 1828) (Figures 2.2m, 2.3m)**

Common Tent Tortoise

Size: small, 100 – 150mm, weighing 150 – 450g. Habitat: varied, including sandy desert, scrub karroid brush and succulent Karoo. Distribution: southern to central Karoo. Diet: varied, including grasses and annuals.

***Psammobates tentorius trimeni* (Boulenger, 1886) (Figures 2.2n, 2.3n)**

Trimen's Tent Tortoise

Size, habitat and diet the same as the Common Tent Tortoise. Distribution: Namaquland Northern Cape South Africa to southern Namibia.

***Psammobates tentorius verroxii* (Smith, 1839) (Figures 2.2o, 2.3o)**

Verroux's Tent Tortoise

Size, habitat and diet the same as the Common Tent Tortoise. Distribution: northern Karoo to Bushmanland Northern Cape South Africa into southern Namibia.

***Stigmochelys pardalis babcocki* (Loveridge, 1935) (Figures 2.2p, 2.3p)**

Babcock's Leopard Tortoise

Size: large, 300 – 450mm, weighing 10 – 20kg. Habitat and diet similar to the larger Leopard Tortoise. Habitat: wide and varied, including karroid fynbos, mesic thicket, arid and mesic savannah, thorn scrub and grasslands. Distribution: northern South Africa from the west to the east up into Botswana, Zimbabwe and Mozambique. Diet: varied, including annuals, grasses, succulents, bones and hyaena faeces.

***Stigmochelys pardalis pardalis* (Bell, 1828) (Figures 2.2q, 2.3q)**

Leopard Tortoise

Size: very large, 655 – 750mm, weighing 20 – 40kg. Habitat: wide and varied, including karroid fynbos, mesic thicket, arid and mesic savannah, thorn scrub and grasslands. Distribution: eastern and southern Cape with relict populations in southern Namibia. Diet: varied, including annuals, grasses, succulents, bones and hyena faeces.

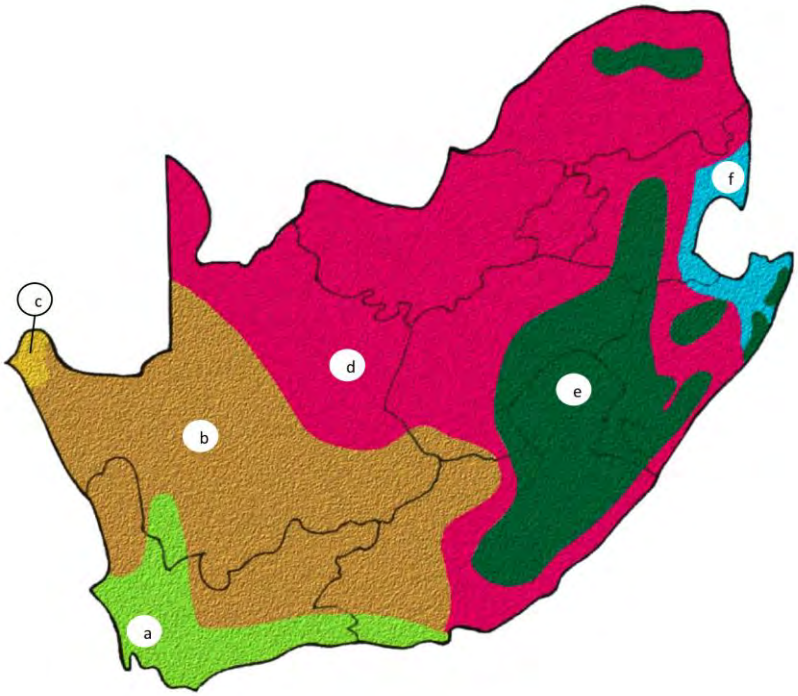


Figure 2.1. Biome distribution: **(a)** fynbos, **(b)** karoo, **(c)** desert, **(d)** savannah, **(e)** montane forest, **(f)** mesic woodland [Boycott and Bourquin (2000) and Branch (2008)].

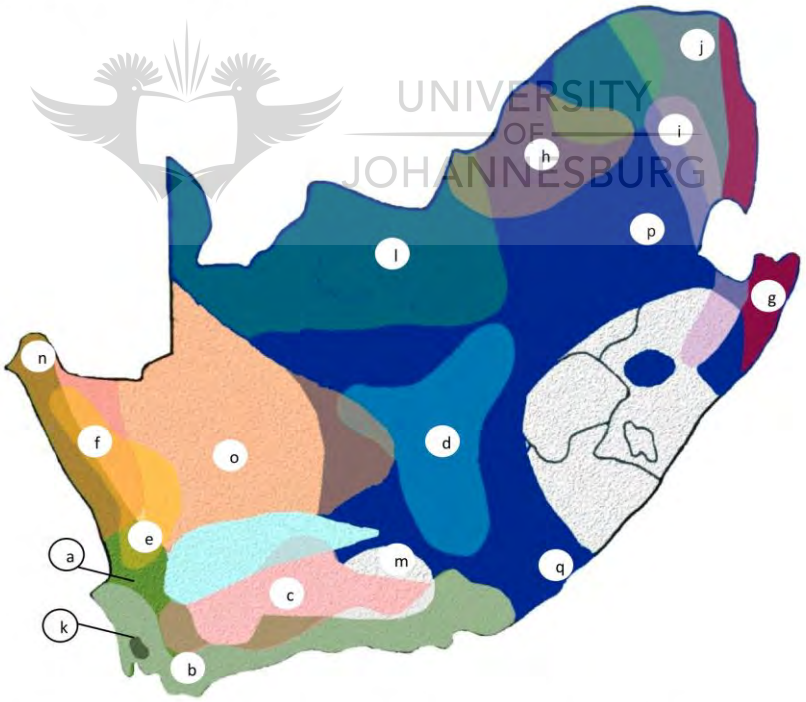


Figure 2.2. Tortoise species distribution: **(a)** *Chersina angulata*, **(b)** *Homopus areolatus*, **(c)** *H. boulengeri*, **(d)** *H. femoralis*, **(e)** *H. signatus cafer*, **(f)** *H. signatus signatus*, **(g)** *Kinixys belliana belliana*, **(h)** *K. lobatsiana*, **(i)** *K. natalensis*, **(j)** *K. spekii*, **(k)** *Psammobates geometricus*, **(l)** *P. oculiferus*, **(m)** *P. tentorius tentorius*, **(n)** *P. t. trimeni*, **(o)** *P. t. verroxii*, **(p)** *Stigmochelys pardalis babcocki*, and **(q)** *S. p. pardalis*. (Taken from Boycott and Bourquin, 2000; Branch, 2008).

Figure 2.3. South African tortoise species. **(a)** *Chersina angulata*, **(b)** *Homopus areolatus*, **(c)** *H. boulengeri*, **(d)** *H. femoralis*, **(e)** *H. signatus cafer*, **(f)** *H. signatus signatus*, **(g)** *Kinixys belliana belliana*, **(h)** *K. lobatsiana*, **(i)** *K. natalensis*, **(j)** *K. spekii*, **(k)** *Psammobates geometricus*, **(l)** *P. oculiferus*, **(m)** *P. tentorius tentorius*, **(n)** *P. t. trimeni*, **(o)** *P. t. verroxii*, **(p)** *Stigmochelys pardalis babcocki*, **(q)** *S. p. pardalis*. Figures a, c-f, h, j-k, m and o: courtesy of Boycott and Bourquin, 2000.

2.2.4. Tortoise evolution

The origin of chelonians is controversial, however, a small rat-sized fossil, *Eunotosaurus africanus*, dated at 260myr before present (bp) from South Africa is thought to be a possible ancestor. The species had thickened ribs, which may have been the preliminary stage of carapace formation (Branch, 2008). The oldest known Testudines (Batsch, 1788), synonym Testudinata Klein, 1751, evolved as early as the late Triassic (~200myr. bp) with evidence showing that the extant species did not occur until the late Jurassic (161-145myr. bp). It is thought that the group originated in Asia (Danilov and Parham, 2008), terrestrial tortoises (Testudinidae Gray, 1825) originating in the early Cenozoic (~60myr. bp) and rapidly dispersing to Europe, Africa and the New World (Parham *et al*., 2006). However, eight species of extinct tortoise have been reported from the middle Jurassic (175-161myr ago) worldwide; four of these were from China, one from Africa (Morocco), three from Europe (UK and Russia) and one from Thailand (Danilov and Parham, 2008).

The discrepancies in chelonian evolutionary time scales are largely attributable to difficulties in analysing these organisms phylogenetically, because of the inability to develop an accurate evolutionary genetic/molecular clock. Phylogenies based on mtDNA (mitochondrial DNA) show that chelonians have reduced levels of variability and differentiation compared with other vertebrate and invertebrate species, the mean micro-evolutionary rate being several fold lower than the standard mtDNA clock for other species (Avise *et al*., 1992). Divergence rates for chelonians are ~0.25%/myr compared to the standard ~2%/myr, which may be attributable to the long life span of most Testudines. The long life span decreases the opportunity for mutational errors and the slow metabolic rate reduces oxygen utilisation, and therefore the number of DNA damaging free oxygen radical events. Finally, the conservative chromosome characteristics of chelonians may be responsible for low divergence rates (Avise *et al*., 1992). Another interesting factor contributing to the reduced mtDNA micro-evolutionary rate may be linked to how the sex of the animals is determined. Depending on the climate of a given year there may be fewer or more female animals produced in a clutch, determined by the temperature of the nest in which the embryos developed. Fewer females mean fewer mtDNA contributions (Caconne *et al*., 2004). The variable mtDNA contributions and in general reduced micro-evolutionary rate makes the rate of genetic evolution difficult to assess and phylogenetic placement of species complicated as the genetic/molecular clock can only be estimated.

Fossil evidence suggests that giant tortoises existed on every continent except for Antarctica and Australia. The earliest Testudinidae fossils date back to the Eocene (55-35myr. bp) (Palkovacs *et al*., 2002) and the oldest giant tortoise fossil from Africa (Egypt), *Gigantochersina ammon*, was dated at

35.4-35.6myr ago (late Eocene) (Holroyd and Parham, 2003). This type of ancestor is thought to have given rise to the modern "*Geochelone* complex", a radiation already diverse in Africa by ~20myr. bp (Parham *et al*., 2006). This complex may have included South African *Stigmochelys* and Malagasy *Asterochelys* and *Pyxis*.

Gigantism in tortoises is an important aspect in their radiation, as this characteristic may have allowed them to cross small spans of open water or inundated land bridges. Their increased size allows for better flotation (Caconne *et al*., 2000), slower metabolic rate allows for conservation of energy and the ability of some species' females to store sperm from multiple males favours colonizing new lands. Tortoises are known to have been able to re-colonize using floatation (see Caconne *et al*., 2004).

2.2.5. Tortoises as hosts

The above characteristics undoubtedly contribute to the distinctive parasite fauna found residing in and on the animals themselves. This study will focus on the blood apicomplexans of tortoises, as well as the vectors associated with their transmission.

Before making assumptions, or forming hypotheses on the transmission of parasites in tortoises, it is necessary to concentrate on the factors which may influence these aspects.

Since tortoises are from an old lineage (see above), their host-parasite relationships may be also long-lived and their parasites well adapted to their hosts. The rate of evolution or change of tortoises is also clearly slow, placing possibly little pressure on parasites to change. The ability of giant tortoises to move and colonize also allows opportunity for associated parasites to do the same. Morphologically, the shell of the tortoise may offer protective armour; however, its weight limits locomotion to a great extent (Branch, 2008) making the animals slow and quick to tire. This affects their diet and tortoises, therefore, usually have a home-range dependent on available sedentary or slow-moving food (Branch, 2008). They are predominantly herbivorous, but as mentioned previously, some species may supplement their diets with millipedes, gastropods and animal faeces (coprophagy) (Boycott and Bourquin, 2000; Branch, 2008) (see section 2.2.3).

Tortoises, unlike cattle and antelope, are inefficient feeders, lacking the specialised gut and bacteria that would aid in cellulose digestion. Tortoises thus need to ingest large quantities of vegetation before their ingested energy content is sufficient (McArthur *et al*., 2004; Branch, 2008). As many tortoises live in arid regions, conservation of body water is important. The animals will avoid the

heat of the day by utilising resting or hiding areas. Personal experience has shown these animals often return to a particular area under vegetation, which they have excavated. Alternatively, under extremely unfavourable conditions, tortoises may enter a period of aestivation in which they hide in burrows, under rock slabs or thick vegetation (Branch, 2008). It is these behavioural features which most likely contribute to their specific parasite fauna.

Comparisons of tortoise behaviour with that displayed by the Australian sleepy lizard, *Tiliqua rugosa* (Gray, 1845), may provide some evidence for likely parasite transmission (Smallridge and Bull, 1999; 2001). These lizards, like tortoises, utilise resting-sites and have home ranges. The lizard rest sites are usually infested with ticks, *Amblyomma limbatum* Neumann, 1899, known to transmit *Hemolivia mariae* Smallridge and Paperna, 1997. A number of different lizards of the same species may use the same resting-sites, allowing for new infections to occur. Tortoises, similarly, may become infected, since in arid regions tortoises share rest sites, which may be infested with ticks (personal observation).

2.3. BLOOD PROTOZOANS OF TORTOISES

2.3.1. General introduction

All blood parasites of tortoises occurring in the current study are classified currently as apicomplexans. The phylum Apicomplexa comprises approximately 5000 (Escalante and Ayala, 1995; Bush *et al*., 2001) fully and partially described species (Escalante and Ayala, 1995). Its biodiversity, however, is the least well known of any group of animals; of all the unicellular organism groups, the Apicomplexa is the largest and it has been suggested that only 0.1% of the total number of species have been named (Morrison, 2009). Even compared to a relatively poorly known group such as the insects, of which 8 – 10% have been described and named, apicomplexans remain the least described group to date (Morrison, 2009).

All species within this phylum are obligate intracellular protistan parasites (Escalante and Ayala, 1995; Morrison, 2009), their characteristic feature being the apical complex which allows for the rapid entry of the infective stages into the host cell. Apicomplexans are unicellular, with a wide environmental distribution (Morrison, 2009). It is thought that all extant animals play host to at least one or several apicomplexan parasite species (Morrison, 2009). Many apicomplexans are of medical and veterinary importance and represent a considerable economic burden (Wasmuth *et al*., 2009).

The life cycles of apicomplexans are complex. Briefly, there are three major stages to the life cycle including the sporozoite, merozoite and gamont/gametocyte (Wasmuth *et al*., 2009). These stages are common to all species of apicomplexan, but differ in mode of transmission and morphology (Morrison, 2009; Wasmuth *et al*., 2009). Some apicomplexans require a single host to complete their life cycle, such as *Cryptosporidium* Tyzzer, 1907 (see Wasmuth *et al*., 2009), and many other species require two, such as *Hemolivia* (see Široký *et al*., 2007), or more hosts, such as some species of *Hepatozoon* (see Telford, 2009).

2.3.2. Classification

Traditionally four groups of apicomplexans are recognised including coccidians, gregarines, haemosporidians and piroplasmids (Morrison, 2009). The groupings are based on phenotypic characters, hosts and/or vectors, as well as the host tissues inhabited by the parasites. Current classification is often conservative and does not take into account modern molecular data (Barta, 1997; Morrison, 2009). The taxonomy of the apicomplexans is thus still the centre of much debate (Bannister *et al*., 2000).

As suggested above, apicomplexans are not well known phylogenetically, with future molecular studies on a number of the species unfortunately unlikely a priority (Morrison, 2009). Many features of the group are indicative of this lack of phylogenetic knowledge; apicomplexans may be difficult to find and have very few easily studied characteristics, these including their life cycles, cyst organisation and ultrastructure (Barta, 1997; Morrison, 2009). However, phylogenetic analysis is important, especially when diseases caused by these organisms need to be treated. For example, *Cryptosporidium* was removed recently from the coccidian group once phylogenetics had contradicted this placement, and this finding elucidated why anti-coccidial drugs were not effective against this genus in the past (Morrison, 2009).

Phylogenies are often based on either phenotypic characters or on multiple genotypic characters, but should be based on a combination of both. The phenotypic characters usually include the life cycle and ultrastructure in apicomplexans. It is hoped in future that progress will be made on the molecular plane, with phylogenetic analyses based on a combination of phenotypes and multiple gene analysis and not solely on one gene, such as those based on just the 18S rRNA gene (Morrison, 2009).

The apicomplexans were, in the past, grouped with the dinoflagellates and the ciliates within the kingdom Protozoa (see Cavalier-Smith, 1999; Lee *et al*., 2000). Subsequently, the apicomplexans were transferred to the infra-kingdom Alveolata under their own phylum Apicomplexa as a synonym of Sporozoa (see Cavalier-Smith, 1999). The phylum subsequently, however, became a subphylum of the Miozoa (see Cavalier-Smith, 2002a; Cavalier-Smith, 2003), now Myozoa, the Sporozoa becoming an infraphylum (see Cavalier-Smith and Chao, 2004). The apicomplexans are now considered to be a sister group to the dinoflagellates based on genetic data from the plastid (see Huang *et al*., 2004; Wasmuth *et al*., 2009) and the Apicomplexa is still widely considered as a phylum rather than a subphylum. The classification of the Apicomplexa is summarised below, together with the placements of several genera studied in this thesis.

(Taken from: Lee *et al*., 2000; Cavalier-Smith, 1993; Cavalier-Smith, 2002b; Cavalier-Smith and Chao, 2004; Adl *et al*., 2005; Roberts and Janovy, 2008; and The Taxonomicon (2012))

Kingdom: Protozoa Goldfuss, 1818
Clade: Chromalveolata Cavalier-Smith, 2002
Infrakingdom: Alveolata Cavalier-Smith, 1991
Phylum: Apicomplexa Levine, 1970
Infraphylum: Sporozoa (Leuckart, 1879) Cavalier-Smith, 1999
Class: Coccidia Leuckart, 1879
Order: Eucoccida Léger & Duboscq, 1910
Suborder: Adeleorina Léger, 1911
Family: Haemogregarinidae (Neveu-Lemaire) Léger, 1911
    Genus: *Haemogregarina* Danilewsky, 1885
    Genus: *Hemolivia* Petit, Landau, Baccam and Lainson, 1990
    Genus: *Hepatozoon* Miller, 1908
Class: Hematozoa Vivier, 1982
Order: Haemospororida Danilewsky, 1885
Family: Haemoproteidae Doflein, 1916
    Genus: *Haemoproteus* Kruse, 1890
Order: Piroplasmorida Wenyon, 1926
    Genus: *Chelonoplasma* Frye, 1981

2.3.3. Apicomplexan evolution

Apicomplexans belong to a clade of chromalveolates (as above). Chromalveolates are suggested to have evolved in a single secondary symbiogenetic event when a unicellular red alga was taken up by a possibly heterotrophic protozoan. It is thought that the resulting chromalveolate ancestor experienced multiple plastid losses along with acquisitions from different green algae (see reference to the apicoplast below), before diverging into the sister groups of the chromists and alveolates (Cavalier-Smith, 2002b). Apicomplexans fall under the second group, and are characterised by cortical alveoli (Archibald, 2009), micropores, and mitochondria with ampulliform or tubular cristae (Moore *et al*., 2008). Besides the Apicomplexa, the Alveolata also comprise the Ciliata and Dinoflagellata. The Apicomplexa differ from the other two by a characteristic set of secretory organelles collectively known as the 'apical complex' (Moore *et al*., 2008). This complex is the main and defining feature of the apicomplexans and includes organelles such as the rhoptries and micronemes, as well as associated structures such as dense granules (Wasmuth *et al*., 2009).

It is hypothesised that the apicomplexan apicoplast organelle was derived from an ancient secondary endosymbiosis within an algal cell (Lang-Unnasch *et al.*, 1998), most likely a species of the cyanobacteria (Huang *et al*., 2004; Wasmuth *et al*., 2009), possibly the photosynthetic alga *Chromera velia* (Janouškovec *et al*., 2010). *Chromera velia* is an organism that demonstrates ultrastructural features typical of alveolates, has been found to be phylogenetically related to the Apicomplexa, but unlike the apicomplexans, contains a photosynthetic plastid (Moore *et al*., 2008). It is difficult, however, to determine the exact origin of the apicoplast due to its genetically divergent nature (Janouškovec *et al*., 2010). The endosymbiosis of the algal cell was, it is suggested, accompanied by a drastic reduction in the gene content of that cell. The algal nucleus and mitochondrion was lost and the plastid genome reduced (Huang *et al*., 2004). Interestingly, the nuclear and plastid genomes of the apicomplexans are apparently co-evolving (Lang-Unnasch *et al.*, 1998). The apicoplast is essential to the survival of the apicomplexan (Lang-Unnasch *et al.*, 1998) as the proteins for the main metabolic pathways are imported into it (Wasmuth *et al*., 2009).

It has also been proposed that the members of the phylum Apicomplexa are ancient, perhaps as old as the three multicellular kingdoms of plants, fungi and animals (Escalante and Ayala, 1995). Leander (2007) hypothesised that apicomplexan parasites may have evolved from parasitic organisms of marine invertebrates, and that their probable evolution may be deduced from that of the hosts (Escalante and Ayala, 1995). In addition, it has been proposed that the gregarines, exclusively parasites of invertebrates, evolved before the other apicomplexan classes, which now infect both invertebrates and vertebrates (Escalante and Ayala, 1995; Leander, 2007). As there are no recorded

fossils of apicomplexans, evolution may only be estimated utilising a molecular clock. The Coccidea, monogenetic parasites of molluscs, marine annelids, mammals and other vertebrates, appear to have radiated approximately 350 - 824myr. bp. Coccidean early hosts may thus not have been chordates or land organisms as these groups had not yet evolved. The group thus existed over 200myr before the origin of modern mammal orders currently parasitized by this group (Escalante and Ayala, 1995).

The haematozoan apicomplexans, with which this thesis is concerned, are digenetic parasites. Maturation of gametes, fertilisation and sporogony occur within the haematophagous invertebrate vector with the life cycle being completed within the vertebrate host. The monogenetic life cycle was most likely the primitive one, the molecular clock indicating that vertebrates were not the original hosts. It would appear, therefore, that the current relationship utilising both an arthropod vector and a vertebrate evolved relatively recently (Escalante and Ayala, 1995).

2.3.4. Haematozoans of reptiles

Most species of haemoparasites have been poorly described, restricted by variable morphological characters described under light microscopy. Compared to mammalian and avian host haematozoans, reptilian blood parasites are not as well described or reported. Furthermore, the size of the reptilian erythrocyte and its prominent nucleus can influence the appearance of parasites occurring within the cell making consistent morphological descriptions challenging (Telford, 2009). The diversity of reptilian haematozoans is higher than that of mammals and birds in both number and genera of species. This applies across all four classes of reptiles (Crocodilia, Rhynchocephalia, Squamata, Testudines) hosting the same types of parasites, namely plasmodiids, haemogregarines and trypanosomatid flagellates. Many reptile species are specific to their habitats and are of great phyletic age, which greatly contributes to the increased taxonomic diversity of their parasites (Telford, 2009).

2.3.5. Haematozoans of tortoises with focus on South African genera

The species of haemogregarines and haemoproteids infecting chelonians worldwide are listed in Tables 2.1 and 2.2.

As reported in Chapter 1, four species of blood apicomplexans have been identified and described from South African tortoises. Two species are classified as haemogregarines, *Haemogregarina fitzsimonsi* Dias, 1953 and *Haemogregarina parvula* Dias, 1953; the third is a haemoproteid, *Haemoproteus testudinalis* (Laveran, 1905) (syn. *Haemoproteus balazuci* Dias, 1953) (see Cook *et al*., 2010a); the fourth is the recently described *Haemoproteus natalensis* Cook, Smit and Davies, 2010 and this new species has been described and named as part of the current research project. In addition to the above species, an unknown species, possibly haemogregarine and therefore referred to as *Haemogregarina* sp. A, has been recorded. Also from work undertaken during the course of the present research, *Haemogregarina fitzsimonsi* has been found infecting five species of tortoises examined within South Africa, while *H. parvula* has appeared more species specific, and found infecting only two tortoise species in a single eco-region (Cook *et al*., 2009a). The unknown *H*. sp. A has only been recorded infecting a single tortoise species from a single locality. *Haemoproteus testudinalis* has been located in two species of tortoise from more than one eco-region and *Hp. natalensis* in a single specimen of one species within one eco-region (see Cook *et al*., 2009a; Cook *et al*., 2010a).

Chelonian species of *Haemogregarina* have been characterised by oocysts containing fewer than 32 naked sporozoites occurring within the gut caeca of the definitive host, a leech, with transmission to the chelonian host occurring through salivary gland transmission by leech bite (see Siddall, 1995). Once within the intermediate host (the chelonian), parasites apparently undergo hepatic and erythrocytic merogony (Smith, 1996). The family Haemogregarinidae Léger, 1911 has two genera known to infect chelonians, namely *Haemogregarina* (*sensu stricto*) Danilewsky, 1885 and *Hemolivia* Petit, Landau, Baccam and Lainson, 1990 (see Davies and Johnston, 2000). *Haemogregarina* (*s. s.*) *stepanowi* Danilewsky, 1885, the type species of the genus *Haemogregarina* (see Davies and Johnston, 2000), has been found infecting many terrapin intermediate hosts from northern and southern America, but typically occurs in Europe (Telford, 2009) with *Placobdella catenigra* Blanchard, 1893, a leech, as the definitive host (Siddall, 1995; Telford, 2009). Siddall (1995) records *Kinixys* species as intermediate hosts for *H. stepanowi*. However, these African-specific terrestrial tortoises often occur in dry terrain where contact with leeches would be unlikely. Therefore, leeches are unlikely the definitive hosts of all tortoise *Haemogregarina* species (see Cook *et al*., 2009a). The genus *Hemolvia* is considered later in this Chapter 2 (see section 2.4.5.1).

The family Haemoproteidae Doflein, 1916 originally had four genera infecting chelonians including: *Haemocystidium* Castellani and Willey, 1904, *Haemoproteus* Kruse, 1890, *Haemamoeba* Simond, 1901 and *Simondia* Garnham, 1966 (see Davies and Johnston, 2000). Wenyon (1915), however,

reassessed the species within the genera *Haemocystidium* and *Haemamoeba*, placing them into the genus *Haemoproteus* or *Plasmodium*, according to the absence or presence of merogony within the intermediate host blood (see Lainson and Naiff, 1998). The genus *Simondia*, equally, has not been widely accepted and therefore Levine and Campbell (1971), Levine (1988) and Paperna and Landau (1991), Lainson and Naiff (1998) placed all known haemoproteids infecting chelonians in *Haemoproteus* (see Davies and Johnston, 2000).

Table 2.1: Haemogregarines infecting freshwater terrapins and terrestrial tortoises from different geographical localities. Recorded below is the locality, haemogregarine species, gamont description, type host and other hosts, along with prevalence and references.

Locality	Species	Mature gamont shape and size (µm)	Type host	Other hosts	Prevalence	References
NORTH AMERICA						
	Haemogregarina balli Paterson and Desser, 1976	Lentiform. Gamonts: (8-14.5) x (3-6.5)	*Chelydra serpentina* (syn. *Chelydra serpentina serpentina*) (Chelydridae)	*Chrysemys picta marginata, Glyptemys insculpta* (syn. *Clemmys insculpta*) (Emydidae)	37/37 *C. serpentina*, 6/11 *C. picta marginata*	Telford (2009)
	Haemogregarina macrochelysi Telford, Norton, Moler and Jensen, 2009	Slender, recurved. Gamonts: (29-35) x (3-4.5)	*Macrochelys temminkii* (Chelydridae)	*Graptemys barbouri* (Emydidae)	115/115 *M. temminkii*, 2/11 *G. barbouri*	Telford (2009)
AFRICAN						
	Haemogregarina sp. A.	Globular. Gamonts: (17.6 x 9.7)	*Stigmochelys pardalis* (Testudinidae)	None known	2/121 *S. pardalis*	See this thesis (Chapter 6, section 6.2)
	Haemogregarina bruneti Commes, 1919	Slender. (22) x (8)	*Kinixys homeana* (syn. *Cinixis homeana*) (Testudinidae)	None known	Unknown	Commes (1919)
	Haemogregarina fitzsimonsi Dias, 1953	Slender. Gamonts: (15.8-18.9) x (3.6-5.7)	*Kinixys belliana belliana* (syn. *Kinixys belliana zuluensis*) (Testudinidae)	*Chersina angulata*, *Kinixys belliana belliana*, *Kinixys lobatsiana*, *Kinixys natalensis*, *Stigmochelys pardalis* (Testudinidae)	6/20 *C. angulata*, 8/16 *K. b. belliana*, 3/29 *K. lobatsiana*, 2/3 *K. natalensis*, 18/89 *S. pardalis*	Dias (1953), Cook *et al*. (2009a)
	Haemogregarina parvula Dias, 1953	Globular. Gamonts: (9.2-13.2) x (5.7-6)	*Kinixys belliana belliana* (syn. *Kinixys belliana zuluensis*) (Testudinidae)	*Stigmochelys pardalis* (Testudinidae)	4/16 *K. b. belliana*, 1/89 *S. pardalis*	Dias (1953), Cook *et al*. (2009a)
	Haemogregarina pelusiensi Pienaar, 1962	Recurved and lentiform within a translucent, hard capsule. (9-17) x (5-9)	*Pelusios sinuatus* (syn. *Pelusios sinuatus sinuatus*) (Pelomedusidae)	None known	Unknown	Telford (2009)
	Haemogregarina sternotheri Bouet, 1909 [also reported by França (1911)]	Recurved. Gamonts: (12.6) x (1.8)	*Pelusios subniger* (syn. *Sternotherus derbianus*) (Pelomedusidae)	None known	Unknown	Bouet (1909), França (1911)
PALEARCTIC						
	Haemogregaria stepanowi Danilewsky, 1885	Recurved into nearly two equal limbs. Gamonts: (29-37) x (3-5.5)	*Emys orbicularis* (Emydidae)	None known	Unknown	Telford (2009)

Table 2.1. continued

Locality	Species	Mature gamont shape and size (µm)	Type host	Other hosts	Prevalence	References
	Hemolivia mauritanicum (Sergent and Sergent, 1904) Landau and Paperna, 1997	Strongly recurved. Gamonts: (9-12) x (5-7), gamonts in cysts: 12.2 x 6.2	*Testudo graeca* (Testudinidae)	*Testudo marginata* (Testudinidae)	26/40*T. graeca*, 38/47 *T. marginata*	Telford (2009)
ASIAN						
	Haemogregarina choudhuryi Ray and Bhattacharjee, 1984	Lentiform. Gamonts: (8.5) x (2-3.5)	*Lissemys punctata punctata* (Trionychidae)	None known	3/5 *L. p. punctata*	Telford (2009)

Table 2.2: Haemoproteids infecting freshwater terrapins and terrestrial tortoises from different geographical localities. Recorded below is the locality, haemoproteid species, gametocyte description, type host and other hosts, along with prevalence and references.

Locality	Species	Mature gametocyte shape and size	Type host	Other hosts	Prevalence	Reference
NORTH AMERICA						
	Haemoproteus degiustii sp. nov. (syn. *H. metchnikovi*) (Simond, 1901) Hewitt, 1940	Ovoid to halteridial. Gametocytes: (8-18) x (4.5-10)	*Chrysemys picta marginata* (Emydidae)	*Pseudemys rubriventris, Pseudemys suwanniensis* (syn. *Pseudemys concinna suwanniensis*), *Trachemys scripta elegans, Graptemys geographica, G. barbouri, Emydoidea blandingii* (Emydidae), *Apalone s. spinifera, A. s. emoryi, A. ferox* (Trionychidae), *Chelydra serpentina* (Chelydridae)	6/39 *T. s. elegans*, 1/1 *A. s. emoryi*, 1/6 *C. serpentina*, 14/105 *P. rubriventris*, 1/26 *P. c. suwanniensis*	Telford (2009)
NEOTROPICAL						
	Haemoproteus geochelonis Lainson and Naiff, 1998	Polar ovoid. Macrogametocytes: (4.4-8.1) x (3.7-7.4), 35.1 µm^2 Microgametocytes: (5.2-8.0) x (4.4-6.0), 31.8 µm^2	*Chelonoidis denticulata* (syn. *Geochelone denticulata*) (Testudinidae)	None known	1/1	Telford (2009)
	Haemoproteus peltocephali Lainson and Naiff, 1998	Polar amoeboid to ovoid. Young gametocytes: amoeboid. Macrogametocytes: (7.4-12.6) x (6.2-11.1), 74.7 µm^2 Microgametocytes: (6.7-12.5) x (6.0-9.0), 68.4 µm^2	*Peltocephalus dumeriliana* (syn. *Peltocephalus dumerilianus*) (Podocnemididae)	None known	4/8	Telford (2009)
AFRICAN						
	Haemoproteus sp. (Plimmer, 1912)	No details	Unknown	*Stauotypus triporcatus* (Kinosternidae), *Chrysemys picta* (Emydidae), *Kinixys belliana* (syn. *C. belliana*), *Kinixys erosa* (syn. *C. erosa*), *Kinixys homeana* (syn. *Cinixys homeana*), (Testudinidae)	Unknown	Telford (2009)
	H. cajali Pittaluga, 1912 (not reported since)	No details	*Pelomedusa subrufa* (syn. *Clemmys africana*?) (Emydidae)	None known	Unknown	Pittaluga (1912), Telford (2009)
	H. roumei (Bouet, 1909) (not observed since Joyeux, 1913)	Spherical/ovoid: 12.6 Halteridial: (12.6-16.2) x (10.8) Elongate: (14.4) x (3.6)	*Kinixys belliana* (syn. *Cinixys belliana*) (Testudinidae)	None known	Bouet (1909): 1/1 Joyeux (1913): 19/34	Telford (2009)
	Haemoproteus testudinis (Laveran1905) Cook, Smit and Davies,, 2010	Ovoid: (10-12) x (7-8) Halteridial: (20) x (7-8)	*Stigmochelys pardalis* (syn. *Testudo pardalis*) (Testudinidaes)	None known	1/1	Laveran (1905), Telford (2009), Cook *et al.* (2010a)

Table 2.2. continued

Locality	Species	Mature gametocyte shape and size	Type host	Other hosts	Prevalence	Reference
	(syn. *Haemoproteus balazuci* Dias, 1953)	Microhalteridial to halteridial. Dias (1953) Macrogametocytes: (19.1-24.8) x (6.6-7.6) Microgametocytes: (11.9-18.8) x (5.9-8.9); Cook *et al*. (2009a) Macrogametocytes: (23.6 – 34) x (5.2 - 7.6), (130.3 – 190) µm^2 Microgametocytes: (10.4 - 22.6) x (4.9 - 9.5), (70.5 - 105.9) µm^2	*Kinixys belliana belliana* (syn. *Kinixys belliana zuluensis*) (Testudinidae)	*Kinixys lobatsiana, Stigmochelys pardalis* (Testudinidae)	Dias (1953) 2/8 *K. belliana*; Cook *et al*. (2010a) 4/29 *K. lobatsiana*, 1/88 *S. pardalis*	Dias (1953), Cook *et al*. (2010a)
	Haemoproteus natalensis Cook, Smit and Davies, 2010	Young gametocytes: amoeboid. Halteridial to Circumnuclear. Macrogametocytes: (24.9 - 47.3) x (4.2 – 9.3), (109.2 - 305) µm^2 Amoeboid to halteridial. Microgametocytes: (6.5 - 25.8) x (5.2 - 10.1), (51.1 - 117.2) µm^2	*Kinixys natalensis* (Testudinidae)	None known	1/3 *K. natalensis*	Cook *et al*. (2010a)
ASIAN						
	Haemoproteus metchnikovi (Simond, 1901)	Spherical/ovoid: 6-10	*Chitra indica* (syn. *Trionyx indicus*) (Trionychidae)	None known	20/20	Telford (2009)
	Haemoproteus caucasica Krasil'nikov, 1965	Halteridial: (10-18) x (5.5-6), 55-99 µm^2	*Testudo graeca* (Testudinidae)	None known	Unclear	Telford (2009)
	Haemoproteus trionyxii Misra and Choudhury, 1977	Macrogametocytes: (10.2) x (7.5) Microgametocytes: (8.2) x (7.8)	*Aspideretes gangeticus* (syn. *Trionyx gangeticus*) (Trionychidae)	None known	Not stated	Telford (2009)
AUSTRALIAN						
	Haemoproteus chelodinae (Johnston and Cleland, 1909) Mackerras, 1961	Spherical/ovoid: (7-12) x (7-9), 50-99 µm^2	*Chelodina longicollis* (Chelidae)	*Chelodina rugosa* (syn. *Chelodina oblonga*), *Emydura macquarii* (syn. *Emydura. krefftti*), *Myuchelys latisternum* (syn. *Emydura latisternum*), *Elseya dentata* (Chelidae)	½	Telford (2009)

2.3.6. Molecular analysis of reptilian haematozoans with focus on those infecting tortoises

The diversity of haematozoans infecting reptilian hosts is vast and in the past, such species were described on morphological and life cycle stages alone (Telford, 2009). Telford (2009) compiled a detailed reference book with information on a large number of these species which ranged from plasmodiids and haemoproteids, to the haemogregarines, piroplasmids and trypanosomes. All these species, however, were described and named on morphological characteristics.

Descriptions based on morphological characteristics alone may result in an inaccurate account of true species diversity (Perkins and Schall, 2002; Vilcins *et al*., 2009a). This is especially true when descriptions are based on peripheral blood stages alone. Parasite life cycle stages may become distorted during preservation (Perkins and Schall, 2002) or may vary in morphology within and between different host species (Perkins, 2000; Telford *et al*., 2001; Maia *et al*., 2011). Also, the ability to detect cryptic species of haematozoans, as well as latent infections, is greatly reduced when relying solely on morphological characteristics or smears (Perkins, 2000 and Fallon *et al*., 2003 respectively). In the past, phylogenetic analyses incorporated morphological character states (see Siddall, 1995; Smith and Desser, 1997; Mathew *et al*., 2000; Jakes *et al*., 2003), relying on these states quite heavily or even solely. This led to the systematics and taxonomy of many haematozoan species being phylogenetically inconsistent (see Mathew *et al*., 2000). The use of small subunit ribosomal RNA genes, such as 18S (Mathew *et al*., 2000), and mitochondrial genes, such as cytochrome *b* (Ujvari *et al*., 2004), allowed for the molecular characterisation of a number of these haematozoans. However, molecular characterisation concerned mostly medically and economically important species such as those of the genera *Plasmodium*, *Babesia* and *Toxoplasma* (Maia *et al*., 2011).

A good number of reptile haematozoan species have undergone molecular characterisation, but they belong mostly to only two genera, *Hepatozoon* (see Mathew *et al*., 2000; Ujvari *et al*., 2004; Vilcins *et al*., 2009a; Herbert *et al*., 2010; Harris *et al*., 2011; Maia *et al*., 2011) and *Plasmodium* (Austin and Perkins, 2006). In the process of developing a haemogregarine-specific primer, Perkins and Keller (2001) sequenced *Hemolivia mariae*, but unfortunately, the sequence was not uploaded onto the NCBI website.

As haemogregarines, in particular *Hepatozoon* species, are the most prevalent blood parasites infecting reptiles (Sloboda *et al*., 2007; Vilcins *et al*., 2009a), most current molecular work has focused on these parasites (see Harris *et al*., 2011; Maia *et al*., 2011). There are examples, however,

of other genera such as *Plasmodium*, where Austin and Perkins (2006) determined the parasite diversity within skinks through sequencing, but these types of studies remain rare in comparison to the work done on *Hepatozoon* species. The majority of *Hepatozoon* infections are within snakes, representing over 200 species, but most of these species accounts are based on morphological characteristics alone, and many of them lacking life cycle stage descriptions within the definitive host. Since the genus is one which requires the description of gamont morphology and sporogonic characters to be relatively accurate, many of these species may be invalid (Sloboda *et al*., 2007). Molecular work done so far on *Hepatozoon* infections of reptiles focuses on determining whether or not species diversity, such as the above, is valid (see Sloboda *et al*., 2007; Harris *et al*., 2011) as well as attempting to infer vectors (see Vilcins *et al*., 2009a).

The most recent *Hepatozoon*-focussed molecular work, involves reptile species of island communities, such as the Seychelles (see Harris *et al*., 2011), and the North (Algeria, Morocco and Tunisia) of Africa (see Maia *et al*., 2011). However, neither of these studies has included the haemogregarines of tortoises. It appears that *Hepatozoon* species have not been described from tortoises (see Table 2.1), with the exception of *Hepatozoon mauritanicum*, which was later transferred to the genus *Hemolivia* (see Landau and Paperna, 1997; Široký *et al*., 2007; Telford, 2009). Thus, it would seem that to date no haemogregarine or haemoproteid infecting tortoises has been characterised by molecular means. Possibly, the only apicomplexan parasites sequenced, and those sequences published and uploaded onto GenBank, from tortoises are species of *Cryptosporidium*, gastrointestinal parasites of medical and economic importance (see Traversa *et al*., 2008; Griffin *et al*., 2010).

2.4. HAEMATOPHAGOUS, METAZOAN ECTOPARASITES OF TORTOISES

Haematophagous, metazoan ectoparasites of land tortoises include mainly ticks (McArthur *et al.*, 2004). Even though such tortoises may submerge themselves in shallow water in an attempt, presumably, to rid themselves of ectoparasites, contact with leeches is likely minimal or non-existent. Only in tropical environments such as those in central Africa, with permanent bodies of water and moist, damp detritus may leeches be factored in as ectoparasites of terrestrial tortoises. From personal experience of sampling in South Africa, no leeches have been collected from terrestrial tortoises, even in subtropical KwaZulu-Natal (see Cook *et al*., 2009a).

The tick, *Hyalomma aegyptiam* (Linnaeus, 1758), is a species known, through histological and experimental observation, to transmit apicomplexan haematozoans between the Palearctic tortoise

species *Testudo graeca* Linnaeus, 1758 and *Testudo marginata* Schoepff, 1792 (see Široký *et al*., 2007). Histological examination of the gut of ticks fed on infected tortoises revealed infective stages of the apicomplexan *Hemolivia mauritanicum*, previously known as *Haemogregarina mauritanicum* (see Landau and Paperna, 1997).

The above findings suggest that ticks may transmit apicomplexan haematozoans between South African tortoises, and this hypothesis is tested in this project.

2.4.1. General introduction to ticks

Ticks are obligate haematophagous ectoparasites of terrestrial vertebrates, consisting of approximately 869 species divided into three families: the Argasidae (soft ticks); the Ixodidae (hard ticks); and the Nuttalliellidae (consisting of a single African species, *Nuttalliella namaqua* Bedford, 1931). The phylogeny of these families remains unresolved and controversial (Barker and Murrell, 2002; de la Fuente, 2003; Pagel van Zee *et al*., 2007). Ticks are important in both human and veterinary disease, by either causing problems themselves or by serving as vectors for severe pathogens (Roberts and Janovy, 2000). However, despite this, knowledge on many members of the suborder Ixodida, in particular, remains limited (Pagel van Zee *et al*., 2007).

Ticks are epidermal parasites during all three feeding stages (larval, nymphal and adult) of their life cycle. They have been recognised as agents and vectors of disease for centuries, the associated pathogenesis presenting itself as anaemia (blood loss at feeding), dermatosis (inflammation, swelling, ulceration and itching associated with the tick bite, as well as secondary bacterial infections), paralysis (common in mammals and caused by toxic secretions from the tick), otoacariasis (infection of the ear canal by ticks) and other transmitted infections (for instance bacteria, rickettsias, spirochetes, filariae, and protozoans such as the apicomplexans with which this thesis is concerned) (see Roberts and Janovy, 2000).

The basic tick life cycle comprises four stages, the egg, larva, nymph and adult. The life cycle may take from six weeks to three years to complete. Ixodids have in general one nymphal instar, whilst argasids may have as many as five (Roberts and Janovy, 2000; Taylor *et al*., 2007). Copulation of adult ticks occurs on the host, the male depositing a spermatophore into the genital operculum of the female (Taylor *et al*., 2007). In order for the female to produce eggs, a blood meal is required. Once engorged, the female tick drops off the host, deposits the eggs (which may number some 100 – 18000) within soil or humus, after which she will die. Some ticks, however, produce offspring by

parthenogenesis. Hatchlings, the six-legged larvae, will 'quest' (seek actively) hosts using low vegetation. Once a host has been found, attached to, and a tissue or blood meal obtained, the engorged larvae will drop off and moult into an eight-legged nymph. Similarly, the engorged nymph may feed, release, and moult into an adult, after which it will reattach to a host for courtship and mating (Taylor *et al*., 2007). If all stages undergo moulting on the same host species, the tick species is a one-host tick species, for example in the subgenus *Boophilus* (genus *Rhipicephalus*) (see Roberts and Janovy, 2000) responsible for the transmission of the Texas fever pathogen *Babesia bigemina* (see de la Fuente, 2003). If the nymph detaches from one host and attaches as an adult on a second host of possibly a different species, the tick species may be described as a two-host tick species. However, most ixodid tick species are three-host species, leaving the host between all three life stages. It is suggested that the two to three-host system may be an adaptation to feeding on a wide host range, and may provide increased opportunities for the transmission of pathogens and possibly, in the current study, apicomplexan protozoans. A few tick species are host-specific but most species are opportunists.

Ticks can be hardy and may survive starvation for up to 16 years. They also exhibit complex behaviour controlled largely by pheromones (Roberts and Janovy, 2000). The main pheromones necessary and responsible for a number of these complex behaviours, especially during mating, are as follows: guanine for aggregation; *o*-nitrophenol for searching and aggregation; methyl salicylate and pelargonic acid for clasping and attachment during mating; 2,6-dichlorophenol for attraction and potential mate recognition in male ticks; cholesteryl oleate for mounting; and lastly 20-hydroxyecdysone for copulation. These pheromones radiate from the anus, coxal glands and female genital aperture (Roberts and Janovy, 2000).

Attachment-site studies such as those of Fielden and Rechav (1994), for the tick species *Amblyomma marmoreum* on the tortoise *Stigmochelys pardalis* illustrate this complex tick behaviour. Recordings of ticks were taken from 162 tortoises housed over a period of 19 months at the National Zoological Gardens in Pretoria, South Africa. Eighty percent of larvae and 87% of nymphs were found attached to the anterior parts of the tortoises, especially the head, neck and anterior leg region. On the contrary, 90% of adult ticks were recorded in the posterior regions, females mostly around the tail and males split equally between the tail, posterior leg and axillar regions. Tick segregation may result from firstly, niche segregation as a result of inter-specific interactions, secondly, from attempts to maximise mating and feeding success, and thirdly, from protection from physical disturbance (Fielden and Rechav, 1994). Often, *A. marmoreum* constitutes the highest density population of a tick species on tortoises such as *S. pardalis* and therefore inter-specific competition

is not a contributing factor in *A. marmoreum* segregation. In this case, intra-specific competition may be more important, but closer inspection of the dynamics of primary attachment reveals that these ticks are more strongly influenced by factors relating to successful mating and feeding, and minimisation of disturbance, than by competitive exclusion (Fielden and Rechav, 1994).

Reptile-infesting ticks often assume a "sit and wait" approach, possibly as a result of their short-range host detection capabilities. Tortoises do not generally return to the same refuge site after periods of activity (Boycott and Bourquin, 2000; Branch, 2008) and therefore tick life stages most likely wait for a host to pass by, attaching to the anterior regions first. As larvae and nymphs are small and softer than adult ticks, the anterior regions of the tortoise, being well protected, perhaps offer a better environment for feeding. Adult ticks move from the anterior regions of primary attachment to the posterior regions, which even though may not be as well protected, offer a larger space for female adult ticks to expand during blood feeding without being crushed by the host. Adult male ticks will follow females, so as to ensure successful mating (Fielden and Rechav, 1994).

2.4.2. Classification

(Taken from ITIS (2012); The Taxonomicon (2012); Mullen *et al*., 2009; Barker and Murrell, 2004)

Phylum: Arthropoda Latreille, 1829
Subphylum: Arachnomorpha Heider, 1913
Class: Arachnida Cuvier, 1812
Subclass: Acari Leach, 1817
Order: Ixodida Leach, 1815
- Family: Argasidae Koch, 1844
- Family: Ixodidae Dugès, 1834
- Family: Nuttalliellidae Schulze, 1934

Of the argasids, or soft ticks, four genera are recorded (number of species in each genus recorded in brackets):

Argas Latreille, 1795 [57],
Carios Latreille, 1796 [88],
Ornithodoros Koch, 1844 [37],

Otobius Banks, 1912 [3].

Twelve genera of ixodids, or hard ticks, are known (number of species in each genus recorded in brackets):

Amblyomma Koch, 1844 [142],
Anomalohimalaya Hoogstraal, Kaiser and Mitchell, 1970 [3],
Bothriocroton Klompen, Dobson and Barker, 2002 [5],
Cosmiomma Schulze, 1919 [1],
Cornupalpatum Poinar and Brown, 2003[1]
Dermacentor Koch, 1844 [36],
Haemaphysalis Koch, 1844 [166],
Ixodes Latreille, 1795 [249],
Margaropus Karsch, 1879 [3],
Nosomma Schulze, 1919 [1],
Rhipicentor Nuttall and Warburton, 1908 [2],
Rhipicephalus Koch, 1844 [79].

The family Nuttalliellidae has only one species, *Nuttalliella namaqua* Bedford, 1931.

2.4.3. South African genera infecting tortoises

(Compiled from Heyne pers. comm. and Horak *et al*., 2006a)

Five tick species have been recorded infesting tortoises of South Africa (Horak *et al*., 2006a).

Argasidae

Ornithodoros Koch, 1844 (syn. *Ornithodorus* Agassix, 1846)

Ornithodoros compactus Walton, 1962

Ixodidae

Amblyomma Koch, 1844

Amblyomma hebraeum Koch, 1844

Amblyomma marmoreum Koch, 1844

Amblyomma nuttalli Dönitz, 1909

Amblyomma sylvaticum (de Geer, 1778)

***Ornithodoros compactus* Walton, 1962 (see Figure 2.4 for tick anatomy) (Figures 2.5 a-b, 2.6)**

(Tampan)

Description: Both females and males are small, round and oval, grey-beige in colour with short legs and claws; both are moderately hairy with an integument covered with fairly flat mammillae each bearing one or two punctations; scutum and eyes completely absent. Hosts: *Chersina angulata*, *Homopus* species, *Psammobates* species and *Stigmochelys pardalis*. Distribution: Northern Cape Province. Transmittable diseases: May transmit *Borrelia* spp. or relapsing fever (Kettle, 1984).

***Amblyomma hebraeum* Koch, 1844 (very rare and only opportunistic therefore not illustrated)**

(South African bont tick)

Infestations are considered opportunistic and thus are rarely collected off tortoises; they can be collected from *Stigmochelys pardalis* (Heyne pers. comm.). Distribution: Coastal from Port Elizabeth in the Eastern Cape Province through KwaZulu-Natal Province; Gauteng and Mpumalanga; and north of Pretoria (Tswane) into Limpopo and North West Province. Transmittable diseases: *Ehrlichia ruminantium* (syn. *Cowdria ruminantium*), rickettsial heart water infection of wild and domestic ruminants (Peter *et al*., 1998; Peter *et al*., 2000).

***Amblyomma marmoreum* Koch, 1844 (Figures 2.5 c-d, 2.6)**

(South African tortoise tick)

Description: Female is large, ornamentation is dull yellow to beige with pink tinges, with three markings covering scutum; eyes are inconspicuous; scutum has fine punctations which enlarge from scapula to behind the eyes; festoons are defined by a deep marginal groove in unengorged specimens. Males also large, with several markings on conscutum and festoons; eyes inconspicuous, conscutum punctations are fine increasing in size towards festoons which are prominent. Hosts: *C. angulata*, *Homopus* species, *Kinixys belliana belliana*, *Psammobates* species and *S. pardalis*. Distribution: Most of the Western and Eastern Cape Provinces into the north-east of KwaZulu-Natal

Province, through most of the Free State Province into the east of Northern Cape Province and also in the north-east of Mpumalanga Province. Transmittable diseases: *Ehrlichia ruminantium* (Peter *et al*., 2000).

***Amblyomma nuttalli* Dönitz, 1909** **(Figures 2.5 e-f, 2.6)**

(Pan-African tortoise tick)

Description: Females are medium in size, ornamentation is bronze-green-yellow with an orange-pink tinge in three markings over scutum; eyes are yellow or brown; scutum covered in fine punctations which host a small centre tubercle; festoons defined by marginal groove. Males medium in size, ornamentation similar to female but with smaller markings covering almost the whole conscutum and festoons; eyes similar but inconspicuous, punctations similar to females, festoons are large and longer than broad. Hosts: *K. b. belliana* (preferred host). Distribution: north-eastern KwaZulu-Natal Province. Transmittable diseases: *Coxiella burnetii*, the rickettsial agent causing Q-fever in humans (Burridge *et al*., 2000; Burridge and Simmons, 2003).

***Amblyomma sylvaticum* (de Geer, 1778)** **(Figures 2.5 g-h, 2.6)**

(Cape tortoise tick)

Description: Female medium in size, ornamentation yellow on black with three markings, larger than *A. nutalli* but smaller than *A. marmoreum*; eyes are deeply orbited and hemispherical in shape; unevenly distributed punctations medium to large in size; cervical grooves have deep 'S' forms, marginal groove defining only outer most festoons. Males are small and oval, ornamentation consists of a narrow pale strip on marginal ridge extending to the first two festoons, eyes similar to females, punctuations are similar to females but become large and course over the lateral and posterior sections of the conscutum and festoons; festoons clearly defined but not by marginal groove. Hosts: *C. angulata*, *Homopus* species and *Psammobates* species. Distribution: most of the Cape including the Eastern and Western Cape Provinces through to the western Northern Cape Province. Transmittable diseases: none recorded (Burridge and Simmons, 2003)

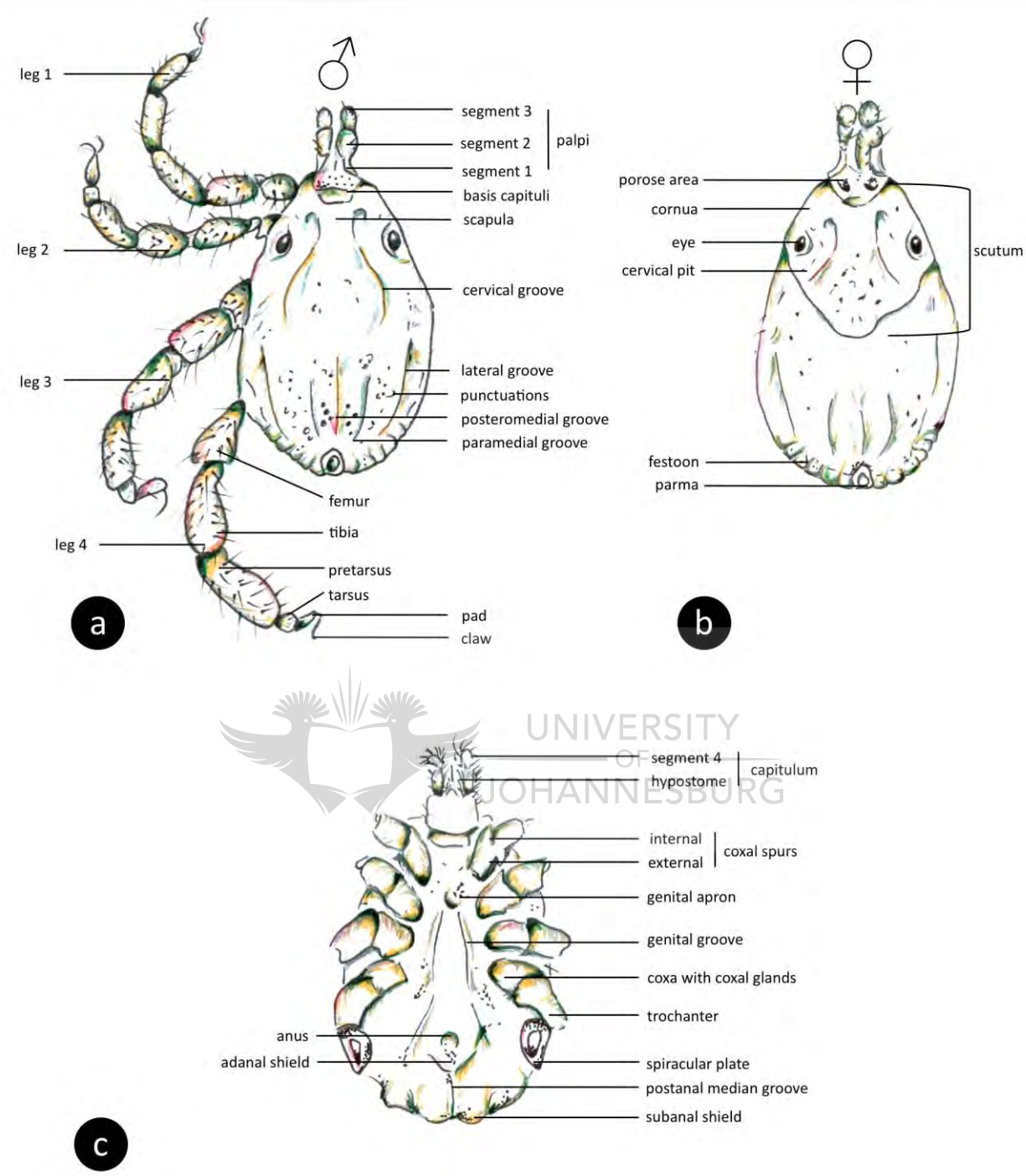


Figure 2.4. Ixodid tick external anatomy. **(a)** dorsal view of male, note absence of scutum as seen in **(b)** dorsal view of female. Dorsal anatomy is shared by both sexes except for the scutum. **(c)** ventral view for both sexes. Redrawn and adapted from Tick Identification Key: www.webpages.lincoln.ac.uk.

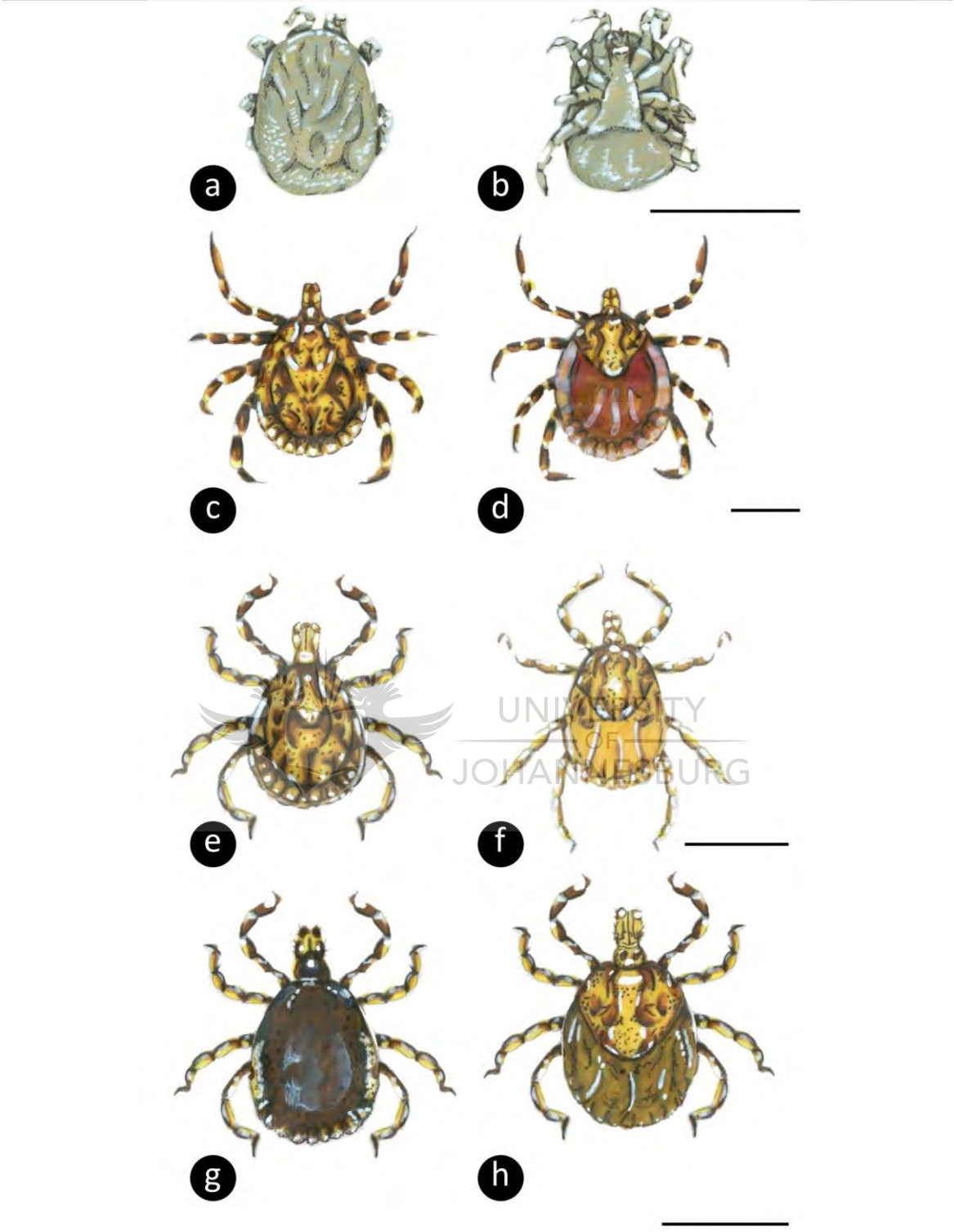


Figure 2.5. Southern African tortoise tick species, redrawn from Onderstepoort Tick Museum. **(a, b)** dorsal and ventral view of *Ornithodorus compactus* Walton, 1962, scutum and eyes completely absent, **(c)** male, **(d)** female *Amblyomma marmoreum* Koch, 1844, **(e)** male, **(f)** female *A. nuttalli* Dönitz, 1909, **(g)** male, **(h)** female *A. sylvaticum* (de Geer, 1778). Scale bars: 5mm.

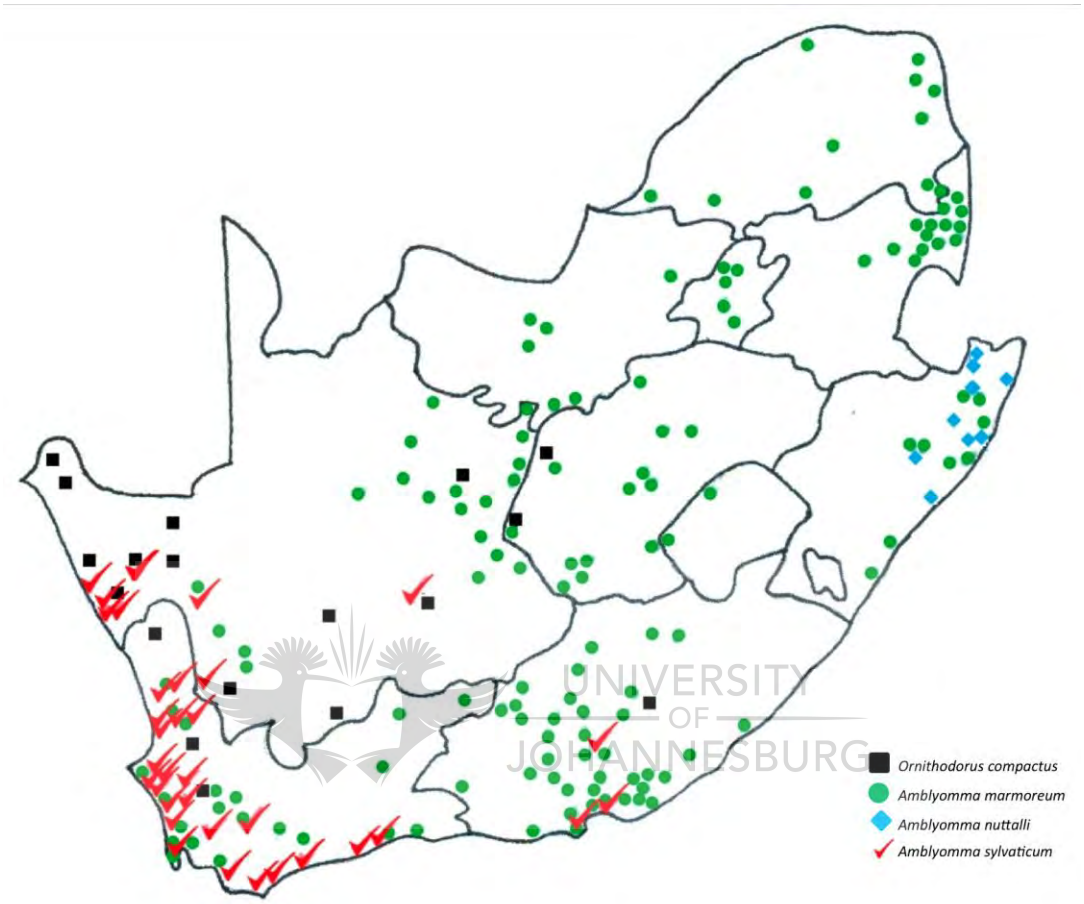


Figure 2.6. South African tortoise tick distribution. Redrawn from Horak *et al*., 2006a.

2.4.4. Tick evolution

Tick phylogeny and evolution remain debateable subjects, despite the application of molecular techniques to these arthropods, and represent continued challenges to acarologists (de la Fuente, 2003; Pagel van Zee *et al*. 2007). Tick fossils provide no further help with these issues, even though vast numbers of tick species have been found within Oligocene deposits, and within the exterior auditory canal of a frozen woolly rhinoceros, and within amber (de la Fuente, 2003). Two main hypotheses regarding tick origin and evolution have been proposed to date. Dobson and Barker (1999) suggested that ticks, especially hard ticks, evolved in Australia or Gondwana on early amphibians in the Devonian period, some 390myr ago. Conversely, Klompen *et al*. (1996, 2000) reviewed a number of theories, proposing, based on revision of the hypotheses of Hoogstraal and Aeschlimann (1982) and Hoogstraal (1985), that the first hard ticks existed much later in the evolutionary time scale, some 120myr.bp. The hypotheses were based on tick-host associations, tick evolution being driven by a broad co-speciation on specific hosts.

It was proposed by Hoogstraal (1985) (see de la Fuente, 2003) that ancestral tick species resembled the present day Argasidae (soft ticks) and that these early ticks evolved on smooth-skinned reptiles during the late Paleozoic-early Mesozoic some 250myr. bp. Ticks of the Prostriata were the first to evolve and then came those of the Metastriata, the Amblyomminae originating from reptiles of the Permian, the Haemaphsalinae on reptiles of the Triassic, and finally the Hyalomminae and Rhipicephalinae on mammals in the late Cretaceous and Tertiary. However, recent analysis of tick-host associations does not support the hypothesis that tick evolution arose through host adaptation, host specificity or co-speciation, but suggests that biogeography, ecological specificity and host size were the driving factors. Therefore, based on molecular and morphological characteristics, Klompen *et al*. (1996, 2000) suggested that ticks evolved from scavengers such as the closely related Holothyrida, placing a possible *Argas*-like ancestor in the late Cretaceous some 120myr.bp. Murrell *et al*. (2001) proposed a more in-depth hypothesis for the origin of ticks, which will not be dealt with any further here, but which supported the hypothesis that ticks did originate in the Cretaceous with most of the radiation occurring in the Tertiary.

Accompanying the controversy regarding tick origins, there is an argument regarding their first hosts. The oldest fossil tick found to date is approximately 90-94myr old, placing it during the Cretaceous. Three hosts have therefore been proposed by differing authors including reptiles by Hoogstraal, amphibians by Oliver, and birds by Stothard and Fuerst (see Barker and Murrell, 2002).

2.4.5. Ticks as vectors

The efficiency of ticks as vectors for disease transmission may be largely attributable to their long attachment times to their hosts, as well as their anti-haemostatic and immuno-modulatory mechanisms (Mans and Neitz, 2003; Kaufman, 2010). This is especially true for ixodid (hard) ticks, which may remain attached for four to 14 days to their hosts, compared to argasid (soft) ticks at under an hour. A longer period of intimate association such as that shown by female ixodid ticks increases the species vector proficiency (Mans and Neitz, 2003; Kaufman, 2010). Transmission of pathogens is commonly through the salivary secretions, but may also occur through coxal fluid during irrigation of the feeding-lesion (Kaufman, 2010). The mode of transmission may change however with species, or even at different stages of the tick life cycle. Bacterial diseases such as *Borrelia duttoni*, transmitted by the argasid, *Ornithodorus moubata*, have a higher chance of transmission through the salivary excretions of nymphs, but conversely through coxal fluid as adults (Kaufman, 2010).

Ticks seem easily utilisable vehicles for transmission of diseases caused by viruses, bacteria and protozoans. However, for such pathogens to survive successfully within ticks, barriers such as tick host immunity need to be overcome. It is also important that the tick itself survives long enough for transmission to occur. Ticks may present suitable reservoir hosts for pathogen long term survival as they can exist in a dormant state until the next blood meal presents itself, possibly as long as 16 years. Ticks, however, when 'questing' (seeking) a suitable host expose themselves to desiccation and predation. They unlikely drink free-standing water to rehydrate, but possess a relatively impermeable integument to reduce water loss, and absorb water directly from the atmosphere given the correct conditions (Kaufman, 2010). Furthermore, ticks only 'quest' during desirable periods when preferred hosts are likely to be most active; they also descend to a moister microhabitat when conditions become unfavourable. The ability to absorb water from the atmosphere is an adaptation shared by the larval and nymphal stages of the ticks; however, during apolysis (moulting) this adaptation is lost. Thus, larval and nymphal stages cannot remain on the intermediate host in moulting.

It is uncertain whether the infection, such as a protozoan infection, persists in an infected moulting larva or nymph until another suitable intermediate host is located. In the case of species of the genus *Babesia* and *Theileria* the infection may persist from infected larva into the next instar, and there may be transovarial transmission, developing eggs in the ovary of the adult female becoming infected (Roberts and Janovy, 2000). If the infection occurs in the juvenile gut epithelium there seems little reason to doubt that a protozoan infection could persist into the next instar. There are

barriers associated with invasion of the tick gut that present themselves to the potential pathogen. The vector's gut, and its luminal contents, such as acids and proteolytic enzymes, may be destructive; penetration of the gut epithelium may pose a physical barrier; the haemolymph may contain cells necessary for signalling of the innate immunity; and finally the chemical content of the saliva when first entering or leaving the intermediate host may pose a danger (Kaufman, 2010). Saliva content includes anti-clotting enzymes, vertebrate host enzyme inhibitors, host protein homologues (tick proteins that mimic those of the host), immunoglobulin-binding proteins and cement components to name a few (Steen *et al*., 2006). The saliva nevertheless may also facilitate the transmission of the potential pathogens (Kaufman, 2010).

Blood-feeding arthropods, such as ticks, have evolved mechanisms to modulate host defences such as the haemostatic and immune systems. The secretion of the salivary gland proteins of haematophagous organisms, collectively known as sialomes, during feeding is important in deactivating and bypassing host defence (Mans *et al*., 2008). Ticks are "pool-feeders" (Mans and Neitz, 2003; Kaufman, 2010) and therefore need to penetrate and damage enough blood vessels for the release of blood. Ixodids are slow feeders, gradually producing a haematoma over a week or more, compared to the argasids which are rapid feeders, choosing deep penetration for a feeding period of 30 to 60 minutes. In order to feed successfully, blood coagulation and platelet aggregation by the host haemostatic system and host immunity (both adaptive and innate) (see Beldomenico and Begon, 2009) need to be overcome. It is important from a protozoan perspective that ixodid feeding involves an initial phase of up to four days in which lymph is taken up before the main blood meal begins. This feeding process increases exposure of both tick and protozoan to host immune defences. This characteristic may be a reason as to why protozoans, such as *Hemolivia*, remain within the gut epithelium of the definitive host (Široký *et al*., 2007) and do not attempt transmission through the salivary glands as do *Plasmodium* species within fast-feeding mosquitoes (Roberts and Janovy, 2000). The pool-feeding and prolonged feeding times of the ixodid may be facilitative to the uptake of the protozoan, but may be costly to its transmission. For the protozoan, it would be more secure and less costly in terms of energy output to remain within a parasitophorous vacuole (PV), avoiding detection by both the definitive and intermediate host immunities (Beyer *et al*., 2002).

2.4.5.1. *Hyalomma aegyptium,* vector of *Hemolivia mauritanica* (Sergent and Sergent, 1904) Landau and Paperna, 1997 (Apicomplexa: Haemogregarinidae)

Hemolivia mauritanica, first described as *Haemogregarina mauritanica*, is a protozoan parasite belonging to the family Haemogregarinidae, with an indirect life cycle involving a definitive-host tick vector and a tortoise intermediate host (Michel, 1973; Landau and Paperna, 1997; Široký *et al*., 2007). Cyst formation (a form of merogony?) (see below) (asexual reproduction) occurs within the parenchymatous organs of tortoises, two Palaearctic species namely *Testudo graeca* Linnaeus, 1758 and *Testudo marginata* Schoepff, 1792. The infective merozoites of *H. mauritanica* enter erythrocytes and develop into gamonts which are transferred to the tick during feeding. Sexual reproduction leading to the formation of oocysts occurs within the tick, in this case *Hyalomma aegyptium*, and sporogony occurs within the intestinal cells, forming sporokinetes. However, even though the oocysts are irregular in shape, they cannot be described as star shaped as with other species of *Hemolivia* such as *Hemolivia mariae* and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1990 (Landau and Paperna, 1997). New gut cells are infected through release of sporokinetes developed within the oocysts, and these then develop into sporocysts containing sporozoites (see Fig. 2.7). Ingestion of the tick by a tortoise is considered to be the only pathway of transmission for this protozoan parasite. Once the tortoise is infected via sporozoites, authors have suggested that there are three pathways of maintaining an infection (Fig. 2.7); that is through cyclic merogony, cyclic cystogony, and merogony via cystozoites or cystogony via merozoites. Cysts, unlike meronts and merozoites which may only be found in the reticulo-endothelial system and erythrocytes, can be found within the parenchymatous organs as well. The cysts have been compared to merogony and have been suggested to be parallel form of merogonic development. It has been proposed by Široký *et al*. (2007) that these cysts may be responsible for the persistence of the infection. This is the only blood protozoan life cycle known for a land tortoise, the natural life cycle having been achieved under experimental conditions (Široký *et al*., 2004; Široký *et al*., 2007).

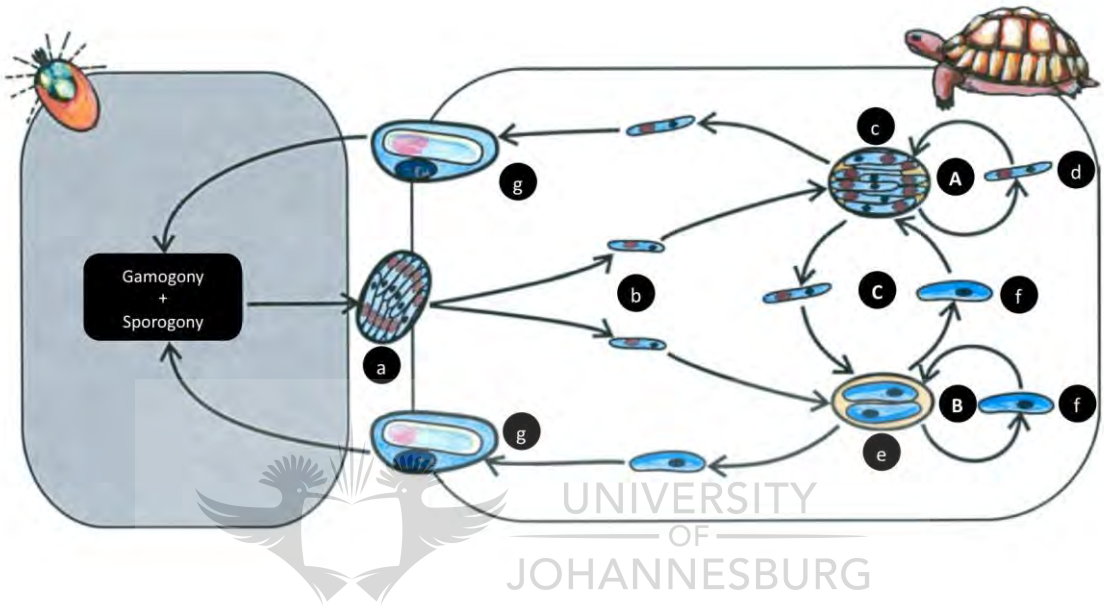


Figure 2.7. Endogenous development of the haemogregarine, *Hemolivia mauritanica*, in the Palearctic tortoise species, *Testudo marginata*. **(a)** sporocysts within the tick to be possibly ingested by the tortoise, **(b)** sporozoites which will enter the reticulo-endothelial system or erythrocytes, becoming either **(c)** meronts containing **(d)** merozoites or **(e)** cysts containing **(f)** cystozoites, **(e – f)** more often found within the parenchymatous organs of tortoises . Infected erythrocytes develop **(g)** gamonts. Authors suggested three pathways of long-term infection: **(A)** cyclic merogony, **(B)** cyclic cystogony, and **(C)** merogony by cystozoites or cystogony by merozoites. [Redrawn from Široký *et al*. (2007)].

2.5. CONSERVATION OF TORTOISES WITH USE OF BIO-INDICATORS, BIOLOGICAL TAGGING AND METAL-FINGERPRINTING

2.5.1. Bio-indicators

Environmental stressors and population densities affect vertebrate immunity. Animals may to a degree be able to 'foresee' certain adverse conditions, such as breeding stress, and compensate for such demands on their immunity. Anthropogenic stress and stress originating from unsustainable densities may not allow for such compensations, and immunity may suffer (Martin, 2009; Beldomenico and Begon, 2009). Parasites may reflect the immunological status of their hosts, decreasing or increasing in intensity and prevalence in concert with changes in host immunity. This may cause, according to Beldomenico and Begon (2009), a cycle leading to an even weaker immunity. Poor body condition with lowered immunity may predispose individuals to infection, further lowering the immunity of the individual and therefore body condition. In high density populations, in which resources are already low, immunity and body condition may translate from an individual level to a population level, leading to population decline. Population decline in endangered species such as the geometric tortoise *Psammobates geometricus* is highly detrimental to the species's continued existence. In this project, tortoise body condition is considered as a possible bio-indicator of environmental stress.

2.5.1.1. *Haemogregarina fitzsimonsi* as a bio-indicator

Cook *et al*. (2009a) found that the haemogregarine, *Haemogregarina fitzsimonsi*, was the most prevalent species of tortoise haematozoan recorded in South Africa, occurring in all the species of tortoise examined, both wild and captive. Haemogregarines and haemoproteids are considered in general non-pathogenic within reptilian hosts, with possibly only mild cases of anaemia occurring; they also reflect supposedly a well-adapted host-parasite relationship (Jacobson, 2007). Thus their effects on body condition might be minimal. However, before *H. fitzsimonsi* can be assessed as a bio-indicator of tortoise body condition and tortoise stress, the species life cycle needs to be elucidated. Life cycle knowledge is vital in determining transmission and therefore the dynamics of the infection.

As mentioned above, *H. fitzsimonsi* is the most prevalent of the South African tortoise haemogregarines (Cook *et al*., 2009a). Blood films examined from 154 tortoises, across five species, from four provinces revealed that ~31% of tortoises were infected with *H. fitzsimonsi* compared to

the other haemogregarine species, *H. parvula*, at 12% of two species of tortoise from a single province. *Haemogregarina fitzsimonsi* trophozoite, immature and mature gamont stages, and possibly dividing forms were seen in blood films. What appeared to be dividing stages, however, may have been the result of closely apposed paired immature gamonts (see Cook *et al*., 2009a). As mentioned previously (see Chapter 1), *Haemogregarina* and *Hemolivia* species may exhibit dividing stages within the peripheral blood, whilst *Hepatozoon* species generally do not. Since examining blood films from tortoises leads to inconclusive evidence about the true identity and development of *H. fitzsimonsi*, molecular analysis of this haemogregarine is necessary. In determining the correct genus to which this haemogregarine belongs, it will it be possible to begin to elucidate the life cycle of this parasite. This will point then to its mode of transmission.

Besides determining the life cycle, it is vital to begin to assess haemogregarine prevalence across as many more of the species of tortoise occurring within South Africa as possible. Five species of tortoise have been assessed (Cook *et al*., 2009a), leaving another eight to be examined. Even though all five tortoise species were found to be infected with *H. fitzsimonsi*, it does not mean this will be true of the remaining eight. Different feeding and habit traits in these other tortoise species may reduce the likelihood of parasitism, and they may not accommodate transmission and persistence of the parasite. However, until these hosts are assessed (as in this thesis), the usefulness of the bio-indicator proposal as an indicator of tortoise body condition and tortoise stress is unknown.

Tortoise body condition will be assessed using the method of Loehr *et al*. (2006). Loehr *et al*. (2006) assessed the relationship between the number of ticks present on the tortoise species, *Homopus signatus signatus*, and tortoise body condition. In this case, it was found that no correlation existed between the above two variables. It is hypothesized in this research that the result may be different. The current research, however, at this point, aims to assess only the relationship between three variables, parasite prevalence, parasitaemia and tortoise body condition. Tortoise body condition is expected to be lower in degraded environments or in times of physiological stress due to lack of nutrition (see Janin *et al*., 2011) and possibly decreased immunity (see Martin, 2009; Beldomenico and Begon, 2009). It is hypothesized that, if *H. fitzsimonsi* were present, the prevalence and parasitaemia of this parasite would be higher in situations such as the above compared to those situations in which tortoise body condition is good.

2.5.2. Biological tagging and metal-fingerprinting

As mentioned in Chapter 1, a large number of tortoises are collected as pets or 'rescued' from their natural environments by citizens (Branch, 2008); however, most of these animals end up in the care of zoological institutions and by law cannot be returned to the wild (Branch pers comm.; Wimberger *et al*., 2009). There has been no real progress in the development of procedures which may aid in the return of tortoises to the wild (Wimberger *et al*., 2009).

Over the last decade there has been increased interest in using parasites as biological tags, a method which may be used to identify a species site of origin or its population by producing a fingerprint. This may be achieved by using either endemic, species-specific parasites or endemic parasite-community structures (MacKenzie and Abaunza, 1998; MacKenzie *et al*., 2008; Mele *et al*., 2010). If apicomplexan blood parasites offer enough diversity between a range of tortoise species and populations, they may be used as biological tags. However, this in future may need to be used in conjunction with molecular techniques due to the difficulty in apicomplexan species identification (as above).

Multi-element analysis may also present a means of identifying the site of origin of species or populations by providing a metal fingerprint (Batista *et al*., 2008), another form of biological tagging. As mentioned in Chapter 1, contaminant accumulation has been documented in recent years in reptiles and the mechanisms of contaminant uptake discussed (Smith *et al*., 2007). Ingestion of contaminated food as well as the indirect consumption of contaminated soil (geophagy), may lead to an accumulation of these contaminants within the tissues of the organism (Gardner *et al*., 2006). The current project is focussed on determining the end result of metal accumulation within the tortoise tissue and if these results are compared to those found in correlating soil samples they may aid in the return of individuals to the wild. The procedure, as mentioned previously, would need to be as non-invasive as possible. Research done in the past, as mentioned in Chapter 1, utilised egg (Guirlet *et al*., 2008), blood and tail clips (Hopkins *et al*., 2001), which were too short lived for the interests of this study. The three longest-lived tissues within a tortoise specimen are bones, nails and scutes. Nail clippings may thus be used from live specimens and all three tissues, if available from dead specimens, can be used potentially for assessing trace element exposure. Nail is not only useful because of its longevity and isolation from other metabolic processes, but also because of its non-invasive collection and stability after collection, not requiring special storage conditions (Batista *et al*., 2008). Scute is also a keratin-based structure (Branch, 2008) and may prove to be of similar value

as nail. Bone was once a living tissue, but it may be difficult to predict its usefulness in the planned methods.

Conclusion

South Africa has a high biodiversity of terrestrial tortoises, 22% of the world's species, which require examination to determine if the same may be said about the diversity of apicomplexan haematozoans that infect them. Thus far, two haemogregarines, *Haemogregarina* (sensu lato) *fitzsimonsi* Dias, 1953 and *Haemogregarina* (sensu lato) *parvula* Dias, 1953, the taxonomy of which is uncertain, and two haemoproteids have been described from South African tortoises. One of these haemoproteids is new to science, *Haemoproteus natalensis* Cook, Smit and Davies, 2010, the description of which is detailed in this thesis, the other, *Haemoproteus testudinalis* (Laveran, 1905) was recently redescribed and synonymised. This diversity of apicomplexans has been described from only 5/14 of the species and subspecies of tortoises occurring in South Africa, which necessitated as many as possible of the remaining 9 species being examined during this study. Tortoise habitat, behaviour and ecology, a result of their evolution, may play an important role in these animals' competence as hosts for haematozoans. It may also provide insight into what appears to be a long-standing association between haematozoan parasites and chelonian hosts, resulting in a well-adapted host-parasite relationship.

Haematophagous ectoparasites such as ticks, common parasites of tortoises, may serve as vectors for the haematozoans, such as the tick *Hyalomma aegyptium* transmitting *Hemolivia mauritanica* (Sergent and Sergent, 1904) of the tortoise hosts *Testudo graeca* and *T. marginata.* Apicomplexan classification remains controversial, and hypotheses on apicomplexan evolution are debatable, these reaching down to genus level for many species such as terrestrial tortoise haematozoans. Tortoise haematozoans have been, besides the above *Hemolivia*, assigned to the genus *Haemogregarina* for lack of life cycle knowledge, and are supposedly transmitted by a leech vector. This leech-vector association is, however, highly doubtful in the case of South African land tortoises, with a tick-vector association hypothesized instead, especially for the most commonly recorded South African tortoise haemogregarine, *H. fitzsimonsi*. Clearly, life cycle elucidation is required for this haematozoan, in addition to molecular characterisation, to aid in determining the correct taxonomic placement of this haemogregarine. Knowledge of the life cycle of this parasite will determine its mode of transmission and how such an infection persists. Data such as this may be helpful when determining the effectiveness and therefore the possibility of *H. fitzsimonsi* as a tortoise stress bio-indicator.

Lastly, wild tortoises are often collected illegally as pets, which due to a number of issues such as disease transmission and genetic contamination of wild populations, does not allow nature conservationists to return them to the wild. As mentioned in the above chapter, biodiversity of tortoise haematozoans between populations and species may provide a method of biological tagging along with the proposed method of metal-fingerprinting for use by authorities as tortoise conservation tools, so that individuals can be properly matched to their sites of origin.

CHAPTER 3

MATERIALS AND METHODS

3.1. Collection sites

Terrestrial tortoises occur throughout South Africa (see Fig. 2.2). The country is divided into a number of provinces (Fig. 3.1) each with their own legislation regarding permit applications (see APPENDIX 2). Tortoises are protected by law and permit grants did not allow sacrifice of animals during the course of this study (see APPENDIX 2). Provinces and sites within them were chosen so that as many different tortoise species as possible could be sampled from high density populations. Where possible, nature reserves were selected (see Fig. 3.1) so that natural prevalence and intensity/parasitaemia of apicomplexan haematozoans could be assessed. Selection factors were differences in biome (see Fig. 2.1) and geographical structure of the sediments (Fig. 3.2), which would in turn present different metal or element compositions to the soils sampled. Along with wild collections, captive tortoise assessments were also done for comparison. The sites are considered below.

3.1.1. Wild tortoise collection sites

Sites are described according to the geology, soil (see Fig. 3.2) and vegetation (see Fig. 2.1, Chapter 2) which occur or dominate the region (taken from DEA (2012): www.environment.gov.za/), as well as the mention of any human impacts (largely based on personal observations).

KwaZulu-Natal (KZN)

Site 1: Mkuze (27°36'S, 32°9'E) (Figures 3.1 a, 3.3 a - b)

A mostly basalt belt, having black and red strongly structured soils. Vegetation of Mkuze comprises of arid lowveld, lowveld and a small area of Zululand thornveld. Human impact only includes traffic from patrons. Tortoise collection and examination occurred during spring (September) and summer (November) 2009.

Site 2: Pongola (27°22'S, 31°53'E) (Figure 3.1 b, 3.3 c)

An area abundant in granite, shale, tillite and quartzite, having red, weakly structured soils. Soils are usually shallow on hard rock with the presence of lime. Vegetation is similar to Mkuze with a small area of tall grassveld. Even though the site is a reserve, it is still impacted by human activities such as

large scale recreational fishing and camping. Tortoise collection and examination were conducted during the same period as that for Mkuze.

Northern Cape (NC)

Site 1: Britstown (30°37'S, 23°30'E) (Figure 3.1 c, 3.3 d - e)

Geology comprises dolerite and shale. Grey and red soils, excessively drained, shallow on hard or weathering rock. Lime present. Kalahari thornveld and shrub bushveld. Human impacts include residential areas and livestock farming. Tortoise collection and examination took place during summer (December) 2010.

Site 2: De Beers Diamond Route Conservancy (30°19'S, 17°17'E) (Figure 3.1 d, 3.3 f - g)

Geology comprises mostly sedimentary and schist rocks. Grey, excessively drained sands. Strandveld of west coast. Even though the area is undergoing rehabilitation, especially regarding the vegetation, residential areas are still present with traffic across the stretch of the conservancy. Tortoise collection and examination occurred during summer (November) 2010.

Site 3: Namaqualand Conservancy (30°0'S, 17°25'E) (Figure 3.1 e, 3.3 h - j)

Gneiss, diamictite, small presence of limestone. Shallow, red or yellow, well-drained sands with areas of rock. Succulent karoo, Namaqualand broken veld, and a small area of mountain-renosterbush veld. Human impacts include only infrequent traffic along sand roads. Tortoise collection and examination took place during the same period as that for De Beers Diamond Route Conservancy.

Site 4: Tswalu Kalahari Private Nature Reserve (27°17'S, 22°58'E) (Figure 3.1 f, 3.3 k - m)

Sand, iron and quartzite geology. Red, weakly structured soil with areas of rock. Vegetation is Kalahari thornveld and shrub bushveld. The reserve is under 20 years old, having previously been a cattle farm. Vegetation appears to have recovered since then, when the land was severely affected by overgrazing (Tswalu authorities, pers. comm.). Unlike Namaqualand, tortoises have access to standing water in the form of man-made watering holes. Tortoise collection and examination were conducted on two separate occasions, both during summer, in November 2009 and February 2010.

Western Cape (WC)

Site 1: Arniston (Waenhuiskrans) (34°33'S, 20°02'E) (Figure 3.1 g, 3.3 n – o, 3.4 a - b)

Limestone and sedimentary geology. Continental, shifting sands, with weakly developed soils and lime present. Coastal fynbos. Human impacts include residential areas and surrounding livestock farmland. Tortoise collection and examination took place on two separate occasions, both during summer (December) 2010 and 2011.

Site 2: De Hoop Nature Reserve (34°26'S, 20°32'E) (Figure 3.1 h, 3.4 c - d)

Limestone and sedimentary geology. Grey, excessively drained sands which are shallow, based on rock formation with lime present. Coastal fynbos. Human impact includes only traffic by patrons and the occasional controlled burning (De Hoop authorities, pers. comm.). Tortoise collection and examination occurred at the end of summer (March) 2010.

Site 3: De Mond Nature Reserve (34°43'S, 20°4'E) (Figure 3.1 i, 3.4 e)

Same as De Hoop Nature Reserve, except that it is situated on an estuary. Human impact includes only very limited recreational activities and fishing. Collection and examination of tortoises occurred during summer (December) 2010.

Site 4: Elandsberg Private Nature Reserve (33°28'S, 19°3'E) (Figure 3.1 j, 3.4 f - g)

Shale dominated geology. Reddish soils with clay accumulation. Coastal renoster-bushveld and fynbos. Human impacts include only controlled burning, traffic is negligible since the reserve is strictly controlled for re-introductory breeding trials of the extinct quagga and as one of three of the last sites for the highly endangered *Psammobates geometricus* (Elandsberg authorities, pers. comm.). Tortoise collection occurred during summer (January) 2011, however since *P. geometricus* is highly endangered and the reserve had recently recovered from a veld fire in which a number of tortoises were killed, only dead specimens could be collected. The likely stress on the remaining individuals meant it was not advisable to disturb them.

Site 5: Gouritzmond (34°20'S, 21°53'E) (Figure 3.1 k, 3.4 h)

Geology a mixture of conglomerate, granite and quartzite. Continental, shifting sands, with weakly developed soils and lime present. Coastal fynbos changing into valley bushveld towards the interior. The site is a residential area with traffic, surrounding areas including farmland. Only small 'pockets' of natural vegetation exist between houses and plots. Collection and examination of tortoises took place during summer (January) 2011.

Site 5: Paarl (33°45'S, 18°56'E) (Figure 3.1 l, 3.4 i)

Granite and lutaceous arenite geology. Clay accumulation, structured or weakly structured, reddish or yellow sands with areas of rock. Fynbos and coastal renosterbush veld. The area in which this site was located was outside of the town of Paarl, but was within farmland, surrounded by heavily trafficked roads. Tortoise collection and examination were undertaken on two separate occasions, both during summer, of December 2010 and January 2011.

Site 6: Paternoster (32°48'S, 17°53'E) (Figure 3.1 m, 3.4 j)

Limestone and sedimentary geology. Continental, shifting sands. Strandveld and coastal fynbos. Human impacts include the residential town with traffic, surrounded by farmland, but bordering the reserve areas of Cape Columbine and West Coast National Park. Collection and examination of tortoises took place during summer (October) 2010.

Site 7: West Coast Conservancy (33°7'S, 18°3'E) (Figure 3.1 n, 3.4 k - l)

Same as Paternoster for both geology and habitat description, as well as season and date of collections.

As can be seen from the above information, wild tortoises were sampled from several sites in each of three provinces (KZN, NC and WC) during spring (September), but mostly in summer (October – March), in the years 2009-2011. Summer, in particular, is the season when these animals are most active, while winter is the time when they tend to hibernate (see Branch, 2008).

3.1.2. Captive tortoise collection sites

Knowledge of the geology, soil composition and vegetation types of these sites were irrelevant as the origin of these captive tortoises was unknown.

Gauteng (GP)

Site 1: Johannesburg Zoological Gardens (26°10'S, 28°2'E) (Figure 3.1 o, 3.4 m)

The zoological gardens receive a number of tortoises of different species through confiscation of these animals from citizens by Nature Conservation, as well as through abandonment by citizens. Such tortoises usually include species such as *Stigmochelys pardalis* and *Kinixys lobatsiana*, which occur in the surrounding country areas. Examination occurred on three separate occasions, twice during winter (June) 2009 and once during summer (November) 2009. Examination was possible

during winter since tortoises were housed in a warm quarantine facility and thus were not hibernating.

Site 2: Private collections within the Johannesburg area (gps coordinates not given for privacy reasons) (Figure 3.1 p)

Collections of this nature belong to citizens, housing the animals within their private gardens. The tortoise species sampled was a single *Stigmochelys pardalis*. Examination took place in summer (March) 2011.

Western Cape (WC)

Site 1: Butterfly World (33°45'S, 18°56'E) (Figure 3.1 q, 3.4 n - o)

This site started as a butterfly park, however, in recent years it has also become a sanctuary for confiscated and abandoned reptiles. Tortoise species housed at the park include *Chersina angulata*, *Homopus areolatus*, *Psammobates tentorius trimeni* and *S. pardalis*. Examination of these tortoises occurred on two separate occasions, both during summer, in December 2010 and January 2011.

3.2. Collection and identification of tortoises

Tortoises were collected during times of the day most conducive to ectothermic activity, that is, late morning, after the reptiles had basked, as well as in the late afternoons, after the hottest period of the day had passed. Temperatures of a number of tortoises as well as their environment were recorded using a handheld infrared thermometer (MOTORTECH®, MT694) to aid in determining when these reptiles were most likely to be found foraging and would thus be easier to capture. On days when the weather was favourable, collection excursions were undertaken by vehicle and on foot. A number of tortoises were collected in and on the shoulders of roads. While examining captured individuals, more specimens were sought in the surrounding vegetation on foot using sticks to push back the undergrowth. Sticks were necessary to safe-guard against venomous snakes, which often find refuge under the same vegetation as the tortoises.

Captive specimens were collected within the enclosures in which they were kept, examination being possible throughout the day.

Tortoises were identified to species level using field guides (Branch, 1998, 2008; Boycott and Bourquin, 2000).

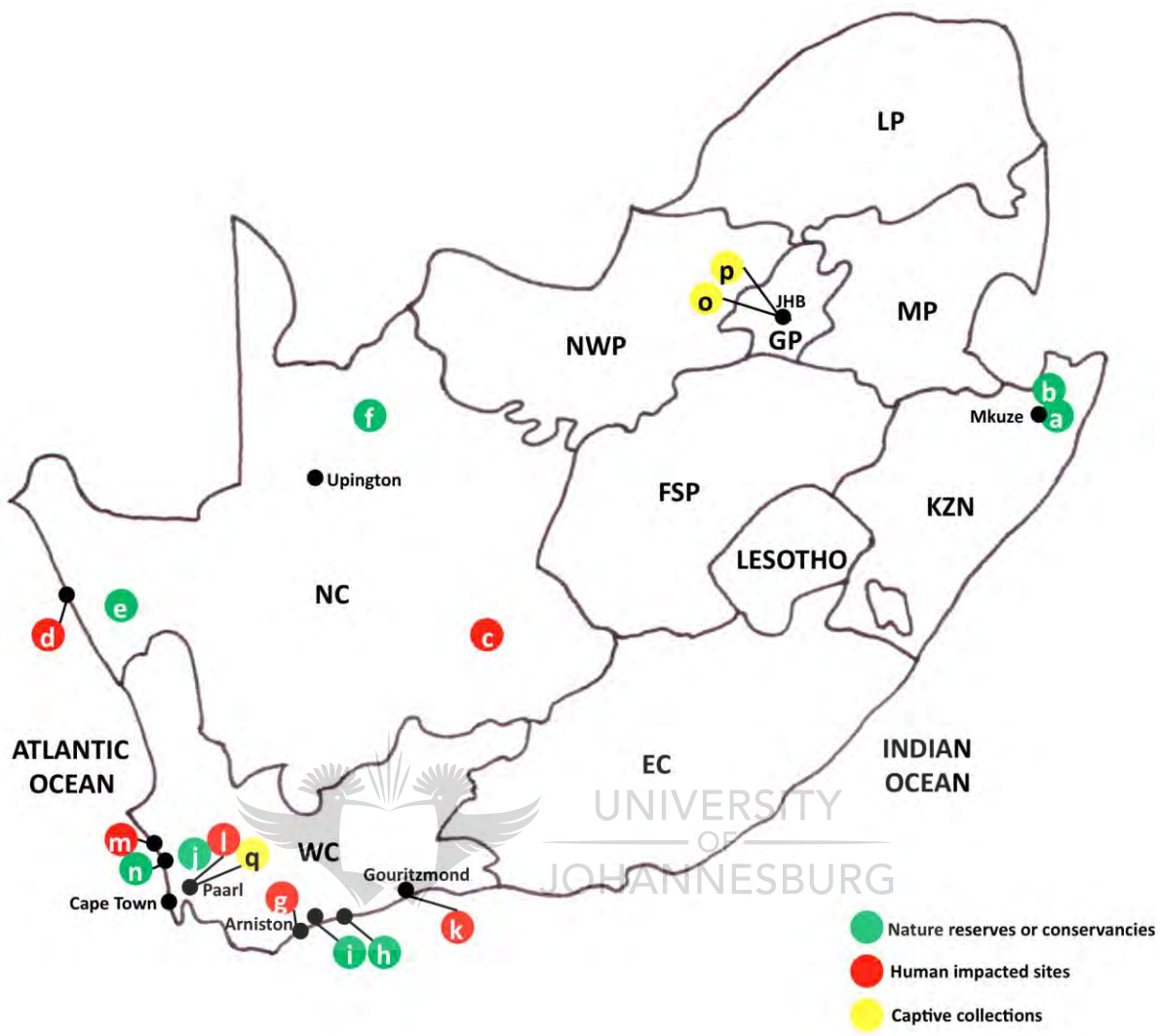


Figure 3.1. Collection sites of both captive and wild tortoises. Provinces are shown, top left to right, LP: Limpopo, NWP: North West, GP: Gauteng, MP: Mpumalanga, NC: Northern Cape, FSP: Free State, KZN: KwaZulu-Natal, WC: Western Cape, EC: Eastern Cape. Four of these provinces were sampled at: **(a)** Mkuze Nature Reserve, **(b)** Pongola Nature Reserve, **(c)** Britstown, **(d)** De Beer's Diamond Route Conservancy, **(e)** Namaqualand Conservancy, **(f)** Tswalu Kalahari Private Nature Reserve, **(g)** Arniston (Waenhuiskrans), **(h)** De Hoop Nature Reserve, **(i)** De Mond Nature Reserve, **(j)** Elandsberg Private Nature Reserve, **(k)** Gouritzmond, **(l)** Paarl, **(m)** Paternoster, **(n)** West Coast Conservancy, **(o)** Johannesburg Zoological Gardens, **(p)** Johannesburg private collections, **(q)** Butterfly World, Paarl. Redrawn and adapted from Cook *et al.* (2009).

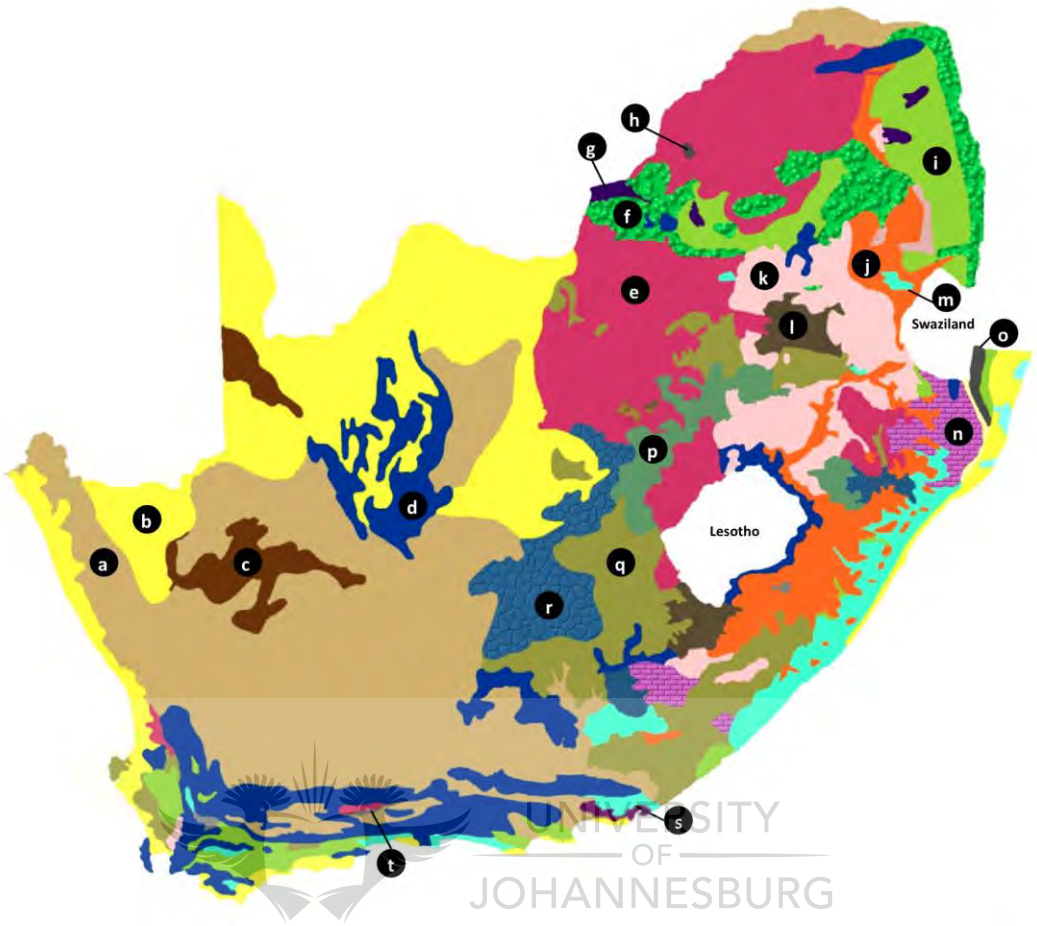


Figure 3.2. Soil map, redrawn and adapted from soil and irrigation research institute (SIRI), Department of Agricultural technical services (1973). **(a)** weakly developed soil on rock with lime, **(b)** sands, continental shifting, **(c)** weakly developed soils with flood basins, **(d)** rock and lithosols, **(e)** neutral sand, red dominant, **(f)** black and red clay, **(g)** red clay, much rocky land, **(h)** red clay, **(i)** weakly developed soils, lime common, **(j)** non-humic, red and yellow latosols, **(k)** acid, red-yellow-grey sands and clays, **(l)** black clays, **(m)** weakly developed soils, lime rare or absent, **(n)** black and red clays, **(o)** black clay with much rocky land, **(p)** black clays and solonetzic soils, **(q)** solonetzic soils, **(r)** red clays and solonetzic soils, **(s)** red porous soils and lithosols on limestone, **(t)** swamps and alluvial plains.

Figure 3.3. Data collection sites. **(a - c)** KZN, **(a - b)** Mkuze Nature Reserve-in camp, **(b)** resident *Stigmochelys pardalis*, **(c)** Pongola Nature Reserve, **(d - e)** Britstown, karoo, NC, **(e)** remains of *Psammobates t. trimeni*, **(f - g)** De Beer's Diamond Route, NC, **(g)** shoulder of road, measuring a live *Chersina angulata*, **(h - j)** Namaqualand Conservancy, NC, **(i - j)** characteristic seasonal vegetation, **(k - m)** Tswalu Kalahari Private Nature Reserve, NC, **(k - l)** watering hole which tortoises frequent, with one of its patrons, *S. pardalis*, respectively, **(m)** characteristic seasonal vegetation after rains, **(n - o)** farms surrounding Arniston (Waenhuiskrans), WC, showing characteristic vegetation.

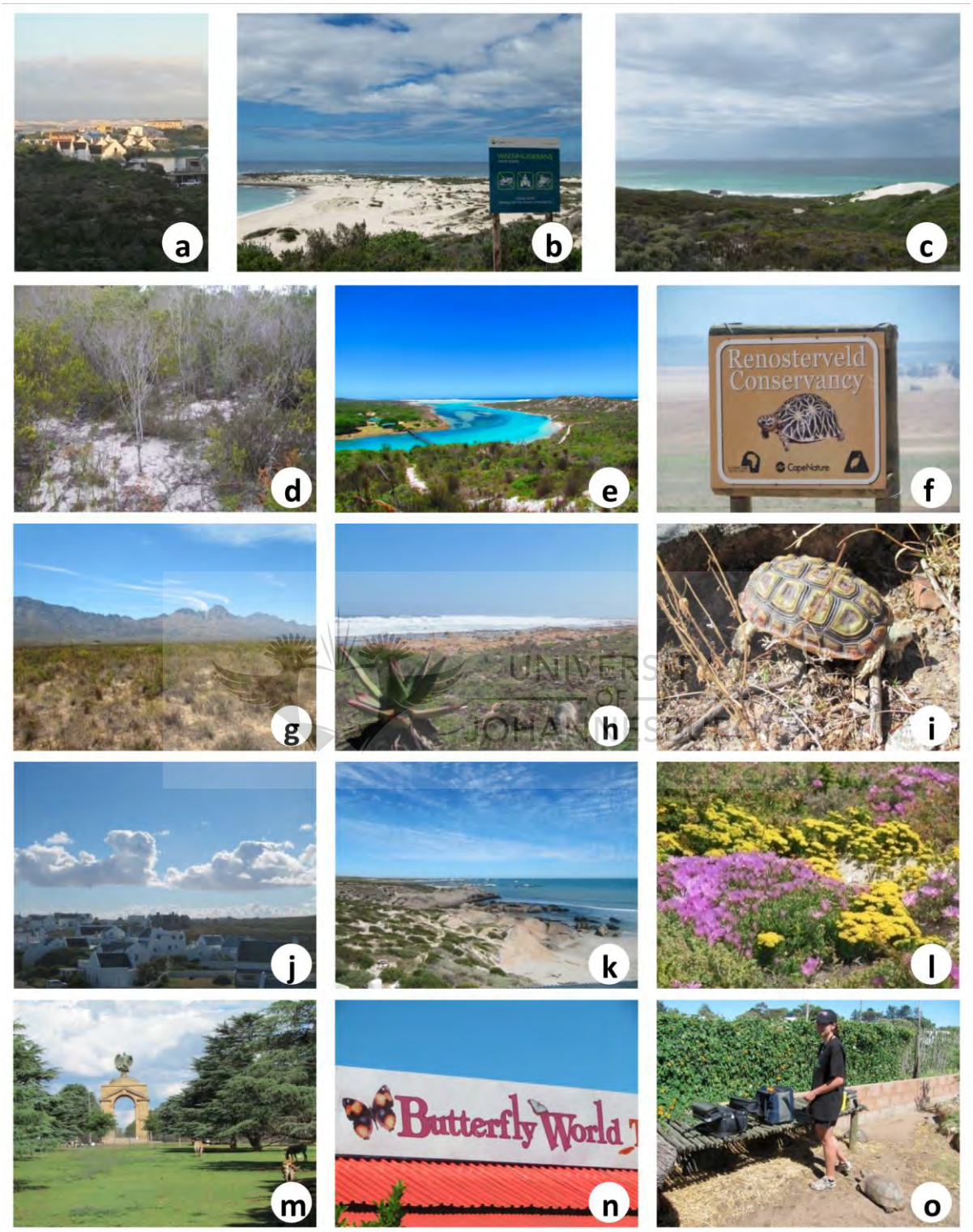


Figure 3.4. Data collection sites. **(a - b)** Arniston (Waenhuiskrans), WC, **(a)** village, **(b)** adjacent cape nature conservancy, **(c - d)** De Hoop Nature Reserve, WC, **(d)** showing coastal vegetation, **(e)** De Mond Nature Reserve, WC, **(f - g)** Elandsberg Private Nature Reserve, WC, with the largest population of endangered *Psammobates geometricus*, **(h)** Gouritzmond, WC, **(i)** Paarl, WC, small holding population of wild *Homopus areolatus*, **(j)** Paternoster, WC, **(k - l)** West Coast Conservancy, WC, **(l)** showing characteristic coastal seasonal vegetation, **(m)** Johannesburg Zoological Gardens, Johannesburg, GP, **(n - o)** Butterfly World, Paarl, WC.

3.3. Blood collection for apicomplexan haematozoans infecting tortoises

Blood, approximately 0.5 – 1ml, was collected from the sub-carapacial sinuses of tortoises (Fig. 3.5 a - b). A portion of the blood was used for thin blood smears and later molecular analysis, the remaining portion, ~0.2 - 0.5ml, for ultrastructural studies.

3.4. Examination of tortoise blood

3.4.1. Light microscopy

Slides were labelled with host/site details and the date, and then thin blood smears were prepared and air-dried. They were then fixed in the field in absolute methanol for 10 minutes and stained for 20 minutes in a modified solution of Giemsa's stain (Sigma). Slides were screened with an Olympus CX21FS1 field light microscope, and images were captured later in the laboratory with a Carl Zeiss Axiocam digital camera attached to a Zeiss Axioplan 2 photomicroscope with a 100x oil immersion objective. Measurements (µm) were taken using AxioVision Release 4.3 (11-2004) software, calibrated to a stage micrometer. Parasite prevalence among each tortoise type was estimated, and parasitaemias/intensities were calculated as infection levels per 100 red blood cells, with ~10^4 erythrocytes examined per blood film (Cook *et al*., 2009a, 2010a).

3.4.2. Transmission Electron Microscopy

Blood collected from tortoises during in field examination was placed in lithium heparinised blood collection tubes and placed on ice in an upright position. This was done to ensure separation of the plasma from the blood cells without the need for centrifugation. Once the excess plasma had been pipetted off, blood cells were fixed with 2% glutaraldehyde in Sorensen's phosphate buffer (Dykstra and Reuss, 2003) for later ultrastructural studies.

Fixed blood cells were transferred from the blood collection tubes to 75mm capillary tubes using disposable glass syringes and centrifuged for 6 min in a Heraeus-Christ micro-haematocrit centrifuge at 5000rpm. Pellets formed were removed and rinsed in 0.13M Millonig's buffer for 15 min and post-fixed in 1% (w/v) osmium tetroxide (O_sO_4) for 1 h. Pellets were dehydrated through a series of graded ethanols (50%, 70%, 80%, 90%, 96% and 2x 100%) for 15 min each. Pellets were then rinsed in 2x 100% propylene oxide for 15 min each and transferred to a 1:1 mixture of propylene oxide and

epoxy embedding resin (EMS products, Set Point Instruments, South Africa) and left overnight. A mixture of 1:3 propylene oxide and epoxy resin followed for 2 h, and thereafter the pellets were transferred to freshly prepared 100% resin and placed under vacuum for 2 h. Resin blocks were prepared using fresh 100% resin and plastic coffin moulds and then polymerised in a Reichert-Jung oven at 70^{o}C for 48 h.

Resin blocks were hand-trimmed using a Reichert-Jung ultramicrotome and Leica disposable microtome blades. Glass knives were prepared using a LKB-Bromma 7800 Knifemaker, with disposable plastic boats (EMS products, Set Point Instruments, South Africa) attached using hot dental wax. Semithin (0.5µm) sections were cut using glass knives on a Reichert-Jung ultramicrotome. Emerald-tinged sections (0.5 µm) were collected off 0.2µm Millipore-filtered dH_2O using an eyelash, floated on filtered dH_2O on a clean, labelled glass slide and dried on a Fried-electric hot plate at 30 - 40^{o}C. Sections were stained using 1% toluidine blue stain in 1% borax, subsequently rinsed using dH_2O, dried on the hot plate, rinsed in xylene, and mounted with Entellen (EMS products, Set Point Instruments, South Africa) and coverslipped, before being viewed under an Olympus light microscope. Ultrathin (90nm) sections of preferred resin blocks were prepared using a 45^{o} ultra DiATOME diamond knife, floated on Millipore-filtered dH_2O and collected on 75-mesh square copper grids (EMS products, Set Point Instruments, South Africa) previously cleaned in 20% acetic acid, rinsed in dH_2O and finally in 95% ethanol. Grids were placed on a non-static silicone grid mat (EMS products, Set Point Instruments, South Africa) for staining. Ultrathin sections were stained and rinsed on droplets using syringes fitted with 0.2µm DynaGard™ nonsterile syringe filters-firstly with Reynold's lead citrate for 30 min (in close proximity to NaOH to reduce precipitation with CO_2), rinsed 6x with dH_2O, thereafter stained with 1% aqueous uranyl acetate for 1 h, rinsed 6x with dH_2O, and finally stained in a second Reynold's lead citrate for 20 min and a final 6x rinse in dH_2O. Dry grids were stored in numbered grid storage boxes (EMS products, Set Point Instruments, South Africa). Sections were subsequently examined at 100 – 110 KV with a JEM – 1010 transmission electron microscope (JEOL) and digital images captured with a MegaView II side mounted digital camera (Olympus) with accompanying TEM imaging platform iTEM software.

3.4.3. Apicomplexan haematozoan morphological measurements of blood stages

Haemogregarines

Parasites were measured during light microscopy examination according to the methods used in Kim *et al*., (1998), Cook *et al*. (2009a) and Telford (2009), that is, the length and width of peripheral

blood stages were measured, and standard deviation was calculated. Further measurements included length and width of the nucleus, as well as the length from the anterior tip of the parasite to its mid-nucleus and the posterior pole to mid-nuclear length (Fig. 3.6 a - f).

Haemoproteids

Haemoproteids were measured during light microscopy examination according to the method used by Lainson and Naiff (1998) and Cook *et al*. (2010a). Length and width were recorded, in addition to the circumference and surface area of the parasite (Fig. 3.6 g - j).

3.5. Collection and identification of ectoparasites

During collection of tortoises, only tick infestations were observed and these were fairly common.

Ticks were collected from the softer areas of the neck and rear by extending the legs of the reptiles outwards, and in some cases off of the plastron (Fig. 3.5 c – d). Ticks were then removed using forceps, taking special care of the angle of removal so as not to leave the hypostome (see Fig. 2.4 c) in the tortoise. Ticks which were damaged during removal were immediately sacrificed for impression smears. Live ticks, on the other hand, were sorted during collection into larvae, nymphs, and male and female adults respectively (Fig. 3.5 e – h). Ticks were placed on ice to reduce activity, and to increase the longevity of the live specimens.

At a later stage, immediately after fixation, but before the 'live' colour was lost, ticks were identified to species level using Howell *et al*. (1978) and keys provided by Heyne (pers. comm.) from the Onderstepoort Museum.

3.6. Examination of ectoparasites

Ticks from infected tortoises were fixed in a solution of 10% neutral buffered formalin (SIGMA) at a refrigerated temperature of 4^{o}C for later histological studies. In addition, a number of dissected ticks were fixed in 70% molecular grade ethanol (SIGMA) for later genetic studies.

3.6.1. Tick tissue impressions

Adult fixed ticks were dissected according to the method of Edwards *et al*. (2009), while fixed larvae and nymphs were squashed whole. Impressions of the organs of adults were prepared, with special attention being observed not to perforate the gut (Fig. 3.7). Such preparations were made on clean microscope slides and left to air dry. Once dry, impressions were fixed in absolute methanol (MERCK) for 10 minutes and then stained for 20 minutes in a solution of Giemsa's modified stain (SIGMA) for detection of blood parasite stages. Screening was done using a field light microscope, an Olympus CX21FS1, and if sporocysts were found, further staining, up to an extra 10 minutes, was usually necessary for the sporozoites to become visible within each cyst. Images of the infective stages were captured using a Zeiss Axioplan 2 photomicroscope with attached camera, as for blood films.

3.6.2. Apicomplexan haematozoan morphological measurements from tick tissue stages

Sporocyst measurements

Sporocysts were measured following Telford *et al*. (2001). Length and width, and standard deviation, was measured for sporocysts. The numbers of sporozoites within each sporocyst was estimated by counting numbers of visible nuclei. When possible, free sporozoites were measured, that is, their length and width, as for haemogregarine gamonts in Fig. 3.6 a - f.

Figure 3.5. Ectoparasite and tortoise tissue collections. **(a)** temporary work station for examination of tortoises, **(b)** blood collection via venepuncture of the subcarapacial sinuses of the tortoise, **(c)** examination for tick infestation, ticks may be seen on the soft area between the neck and forelimb, **(d)** an adult male *Amblyomma sylvaticum* attached to the plastron, **(e)** tick larva, **(f)** tick nymph, **(g)** adult male *A. marmoreum*, **(h)** adult female *A. marmoreum*, **(i - j)** collection of fresh 'roadkill', **(k)** collection of old 'roadkill', **(l - p)** collection of naturally dead specimens ranging from fresh to only bones, **(p)** a large number could be collected sometimes in a single area.

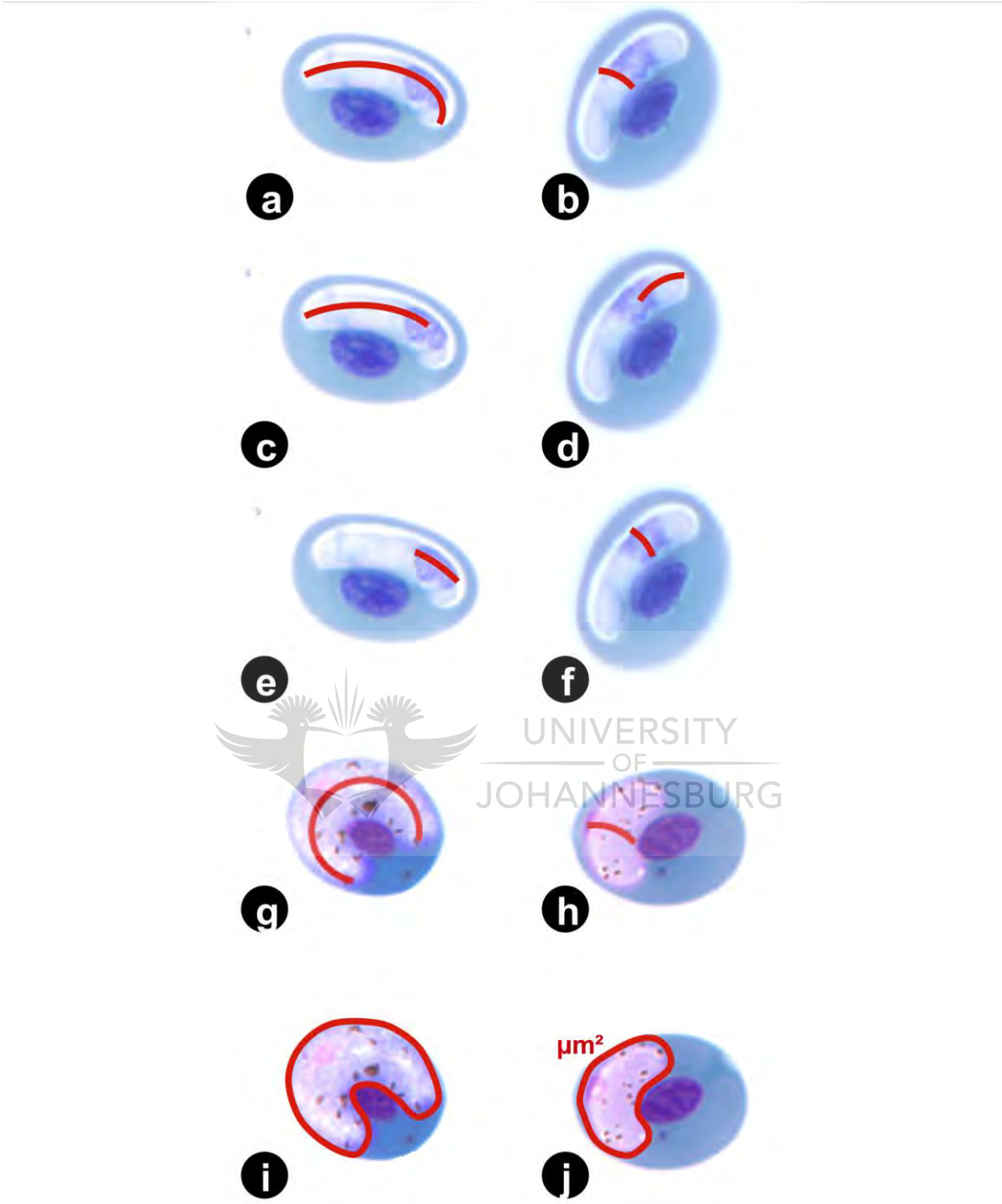


Figure 3.6. Haematozoan morphological measurements. **(a - f)** haemogregarine, *Haemogregarina fitzsimonsi* Dias, 1953, measurements. **(a)** total length, **(b)** width at widest point, **(c)** presumed anterior to mid-nucleus, **(d)** posterior to mid-nucleus, **(e)** nuclear length, **(f)** nuclear width, **(g - j)** haemoproteid, *Haemoproteus testudinalis* (Laveran, 1905), measurements. **(g)** total length, **(h)** total width, **(i)** circumference, **(j)** surface area. All measurements are in µm, except surface area in µm². Measurements according to method in Cook *et al.* (2009, 2010).

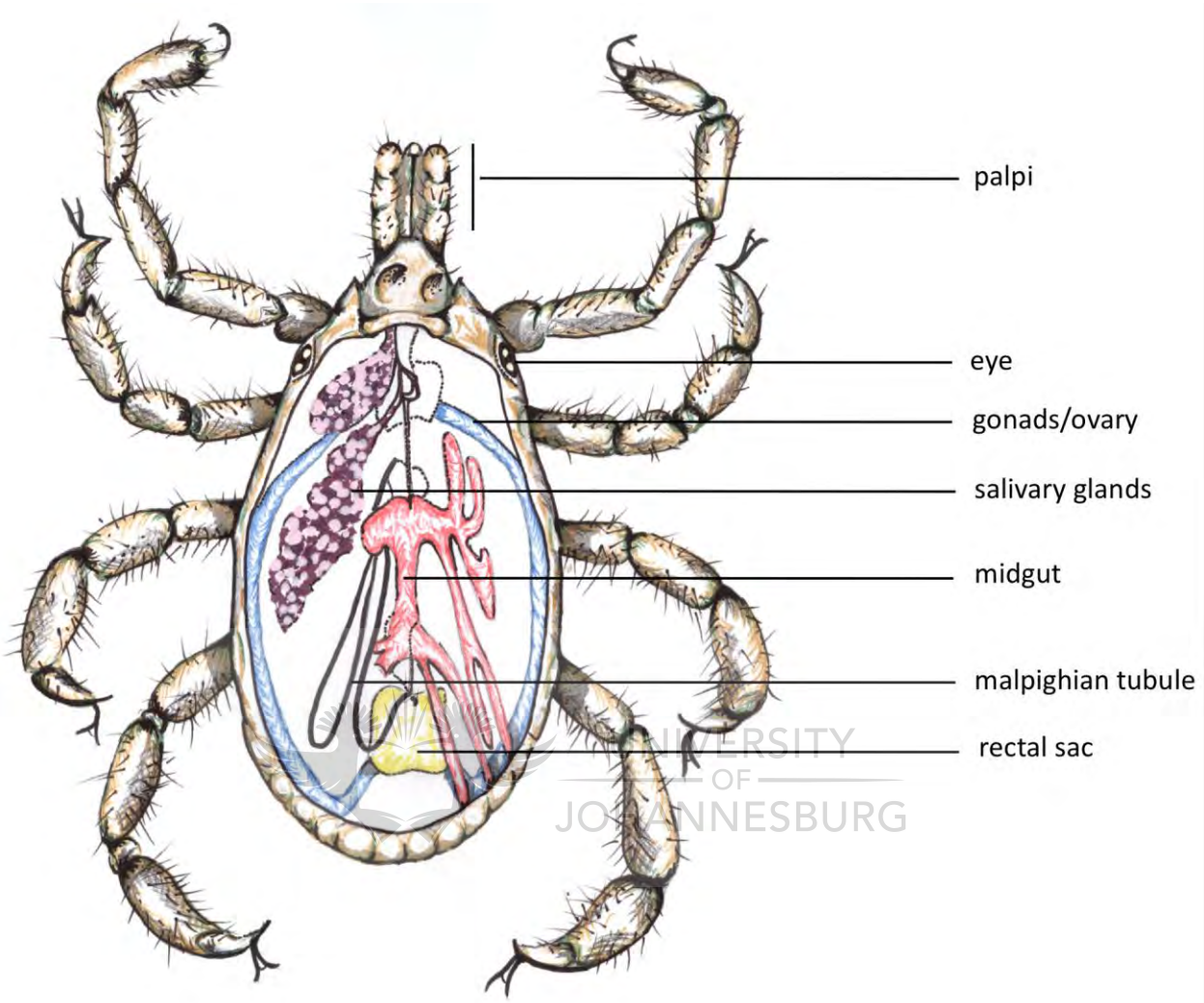


Figure 3.7. Internal anatomy of a tick. Redrawn and adapted from Edwards *et al*. (2009).

3.7. Molecular analysis of apicomplexan haematozoan tortoise blood stages and tick tissue stages

3.7.1. DNA extraction and PCR methods

3.7.1.1. Apicomplexan DNA from tortoise blood slides

To extract total DNA from tortoise blood slides, two methanol-fixed, Giemsa stained blood slides, taken from two specimens of *C. angulata*, from Arniston and De Mond respectively, with reasonably high *Haemogregarina fitzsimonsi* parasitaemias (3.3% and 0.8% respectively) were placed separately on two sterile pieces of foil. The two slides were then each scraped with separate sterile scalpel blades, and the scrapings collected and transferred to individual sterile 1.5ml eppendorf tubes (labelled Hf 1 and Hf 2). Samples were then processed using a DNeasy Animal Tissue Kit (using the spin column protocol) (QIAGEN Ltd., UK). This included adding 180µl ATL buffer and 20µl Proteinase K to each specimen and then vortexing the samples thoroughly for 30 seconds to initiate digestion. Samples were then incubated at 56°C for 2 hours in a GENLAB incubator for adequate digestion to occur. Once incubation was complete, samples were vortexed for a few seconds and 200µl of both AL buffer and absolute molecular grade ethanol were added to each, with mixing by vortexing for a few seconds between each addition. The mixtures were then pipetted separately into DNeasy mini spin columns and centrifuged in a VWR 1814 digital centrifuge at 8000rpm for 1 minute. The flow through was discarded and 500µl of AW1 buffer was added to each sample, and thereafter the samples were further centrifuged for another minute at 8000rpm. Once again the flow through was discarded and 500µl of AW2 buffer added to each sample. This time, the samples were centrifuged for 3 minutes at 13000rpm to dry the membrane of each spin column. The flow through was discarded and the membrane component of each spin column transferred to new, individual sterile 1.5ml eppendorf tubes. Directly onto each of the membranes, 100µl of ddH_2O were then pipetted and the samples centrifuged at 8000rpm for 1 minute. On this occasion, each flow through was retained, since it contained the total DNA extracted from the blood samples.

To amplify parasite DNA from the total DNA extracted, polymerase chain reaction (PCR) sequence runs were undertaken in an AB Applied Biosystems Veriti 96 well thermal cycler, in which three SIGMA primer sets were used. The primer sets are recorded in Table 3.1 and these included HEMO1 and HEMO2 (Perkins and Keller, 2001), HepF300 and HepR900 (Ujvari *et al*., 2004) and 4558F and 2773R (Mathew *et al*., 2000). The HEMO set was designed to amplify apicomplexan 18S ribosomal DNA (rDNA), specifically that of haemogregarines (Perkins and Keller, 2001), and the HEP (Ujvari *et*

al., 2004) and 4558F/2773R sets (Mathew *et al*., 2000) also 18S rDNA, but specifically for members of the genus *Hepatozoon*. 'Master mixes' were made of each of the primer sets by adding 2.5µl of the forward and reverse set (to make up 5 µl of each set) with the addition of 17µl of ddH_2O (making up 22 µl of 'master mix'). To each PCR reaction tube, complete with a PCR Taq bead, (PuReTaq™ PCR Ready-to-Go™,GE Healthcare UK, Ltd), 3µl of each total DNA sample was added along with 22µl of the 'master mix'. Control tubes lacked the DNA samples. PCR conditions are recorded in Table 3.1.

3.7.1.2. Apicomplexan DNA from tick tissue sporocyst slides

Using similar methods as the above, two methanol fixed, Giemsa stained impression slides of *Amblyomma sylvaticum* adult male ticks, collected from two *C. angulata* from De Mond, one with a medium sporocyst infection, the second with a low sporocyst infection, were scraped onto separate foil sheets. Scrapings were transferred to two separate sterile 1.5ml eppendorf tubes (labelled Sp1 and Sp2) and digested using the same DNeasy method as above. Total DNA was then extracted and, the same SIGMA primer sets employed for the blood slides were used to amplify apicomplexan DNA from the ticks. Again, controls lacked the DNA extracts. The PCR conditions, the same as above, are recorded in Table 3.1.

3.7.1.3. Host tick and apicomplexan DNA from tick tissue samples

Ticks of the species *A. sylvaticum*, collected from *C. angulata* from De Mond, fixed in 70% ethanol, were dissected using a Zeiss dissection microscope, separating legs and abdomens. For each leg sample taken there was a corresponding abdomen sample (labelled As L1, As B1; As L2, As B2; As L3, As B3; As L4, As B4). The abdominal contents of further *A. sylvaticum* ticks fixed in 70% ethanol (four males, 1 nymph and 1 larva, from De Mond, as well as two adults from De Mond from *C. angulata*) were also removed for processing. An additional tick sample (labelled Am), in the form of a non-fixed, non-stained impression of the species *Amblyomma marmoreum,* collected from a *S. pardalis* recently acquired by Paarl Butterfly World, was also examined as well as two adults of the same species (from Britstown from a *S. pardalis*). Total DNA from these tick samples was extracted using the DNneasy method described above for both the blood and sporocyst slides (above).

To amplify *A. sylvaticum* tick DNA, As L1, As L2 and AsB3 were combined with SIGMA primer sets for TICKITS2 (Zahler *et al*., 1995) and TICK16S (Black and Piesman, 1994) respectively and subjected to PCR, with control tubes lacking DNA extracts. In a similar manner to the As samples, total DNA extracts of the *A. marmoreum* (Am) tick were combined with the same primers for amplification of Am tick DNA by PCR. The ITS regions on the ribosomal gene show very little intraspecific variation,

but high interspecific variation, allowing them, especially ITS2, to be used to differentiate between closely related species (Barker, 1998; El-Kammah and El-Fiky, 2004; Chitimia *et al*., 2009). The 16S rDNA gene is large, ~1370bp (Black and Piesman, 1994; Kambhampati and Smith, 1995), and portions of this gene are highly conserved among populations, but are not as useful as the ITS regions for differentiating between species (Simon *et al*., 1994).

For PCR of each of the As leg and abdomen samples and the Am samples (above) 3µl of tick total DNA extract was added to a PCR reaction tube, complete with a PCR Taq bead (as detailed above), and 5µl of each of the primers was added (2.5µl F and 2.5µl R) along with 17µl ddH_2O. The tick abdomen samples comprised 3µl of total DNA extract and 22µl of the Hep 'master mix' (15µl of both primers of the primer set). PCR conditions are recorded in Table 3.2.

Finally, extracts from abdomens of the As and Am tick series were combined with the HepF300 and HepR900 (*Hepatozoon*) primers for attempted detection of haemogregarine DNA in the two tick species by PCR (as in Table 3.1).

Table 3.1: The three SIGMA primer sets, HEMO1 and HEMO2 (Perkins and Keller, 2001), HepF300 and HepR900 (Ujvari *et al*., 2004) and 4558F and 2773R (Mathew *et al*., 2000), their corresponding annealing temperatures and the PCR conditions used during apicomlexan DNA amplfication.

SIGMA Primer sets	Annealing temp. (°C)	PCR conditions
HEMO1 'TATTTGGTTTTAAGAACTAACTAATTTTATGATTG' HEMO2 'CTTCTCCTTCCTTTAAGTGATAAGGTTCAC'	52	Step 1: 5 min. at 95°C. Step 2: 4 min. at 95°C, with 1 min. at annealing temperature for 40 cycles. Step 3: 10 min. at 72°C.
HepF300 'GTTTCTGACCTATCAGCTTTCGACG' HepR900 'CAAATCTAAGAATTTCACCTCTGAC'	60	
4558F 'GCTAATACATGAGCAAAATCTCAA' 2773R 'CGGAATTAACCAGACAAAT'	55	

Table 3.2: The three SIGMA primer sets, TICKITS2 (Zahler *et al*., 1995), TICK16S (Black and Piesman, 1994) and HepF300 and HepR900 (Ujvari *et al*., 2004), used during tick DNA amplification, with the corresponding annealing temperatures and the PCR conditions.

SIGMA Primer sets	Annealing temp. (°C)	PCR conditions
TICKITS2	50	Step 1: 5 min. at 95°C. Step 2: 4 min. at 95°C, with 1 min. at annealing temperature for 40 cycles. Step 3: 10 min. at 72°C.
TICK16S	51	

3.7.2. Gel casting of extracted DNA products

Following PCR, and to determine whether PCR products of the correct molecular weight were present in the samples, sub-samples of the products were run on agarose gels. The gel caster was sealed well with masking tape on each pole before a 40% agarose gel was poured into it. Approximately 0.4g of molecular grade agarose (SIGMA) was weighed using a digital balance (Adventurer™ OHAUS) and added to 40ml of 1x TAE buffer (a 50x stock solution of TAE contains 242 g Tris base dissolved in ~ 750 mL deionized H_20, 57.1 ml glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0), adjusted to a final volume of 1 L). The solution was then microwaved in a domestic microwave for 2 minutes, swirling at 30 second intervals until agarose crystals were fully dissolved. The mixture was cooled under running tap water and 8µl of Gel Red (BIOTIUM) fluorescent dye was added before the gel was poured into the caster with an added comb (to create wells in the gel) and allowed to set for approximately 1 hour. Once set, masking tape and gel combs were removed from the caster, and the caster with the solid gel was placed within an electrophoresis tank and the tank filled with TAE buffer to the indicated level. For each PCR product, 5µl was added to 10µl of loading dye before being pipetted into the wells. Molecular hyperladders (BIOLINE) were added to one or two free wells per gel and then electrophoresis was initiated using a BIO-RAD POWER PAC 300. Samples were run on each gel at 70V for 30-45 minutes. Each gel was then viewed under a transilluminator (U-GENIUS) and examined for bands indicating products of the correct molecular weight. Images of gels were then captured using the U-Genius system.

3.7.3. Sequencing of PCR products containing amplified DNA

If discrete bands of the correct molecular weight were detected on gels, the remaining PCR products containing apicomplexan or tick DNA were sequenced at the DNA sequencing facility of the Natural History Museum, London, using the PCR primers with Fluoresent Dye Terminator Sequencing Kits (Applied Biosystems^{TM}) and sequencing reactions were run on an Applied Biosystems 3730XL automated sequencer.

3.7.4. Phylogenetic tree construction

Published sequences derived from apicomplexan and tick DNA were downloaded from the NCBI website *(*2012) and pasted into BioEdit Sequence Alignment (2012) along with sequences originating

from the current project's PCR products. Once sequences had been carefully edited and aligned using the MUSCLE sequence alignment tool (2012), they were processed using the phylogenetic tree constructing program MEGA 5.05 (2012*).* Two types of trees were constructed. A Maximum Likelihood (ML) analysis phylogram, used the Tamura 3-parameter + Gamma model of molecular evolution for *H. fitzsimonsi* and related species, and the GTR + G general time reversal model + Gamma distribution model of molecular evolution for ticks *A. marmoreum* and *A. sylvaticum* with related species. A second type of tree, a Neighbour Joining (NJ) analysis phylogram, used maximum composite likelihood for *H. fitzsimonsi* and related species, and the Jukes Canter model for ticks *A. marmoreum* and *A. sylvaticum* with related species. The out groups employed are recorded in Chapter 5, and nodal support was assessed using bootstraps calculated from 1000 replicates during tree construction.

3.8 Measurements of tortoises for use in bio-indicator assessment

All tortoises were weighed (g) and measured for calculation of the body condition index (BCI). Measurements included straight carapacial length (SCL), straight plastrol length (SPL) (not necessary for the calculation of body condition), greatest width (W), and greatest height (H). Such measurements were used in calculating tortoise volume = $\pi \times SCL \times SH \times SW/6000$, as mm^3. Body condition index (BCI) was calculated using the method of Loehr *et al.* (2006) by dividing the mass of the tortoise with its volume. An average BCI for each species of tortoise examined was determined, to indicate the lowest preferable BCI for each particular species (see Fig. 3.8). Allometric measurements were taken into account for differences in shell shape within species (as a result of age) and between species.

Tortoise BCI was then compared with *Haemogregarina fitzsimonsi* prevalence and peripheral blood stage parasitaemia (which was calculated during light microscopy of tortoise blood slides, see section 3.4.1). The significance of the relationships between these variables was then tested using the Student's T-Test ($P \leq 0.01$ = significant) and regression analyses ($R^2 \geq 0.05$ = significant).

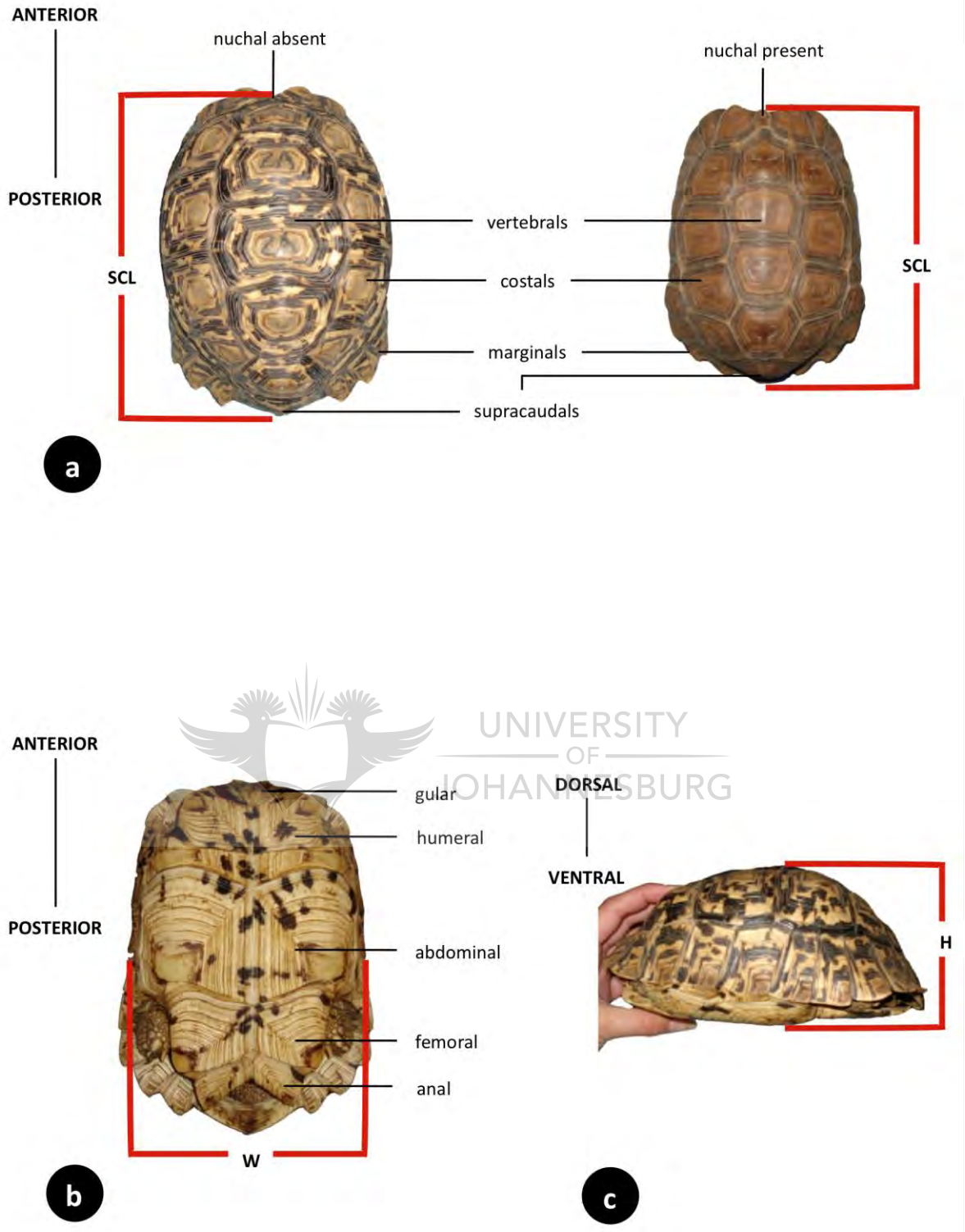


Figure 3.8. Diagramatic representation of tortoise body condition index (BCI) measurements. **(a, left to right)** carapace (top side), of *Sigmochelys pardalis* (Bell, 1828) and *Kinixys lobatsiana* (Power, 1927) , SCL: straight carapacial length from nuchal scute (if present) to end of supracaudal scute, **(b)** plastron (under side), of *S. pardalis*, W: straight width taken at widest region of body (does not include flared marginals), **(c)** lateral view, showing shell height at highest point. Photographs taken and adapted from Cook (2008).

3.9. Collection of geological and tortoise tissue samples for metal-fingerprint analysis

Geological material (soil samples), from the sites of origin of tortoises, and tissue samples from the same wild tortoises (that is, from both reserve and human impacted sites as listed previously in section 3.1.1), were collected (Fig. 3.1). No soil and tissue samples were collected from captive tortoise sites (Fig. 3.1) since origin of tortoises is unknown. GPS co-ordinates were taken at sites of collection of wild tortoises (see above, section 3.1.1). Sampling for this particular section focussed on the provinces (Fig. 3.1) and geology (Fig. 3.2) of the collection sites as well as on the tortoise tissue samples. Since provinces are large and the geology of each is different (see Figs. 3.1 and 3.2), it was deduced that soil metal composition would be comparatively different for the different sites. Knowledge of human impact and overall biome information was not essential for metal-fingerprinting and is therefore omitted from the following section.

3.9.1. Collection of soil

The top 5cm of soil or sediment was collected using acid-washed 15ml falcon tubes and subsequently stored in these tubes in a cool, dry environment until processing for ICP-MS and ICP-OES analysis.

3.9.2. Collection of tortoise tissue

Tissue samples included tortoise nail clippings (0.001 – 0.05g), taken using a standard pet nail-clipper, from live and deceased specimens. Scute (0.05 – 1g) and bone (0.05 – 1g) samples were taken only from deceased specimens collected either from 'road-kill' or natural death within the surrounding wilderness (Fig. 3.5i – p). Tissue specimens were stored dry in 1.5ml plastic eppendorfs.

3.9.3. Soil digestion and element analysis

Soil samples were weighed (*ca.* 0.1 – 0.5g) using a digital balance (Vicon top balance, BT200). Samples were transferred to the clean and dry teflon bombs for later digestion in a Milestone Ethos microwave digester. To the bombs was added 3ml 65% nitric acid (Fluka analytical standard) and 9ml 30% hydrochloric acid (Merck analytical standard). Microwave digestion followed a modified

protocol of the Soil Total Digestion method (Environmental Application Note DG-EN-14, http://milestonesci.com/).

Table 3.3: Microwave digestion protocol for total soil digestion (Environmental Application Note DG-EN-14, http://milestonesci.com/)

Procedure	Step	Power (W)	Time (min)	Temperature (^{o}C)
Milestone Ethos Plus Labstation (Soil)	1	1000	20	200
	2	1000	15	200
	3	1000	20	cooling

Following digestion, samples were diluted with 15 ml 1% nitric acid (MERCK Suprapure) and transferred to Erlenmeyer flasks and further diluted to 50ml using ultrapure water from a Milli-Q filtration system. Samples were subsequently filtered (0.45µm) into 15ml analysis tubes and stored in the refrigerator until ICP-MS (for trace and ultra-trace element, <1mg/l and <1µg/l respectively) and OES (for trace and macro-element, <1mg/l and <100mg/l) analysis. The ICP readings required that calibration standards for the different tissue types be prepared and the matrix matched as far as possible, which included single-element stock solutions per element (MERCK, Germany) added to the microwave-digestion mixture standards for the different tissue types. Elements included in single-element calibration standards for analysis were: ^{52}Cr, ^{55}Mn, ^{59}Co, ^{60}Ni, ^{65}Cu, ^{66}Zn, ^{75}As, ^{82}Se, ^{107}Ag, ^{111}Cd, ^{206}Pb, ^{207}Pb, ^{208}Pb, ^{238}U trace-elements for MS; and Ag, Al, Cd, Co, Cr, Cu, Fe, Mn, Ni, Pb, U, Zn, Ca, K, Mg, Na, Si macro-elements for OES. A separate single-element internal standard stock solution was also prepared and diluted for use in all analyses. Internal standard elements were ^{115}In ^{9}Be, ^{89}Y, ^{159}Tb and ^{209}Bi. For the analysis of all dilution and microwave-digested samples an internal standard was added and filled to volume in volumetric flasks and necessary blanks of tissue types incorporated. All trace element analyses were done using a Thermo Electron X-Series 2 Inductively Coupled Plasma Mass Spectrometer (ICP-MS) equipped with a Cetac auto sampler. The ICP-MS was tuned before each analysis for maximum indium (^{115}In) ion counts per second, maximum signal stability, and as low as possible (preferably 0) background and oxide levels (between 0.03 – 0.04). Improved auto tuning sequences were obtained from Thermo Fischer Scientific to advance signal stability. The rinse time between sample readings was increased to 100 seconds to decrease the possibility of contamination between readings. All macro-element analyses were done using a Spectro ARCOS Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES) equipped with

a Cetac ASX-520 auto sampler. Calibration standards were prepared similarly to those used for analyses with the ICP-MS, but no internal standards were used for analyses with the ICP-OES.

3.9.4. Tortoise tissue digestion and element analysis

3.9.4.1. Nail and scute

Nail and scute samples were weighed (*ca.* 0.001 – 0.09g). Samples were transferred to 45ml plastic tubes and rinsed briefly with acetone to remove debris adhering to the surface of the samples. Digestion followed a modified method of Batista *et al*. (2009), 1ml of 25 wt. % TMAH (tetramethylammonium hydroxide solution, Sigma) was added and vortexed for a few seconds. Samples were left to digest for 1 week at room temperature after which 9ml of 1% nitric acid (MERCK Suprapure) was added and samples vortexed for a few seconds. Once again, samples were left to digest for a further 3 days and there after diluted to 15ml with 1% analytical grade nitric acid (MERCK Suprapure) and filtered using ordinary filter paper. Samples were refrigerated until ICP analysis could be done.

3.9.4.2. Bone

Bone samples were ground into a fine powder using a marble mortar and pestle, which was cleaned between each sample by rinsing under tap water and then wiping with 1% nitric acid (MERCK Suprapure). Each sample was weighed (*ca.* 0.1 – 0.26g) and transferred to clean and dry teflon bombs for later digestion in a Milestone Ethos microwave digester. To the bombs was added 3ml of 65% nitric acid (Fluka analytical standard), 2ml 30% hydrogen peroxide (MERCK Suprapure) and 3ml Milli-Q water (following a modified method from Ashoka *et al*., 2009). The bomb was sealed and placed into the digester and run on the following procedure.

Table 3.4: Microwave digestion protocol for bone digestion (modified from the method of Ashoka *et al*., 2009).

Procedure	Step	Power (W)	Time (min)	Temperature (^{o}C)
Milestone Ethos Plus Labstation (Bone)	1	1000	5	180
	2	1000	10	180
	3	1000	20	cooling

Thereafter, samples were removed from the digester, allowed to cool and then transferred to plastic 50ml Erlenmeyer flasks. Samples were then further diluted to 50ml with 1% nitric acid (MERCK Suprapure) and subsequently filtered before being analysed with the ICP-MS and ICP-OES.

3.10 Statistical analyses

As mentioned above in section 3.8., significance of the relationship between body condition index (BCI) and parasitaemia (%) of *H. fitzsimonsi* within tortoise peripheral blood samples was tested using the Student's T-Test (where $P \leq 0.01$ = significant) and a linear regression analysis (where $R^2 \geq 0.05$ = significant). The above was done along with the construction of XY and grouped graphs using the statistical analysis computer package GraphPad Prism® 5.

Differences of metal concentrations (µg/g dry weight) between sites per different tissue were analysed and tested for significance using the statistical analysis computer package SPSS version 17. Statistics used included the calculation of means, maximum values, minimum values, standard deviations, percentiles and significant differences ($P < 0.05$). Differences of metal concentrations (µg/g dry weight) between the tortoise tissues (nail, scute and bone) of the same species at different sites and differences of metal concentrations between sites for a single tissue per species was analysed and tested using a principal component analysis (PCA) by use of the statistical program PRIMER 6.

CHAPTER 4

OBSERVATIONS ON SOUTH AFRICAN TORTOISE BLOOD PROTOZOAN BIODIVERSITY

Chapter 4 details the species of wild and captive tortoises examined for apicomplexan haematozoans from four South African provinces and 17 sites within these, including some of un-impacted (reserve) status and others of anthropogenically impacted standing. It provides information on the tortoise species infected with haematozoan parasites, imparting prevalence and parasitaemia data on three species of haemogregarine, *Haemogregarina fitzsimonsi* Dias, 1953, *Haemogregarina parvula* Dias, 1953, and a previously undescribed intraleucocytic haemogregarine, *Haemogregarina* sp. A. It also provides information on two species of haemoproteid, *Haemoproteus testudinalis* (Laveran, 1905), and *Haemoproteus* sp. A (described in published work resulting from research recorded in Chapter 7, as *Haemoproteus natalensis* Cook, Smit and Davies, 2010). The tick species found infesting the South African tortoises are listed, including information on the life stages collected (larvae, nymphs and adults) and locality of collection (as listed and described in section 3.1.1.). Finally a discussion of the results and their implications is provided.

4.1. General observations on tortoises, their ectoparasites and haematozoans

(Refer to Appendix 1)

In total, 367 tortoises were collected, both wild (287) and captive (80), 270 live and 97 dead, from four provinces, including Gauteng (GP), KwaZulu-Natal (KZN), the Northern (NC) and the Western Cape (WC). Live tortoises were of 10 species: *Chersina angulata* (Schweigger, 1812); *Homopus areolatus* (Thunberg, 1787); *Kinixys belliana belliana* Gray, 1830; *Kinixys lobatsiana* (Power, 1927); *Kinixys natalensis* Hewitt, 1935; *Psammobates geometricus* (Linnaeus, 1758); *Psammobates oculiferus* (Kuhl, 1820); *Psammobates tentorius trimeni* (Boulenger, 1886); *Stigmochelys pardalis* (Bell, 1828); and *Testudo graeca* Linnaeus, 1758, a species alien to South Africa. However, as recorded in the previous chapter, it was impossible to collect blood and other material from the live *P. geometricus* (three individuals), as this species is considered highly endangered, and its handling during the dry season (a period of aestivation) would have greatly reduced its chance of survival. Therefore, the number of live, endemic, South African tortoise species examined for haematozoans during the study was eight, with one alien tortoise species also investigated. Dead specimens examined included specimens of *C. angulata*, *H. areolatus*, *P. geometricus*, *P. oculiferus*, *Psammobates tentorius tentorius* (Bell, 1828), *P. t. trimeni* and *S. pardalis*. Deceased specimens will not be discussed further in this chapter, but in Chapter 8.

Of the eight endemic tortoise species, the highest number collected and examined were 5 from the WC (63%), including *C. angulata* (22 captive, 58 wild), *H. areolatus* (6 captive, 20 wild), *K. lobatsiana* (1 captive), *P. t. trimeni* (1 captive) and *S. pardalis* (31 captive, 2 wild). The NC had the second highest species count with 4 of the 8 species, including *C. angulata* (19 wild), *P. oculiferus* (10 wild), *P. t. trimeni* (2 wild) and *S. pardalis* (61 wild). Thirdly came KZN with 3/8 (38%) species, including *K. b. belliana* (3 wild), *K. natalensis* (1 captive, 1 wild) and *S. pardalis* (16 wild). Gauteng Province only had 2 of the 8 (25%) tortoise species, including *K. lobatsiana* (6 captive) and *S. pardalis* (11 captive) (Appendix 1 a, b).

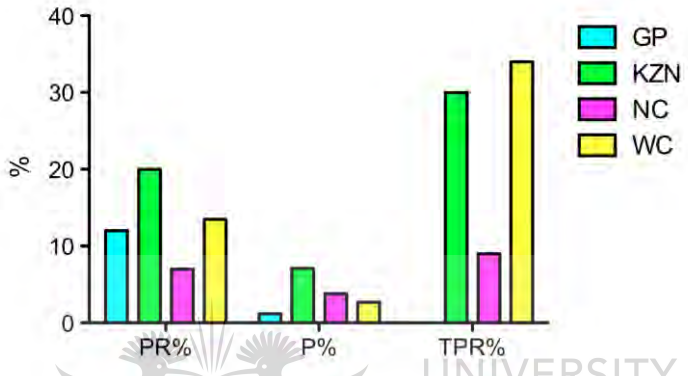


Figure 4.1. Haematozoan prevalence (PR%) and mean parasitaemia (P%) as well as the tick prevalence (TPR%) of the four provinces in which tortoises were collected and examined.

Tortoises in KwaZulu-Natal (KZN) had the highest haematozoan prevalence (20%) and parasitaemia (~9.1%), and the second highest tick prevalence (30%) of all four provinces (Fig. 4.1). The WC tortoises had the second highest haematozoan prevalence (13.5%), with the highest tick prevalence (34%), but had the second lowest parasitaemia (~2.7%). Gauteng Province and NC tortoises had the lowest haematozoan (12% and 7% respectively) and tick (0% and 9% respectively) prevalence with parasitaemias of 1.2% and 3.8% respectively.

Of the live tortoises (wild and captive) (Appendix 1 a, b), 40/270 (14.8%) had haemogregarines. These included 36/270 (13.3%) with *H. fitzsimonsi*, 2/270 (0.7%) with *Haemogregarina parvula*, and 2/270 (0.7%) with *Haemogregarina* sp. A. A further 3/270 (1.1%) of the live tortoises had haemoproteids. These were 2/270 (0.7%) with *Haemoproteus testudinalis* and 1/270 (0.4%) with *Haemoproteus* sp. A.

Haemogregarina fitzsimonsi was recorded infecting 23/97 (23.7%) *C. angulata* (all wild), with parasitaemias of ~7.1%, 2/3 (66.7%) *K. b. belliana* (all wild) with parasitaemias of ~7.7% and 12/121 (9.9%) *S. pardalis* (5 captive, 7 wild) with parasitaemias of ~6.5%. Provincial prevalence and parasitaemias for *H. fitzsimonsi* were: from GP, 2/11 (18%) *S. pardalis* (all captive), parasitaemia ~0.6%; KZN, 2/3 (67%) *K. b. belliana* (all wild) and 6/16 (38%) *S. pardalis* (all wild), parasitaemias ~10.6%; the NC, 6/19 (32%) *C. angulata* (all wild), parasitaemias ~3.8%; and the WC, 16/78 (21%) *C. angulata* (all wild), 4/32 (13%) and *S. pardalis* (all captive), parasitaemias ~2.7% (Appendix 1 a, b).

Haemogregarina parvula was found infecting 2/3 (67%) *K. b. belliana* (all wild) from only KZN with parasitaemias ~10.8%. Similarly, *Haemogregarina* sp. A. was also only found in KZN infecting 2/121 (1.7%) *S. pardalis* (both wild) with parasitaemias of ~0.004% (Appendix 1 a, b).

Haemoproteus testudinalis was recorded from only 2/6 (33%) of captive *K. lobatsiana* specimens from GP (Johannesburg National Zoological Gardens) with parasitaemias ~1.7%. (Appendix 1 a, b).

Haemoproteus sp. A (parasitaemia ~15%) was recorded during a follow up study of a single captive *K. natalensis* housed at a sanctuary in Ballito (KZN), collected and examined first by Cook (2008). Additional stages were observed during the follow up study, which resulted in a publication in which the species was named *Haemoproteus natalensis* Cook, Smit and Davies, 2010 (Cook *et al*., 2010). The species will be discussed in further detail in Chapter 7.

The tick species *Amblyomma marmoreum* Koch, 1844 was collected from only wild *S. pardalis* and wild *H. areolatus*, and *Amblyomma sylvaticum* (de Geer, 1778) from wild *C. angulata*. *Amblyomma marmoreum* was found at: Mkuze and Pongola (KZN) on 5/16 (31%) *S. pardalis*; Britstown (NC) on 1/1 (100%) *S. pardalis*; Tswalu (NC) on 14/60 (23%) *S. pardalis*; and Paarl (WC) on 14/20 (70%) *H. areolatus*. *Amblyomma sylvaticum* was located at: Arniston (WC) on 10/20 (50%) *C. angulata*; De Hoop (WC) on 1/4 (25%) *C. angulata*; De Mond (WC) on 4/4 (100%) *C. angulata*; Paternoster (WC) on 18/19 (95%) *C. angulata*; and West Coast (WC) on 6/6 (100%) *C. angulata*. *Ornithodorus compactus* Walton, 1962, was collected from only 1/10 (10%) wild *P. oculiferus* and 1/60 (1.7%) wild *S. pardalis* from Tswalu (NC). All life stages including the larval, nymphal and adult stages were

observed for *A. marmoreum* and *A. sylvaticum*, but only adults for *O. compactus* (Appendix 1a). Tick infestations ranged from ~ 5 to >50 per tortoise, high infestations comprising mostly larvae. It was difficult to count exact numbers of ticks per tortoise, especially on large tortoises, due to their strength and habit of retreating into their shells. However, the average number collected per infested tortoise was ~ 20, including all life stages.

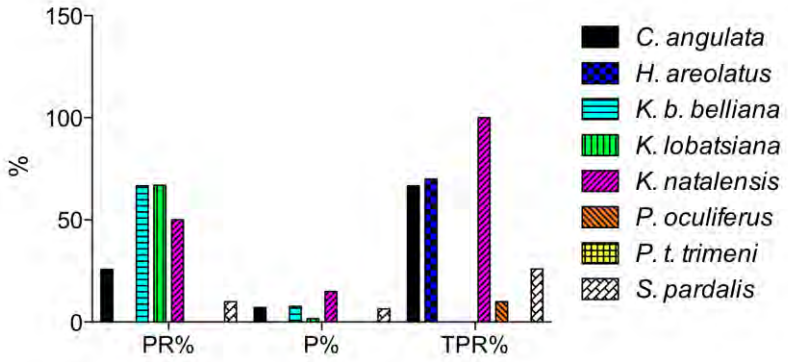


Figure 4.2. Haematozoan prevalence (PR%) and mean parasitaemia (P%) as well as the tick prevalence (TPR%) of the eight tortoise species examined.

Kinixys lobatsiana (all captive) had the highest prevalences of haematozoans at 67% with parasitaemias of ~1.7%, followed closely by *K. b. belliana* (all wild) at 66.7% with the second highest parasitaemias of ~7.7% (Fig. 4.2). The highest parasitaemias were observed from *K. natalensis* (1 captive) at ~15%, with a prevalence of 50%. This species, the wild specimen, also had the highest tick prevalence at 100% (Fig. 4.2), but this was based on a small sample size of tortoises (n = 2)(Appendix 1 a, b). Similarly, the sample size of *K. b. belliana* (n = 3) (all wild) and *K. lobatsiana* (n = 7) (all captive) was small. On the other hand, large sample sizes were obtained for *C. angulata* (n = 97; 22 captive, 75 wild) and *S. pardalis* (n = 121; 42 captive, 79 wild). *Chersina angulata* had a haematozoan prevalence of 26%, parasitaemias of ~7.1% and a tick prevalence of 67%, while *Stigmochelys pardalis* showed a haematozoan prevalence of 10%, parasitaemias of ~6.5% and a tick prevalence of 26%. Even though wild *H. areolatus* had a high tick prevalence (70%) it was not found to be infected with haematozoans (Fig. 4.2). The *Psammobates* species also lacked detectable haematozoans (1 captive, 12 wild) (Appendix 1 a, b).

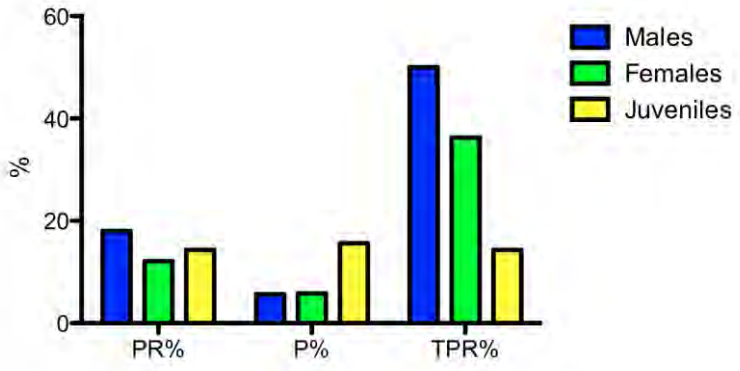


Figure 4.3. Haematozoan prevalence (PR%) and mean parasitaemia (P%) as well as the tick prevalence (TPR%) of adult male and female, as well as juvenile tortoises. Juvenile tortoises are those that, due to their young age, could not be sexed.

Collectively, all species of wild adult male tortoises (n = 82) showed the highest tick (both *A. marmoreum* and *A. sylvaticum* collectively) and haematozoan prevalences at 50% and 18% respectively (Fig. 4.3). Collectively, all species of wild adult female tortoises (n = 94) in comparison had tick and haematozoan prevalences of 36% and 5.8% respectively. Tick and haematozoan prevalences, and parasitaemias appeared high in wild juveniles of all species (Fig. 4.3), but few were examined; 1/7 juveniles (a *C. angulata*) had *H. fitzsimonsi*, and this particular individual had a parasitaemia of 15.6% with a heavy infestation of *A. sylvaticum* (Appendix 1a).

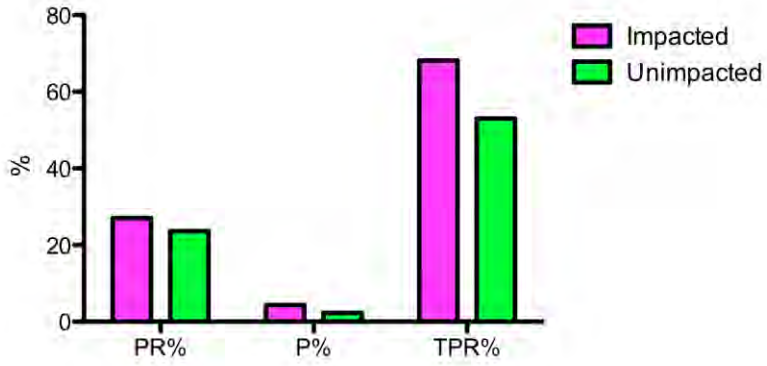


Figure 4.4. Comparison of *Haemogregarina fitzsimonsi* prevalence (PR%) and mean parasitaemia (P%), as well as tick prevalence (TPR%) of impacted versus un-impacted (reserve) areas.

Haemogregarina fitzsimonsi had the widest distribution and host range of all the haematozoans observed within this study (Appendix 1 a, b). Thus comparisons of the prevalence and parasitaemia between human impacted areas such as (Arniston, Gouritzmond, Paarl, Paternoster, Britstown and De Beers Diamond Route) and un-impacted (reserve) areas such as (Mkuze, Pongola, De Hoop, De Mond, West Coast conservancy, Namaqualand and Tswalu) were undertaken using this parasite species. The prevalence of *H. fitzsimonsi* and ticks (both *A. marmoreum* and *A. sylvaticum*), as well as *H. fitzsimonsi* parasitaemia appeared higher in impacted than in un-impacted (reserve) areas (Fig. 4.4). This will be discussed in further detail in Chapter 8.

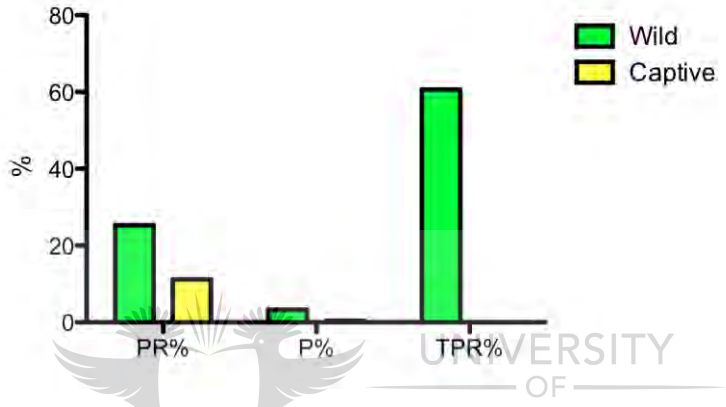


Figure 4.5. *Haemogregarina fitzsimonsi* prevalence (PR%), mean parasitaemia (P%) and tick prevalence (TPR%) of wild versus captive tortoise specimens.

Haemogregarina fitzsimonsi had the highest prevalence and parasitaemia for wild tortoises. Average tick prevalence for captive specimens was 0% (Fig. 4.5).

4.2. Discussion

Four different provinces were sampled (see Fig. 3.1), each representing a different biome (see Fig. 2.1). KwaZulu-Natal was thus subtropical, GP temperate, the NC arid semi-desert, and finally the WC a region of endemic coastal fynbos. Results (Appendix 1 a, b) indicate that tortoise populations in KZN (subtropical) had the highest prevalence and parasitaemias of apicomplexan haematozoans (Fig. 4.1). This province also recorded the highest biodiversity of blood parasites (three species) compared to the others in which only two (GP), or a single species was recorded (NC and WC). Also, KZN appeared to have two endemic haematozoans, *Haemogregarina parvula* and *Haemogregarina* sp. A., which were not recorded from any other province. Since ticks are the suggested vectors of

tortoise haemogregarines (see section 2.3.5.), tick prevalence was expected to be highest in KZN, but in reality it was second to the WC (Fig. 4.1). The WC is arid compared to subtropical KZN, possibly resulting in tortoises requiring larger territories for feeding (Branch, 2008). Tortoises in the WC may thus be more active than in KZN and encounter tick infested resting sites more frequently.

Gauteng Province tortoises (only captive specimens) showed the third highest prevalence of blood parasites (Fig. 4.1), but the origin of these animals was unknown. These GP tortoises most likely originated from the North West Province (see Fig. 3.1) where rural market trade in these animals (see Cook *et al*., 2009a) is still active (personal observation). Members of the public often buy such tortoises as pets or to 'save' them (Branch, 2008; personal observation), only at a later stage having them confiscated by authorities or surrendering them to zoological institutions. Evident, however, are the low parasitaemias of captive tortoises (Fig. 4.1), possibly resulting from removal of ticks in such surroundings.

The NC had the lowest prevalences of blood parasites and parasitaemias in wild tortoise populations, along with the lowest tick prevalence (Fig. 4.1), perhaps a result of the aridity of the region. As mentioned above for the WC, wild tortoises would need a larger territory for feeding in the NC, but due to the increased aridity of the NC compared to that of the WC, the NC would likely have a decreased carrying capacity for tortoises (Cang, 2006; BenDor *et al*., 2009). A lower abundance or density of tortoises per given area may perhaps lead to particular tick infested resting sites being used less frequently and thus the chance for transmission to be greatly reduced.

Of the five tortoise genera that occur in South Africa, representatives of all five (100%) were examined. Within these five genera, 9 species of indigenous tortoise were collected, with one species being highly endangered (*Psammobates geometricus*), the tenth species being exotic (*Testudo graeca*). Since peripheral blood could not be examined from wild *P. geometricus*, only eight of South Africa's 14 (57%) indigenous species were studied in detail. Furthermore, although two captive *T. graeca* were examined, the Palearctic haemogregarine species, *Hemolivia mauritanica* known to occur in this tortoise (see sections 2.3.4. and 2.3.5; Široký *et al*., 2007), was not detected.

In general, wild tortoise species in which none of the five haematozoans were recorded showed low tick prevalence (Fig. 4.2). Wild *P. oculiferus* and *P. t. trimeni* in particular showed low to no tick prevalence and no recorded blood infections. However, both the above species occur in arid regions of the NC, a province which had the lowest recorded tick prevalence for wild specimens (Fig. 4.1).

Kinixys lobatsiana (all captive) showed a high prevalence (Fig. 4.2) of *Haemoproteus testudinalis*, which is assumed to be transmitted by biting flies (Telford, 2009). However, no biting flies were recorded on tortoises, or collected during the current study. *Homopus areolatus* (wild) also had high tick prevalences, however the sampling site was isolated (see section 3.1.1.), with many tortoises having to share resting sites in a small area. High tortoise densities such as these may explain high tick prevalences, but *H. fitzsimonsi* and other blood parasites were absent, possibly due to the isolated nature of the site, with infected tortoises and infected ticks never entering the area.

Even though results indicate that tick prevalence was highest for *Kinixys natalensis* (Fig. 4.2), data were collected from only two specimens (1 captive, 1 wild). Results show that wild *K. b. belliana* had the highest haematozoan prevalence and parasitaemias, followed by *C. angulata* (wild specimens only) and *S. pardalis* (captive and wild), respectively (Fig. 4.2). Again, this may result from the subtropical environment of KZN in which *K. b. belliana* occurs and the high activity levels of the WC tortoise species, *C. angulata*. No ticks were collected from *K. b. belliana*, but this most likely is due to the low sample number during this study (n = 3). *Kinixys belliana belliana*, *C. angulata* and *S. pardalis* had high prevalences and parasitaemias of haemogregarines (Appendix 1a). Tick prevalence was high in both KZN and the WC (Fig. 4.1), providing additional support that ticks may be vectors for *H. fitzsimonsi* and other tortoise haemogregarines.

Adult male tortoises showed the highest tick prevalences. Adult female tortoises in comparison had lower tick prevalences, but very similar haematozoan prevalences to male tortoises (Fig. 4.3). Adult male tortoises are known to maintain a territory, which they frequently peruse to protect it from other males and to check for breeding females (Boycott and Bourquin, 2000; Branch, 2008). Therefore, they may pass through tick infested resting sites more frequently than females. Tick and haematozoan prevalences and parasitaemias appeared high for juveniles, but only seven juveniles were examined and only one of these was infected with *H. fitzsimonsi* at a parasitaemia of 15.6% (Appendix 1a).

The current study recorded *Haemogregarina fitzsimonsi* infecting 3/8 (38%) of the tortoise species examined and, with those species recorded by Cook (2008) and Cook *et al*. (2009a), infected a host species total of 5/8 (63%). *Haemogregarina fitzsimonsi* was the haematozoan with the highest prevalence among captive and wild tortoises, forming on average the highest parasitaemias, and being the most widely distributed haematozoan over all four provinces and therefore biomes. This suggests it may be opportunistic in relation to its intermediate and definitive hosts (see Appendix 1

a, b).

Tick prevalence was especially high in the WC for wild tortoises (Fig. 4.1), and comprised two species, *Amblyomma marmoreum* and *Amblyomma sylvaticum*. *Amblyomma marmoreum* is found infesting tortoises collected inland, whilst *A. sylvaticum* infests tortoises inhabiting coastal areas (Horak *et al*., 2006a). *Amblyomma sylvaticum* is also only found in the Cape regions and *A. marmoreum* is more widely distributed throughout South Africa, except for the coastal areas (see Fig. 2.6). Comparing the distribution of both tick species and that of *H. fitzsimonsi*, the latter is also widely distributed, covering both inland and coastal areas, suggesting that it may infect both species of tick, if these ticks are its definitive hosts. The tick species *O. compactus* is restricted to arid regions such as the Kalahari from which all specimens recorded within this research were collected. Haematozoan infections were not observed in tortoises from the area (Tswalu) from which these ticks were collected.

Comparisons of un-impacted nature reserves (Mkuze, Pongola, De Hoop, De Mond, West Coast conservancy, Namaqualand and Tswalu) with anthropogenically impacted sites (Arniston, Gouritzmond, Paarl, Paternoster, Britstown and De Beers Diamond Route) suggest that *H. fitzsimonsi* prevalence and parasitaemia, as well as tick prevalence are greater in impacted areas (Fig. 4.4). However, taken individually, some impacted and un-impacted areas do not produce the same results. For example, comparing De Mond (clean reserve) (section 4.1.1.3.), and the adjacent farmland near Arniston (impacted) (section 4.1.1.3.) tick infestations are higher in De Mond tortoises, along with *H. fitzsimonsi* prevalence (Appendix 1a). An explanation for this may be vegetation burning frequency. De Mond burning occurs approximately only every 17 years, while burning occurs annually on the adjacent farmlands (De Mond authorities pers. comm.). Burning affects both ticks and their hosts, likely killing questing ticks (actively seeking hosts) as well as potential hosts. Besides the fatalities that occur, burning also alters the host environment, especially the food sources, and changes tick and tortoise spatial distribution and density (Horak *et al*., 2006b).

As a whole, however, comparing reserves and impacted areas, wild tortoises in impacted sites have a higher prevalence of ticks and *H. fitzsimonsi* along with greater *H. fitzsimonsi* parasitaemias (Fig. 4.4). Impacted sites may alter the ability of tortoises to move around freely, through physical barriers such as roads and fences confining individuals to specific areas. In these conditions, new infections may occur more readily and if frequently resulting in immuno-suppression, may induce higher parasitaemias (Jacobson, 2007). Additionally, this fragmentation of populations may cause a

decrease in the gene pool, as well as decreasing the nutritional worth of the particular area by overgrazing (BenDor *et al*., 2009), resulting in malnourishment and further immuno-suppression. Tick populations may be forced to utilize the same tortoise(s), possibly leading to a higher prevalence of infection within the vectors themselves.

Tick prevalence is higher for wild tortoise specimens along with prevalence and parasitaemias for all haematozoans (Appendix 1a) (Fig. 4.5). Staff within captive sites in the current study removed ectoparasites, such as ticks (Appendix 1b). Previous studies (Cook, 2008) sampled captive sites that did not share the same policies on removing ectoparasites, therefore not clearing ticks. In the previous case, prevalence and parasitaemia of *H. fitzsimonsi* were found to be higher in captive individuals than in wild specimens. Findings such as these, yet again, suggest that ticks are vectors for *H. fitzsimonsi*. The likelihood of parasite transmission and proliferation is greatly increased in captive situations in which animals are kept in close proximity (Jacobson, 2007; McArthur *et al*., 2004). If, however, vectors and survival stress are removed through better husbandry and veterinary attention, parasitaemias may decrease or become latent as host immunity improves.

In summary, the subtropical region of KZN proved to have the highest biodiversity of haematozoan parasite species along with high tick prevalence. Areas which were more arid in nature, such as the NC, presented with a lower biodiversity of haematozoans and a lower prevalence of ticks. Tick species were prevalent in areas in which *H. fitzsimonsi* prevalences and parasitaemias were high, and no other haematophagous ectoparasites were detected, strongly suggesting that ticks are the vectors of this haemogregarine. Tick prevalences, and *H. fitzsimonsi* prevalences and parasitaemias on a whole were higher in human impacted areas, suggesting the haematozoan species future use as a bio-indicator (discussed in further detail in Chapter 8, section 8.1). Captive collections of tortoises, from which ticks had been removed, presented with lower prevalences of infection and parasitaemias than those of their wild counterparts, further suggesting ticks to be the vectors of *H. fitzsimonsi*, but also emphasising the importance of ectoparasite control within captive collections.

CHAPTER 5

MORPHOLOGICAL AND MOLECULAR ANALYSIS OF *HAEMOGREGARINA* (SENSU LATO) *FITZSIMONSI* DIAS, 1953 AND ASSOCIATED VECTORS

In Chapters 2 (sections 2.3.3. and 2.3.5.) and 4 (section 4.2.) the likelihood of *Haemogregarina fitzsimonsi* being transmitted by a tick vector was discussed, and results presented in Chapter 4 showed that two different species of tick, namely *Amblyomma marmoreum* Koch, 1844 and *Amblyomma sylvaticum* (de Geer, 1778) were probably involved. Siddall (1995) placed all chelonian haemogregarines within the genus *Haemogregarina* (sensu stricto), transmitted solely by leech vectors. Since the only ectoparasites collected from terrestrial tortoises in the current study were ticks, this finding supported the need for *H. fitzsimonsi* to be taxonomically reassessed. The first aim of this section of the present study was to examine the peripheral blood stages of the parasite within its tortoise host and the second, to attempt to link tortoise blood stages to stages recorded within the ticks.

5.1. Morphological analysis of *Haemogregarina* (sensu lato) *fitzsimonsi* Dias, 1953

5.1.1. Light microscopy of tortoise peripheral blood stages of *Haemogregarina fitzsimonsi* (Figure 5.1 a - l, o)

Stages of *H. fitzsimonsi* observed in Giemsa stained peripheral blood films of tortoises during this study (2009 – 2011) were compared with those reported for *H. fitzsimonsi* by Cook *et al*. (2009a). They included trophozoites or merozoites, immature and mature gamonts, and all were intraerythrocytic with the exception of extracellular gamonts (Fig. 5.1 a – l, o). Mature gamonts often occurred singly within mature erythrocytes when parasitaemias were low. However, less commonly, when parasitaemias were higher at between 14 – 60% of erythrocytes parasitized (Appendix 1a), paired mature gamonts occurred in single erythrocytes (Fig. 5.1 e - i). Extracellular gamonts were not commonly observed (Fig. 5.1 j – l).

Trophozoites/merozoites: these lay singly within the immature erythrocyte cytoplasm (Fig. 5.1 a – c), and measured 4.7 ± 1.0 (4.2 – 5.9) µm long by 2 ± 0.1 (1.9 – 2.2) µm wide; their cytoplasm was granular, staining pale purple and exhibiting vacuolation at the poles; nuclear chromatin was loose, and the nucleus lay centrally, measuring 1.6 ± 0.1 (1.5 – 1.7) µm long by 0.8 ± 0.1 (0.7 – 0.8) µm wide, and staining dark pink; no parasitophorous vacuole was visible (n = 3).

Immature gamonts: these lay singly (Fig. 5.1 b – c [centre; left respectively]) within the mature erythrocyte cytoplasm; they were slender and elongate, and recurved at the poles (Fig. 5.1 b), measuring 16.2 ± 1.7 (14 – 18.2) µm long by 2.1 ± 0.6 (1.5 – 2.8) µm wide (n = 5); their cytoplasm stained whitish blue-purple without vacuolation; the rectangular nucleus with condensed chromatin, stained purple, always lay closer to one pole of the gamont than the other, and measured 2.0 ± 0.4 (1.4 – 2.4) µm long by 0.8 ± 0.1 (0.7 – 0.9) µm wide (n = 5).

Mature gamonts: these occurred singly (Fig. 5.1 d), or in pairs (Fig. 5.1 e - i) within the mature erythrocyte cytoplasm, and measured 17.2 ± 0.2 (17 – 17.5) µm long by 3.8 ± 0.3 (3.4 – 4.2) µm wide (n = 15); they were also occasionally extracellular (Fig. 5.1 j - l). Mature gamonts were also elongate and curved, but broader than immature gamonts, especially at one pole (anterior?), with the opposing pole having a small recurved tail (Fig. 5.1 k [top centre]); cytoplasm stained whitish-blue and was without vacuoles or granules; the nucleus stained sometimes pink (Fig. 5.1 i), but more often purple (Fig. 5.1 d – h, j – l), was square or oval in outline, measured 4.6 ± 0.2 (4.3 – 5) µm long by 2.8 ± 0.2 (2.5 – 3.1) µm wide (n = 15), and usually lay nearer one pole than the other, anterior (?) to mid-nucleus 10.4 ± 0.7 (9.8 – 10.6) µm and posterior (?) to mid-nucleus 5.3 ± 0.9 (4.6 – 6) µm; the parasitophorous vacuole of the intracellular gamont was narrow (Fig. 5.1 j – l). Paired gamonts were positioned on either side of the host cell nucleus, apparently lying in opposite directions (Fig. 5.1 e - f), or on the same side of the host cell nucleus, lying in the same (Fig. 5.1 g) or opposite planes (Fig. 5.1 h - i). Both immature and mature gamont stages often appeared to displace and compress host cell nuclei.

5.1.2. Remarks

During the current study, the tortoise peripheral blood stages of *H. fitzsimonsi* observed included possible trophozoite or merozoite stages, and immature and mature gamonts (Fig. 5.1 a – l). They were morphologically and morphometrically similar to those stages described by Dias (1953), and then by Cook (2008) and Cook *et al*. (2009a). Previously in Cook (2008) and Cook *et al*. (2009a), meronts were reported and these were possibly binucleate and, in the process of division, with two or more merozoites observed in each parasitized erythrocyte. However, in the present study such meronts were not identified. The stages found in the current research (Fig. 5.1 a – c) appeared intermediate between the

trophozoite and merozoite stages reported in Cook *et al*. (2009a). They were thus larger than the trophozoite stages of Cook *et al*. (2009a) and were nearer to merozoites in size, but still showed cytoplasmic vacuolation characteristic of trophozoites. Of the 36/269 (13.4%) tortoises infected with *H. fitzsimonsi* only a single *Kinxys belliana belliana* Gray, 1830 specimen from KZN presented with trophozoite/merozoite stages. Cook *et al*. (2009a) reported that these stages were rare, but also that they were found in five tortoises, three *Stigmochelys pardalis* (Bell, 1828) from Gauteng (GP) and two *Chersina angulata* (Schweigger, 1812) from Western Cape (WC). A rarity or absence of detectable dividing stages within the peripheral blood is characteristic of the haemogregarine genus *Hepatozoon* (Miller, 1908) (see Davies and Johnston, 2000).

As mentioned previously, *H. fitzsimonsi* was found concurrently with *Haemogregarina parvula* infecting *K. b. belliana* (see Figure 5.1 m – o) and the latter species is discussed in further detail in the following chapter.

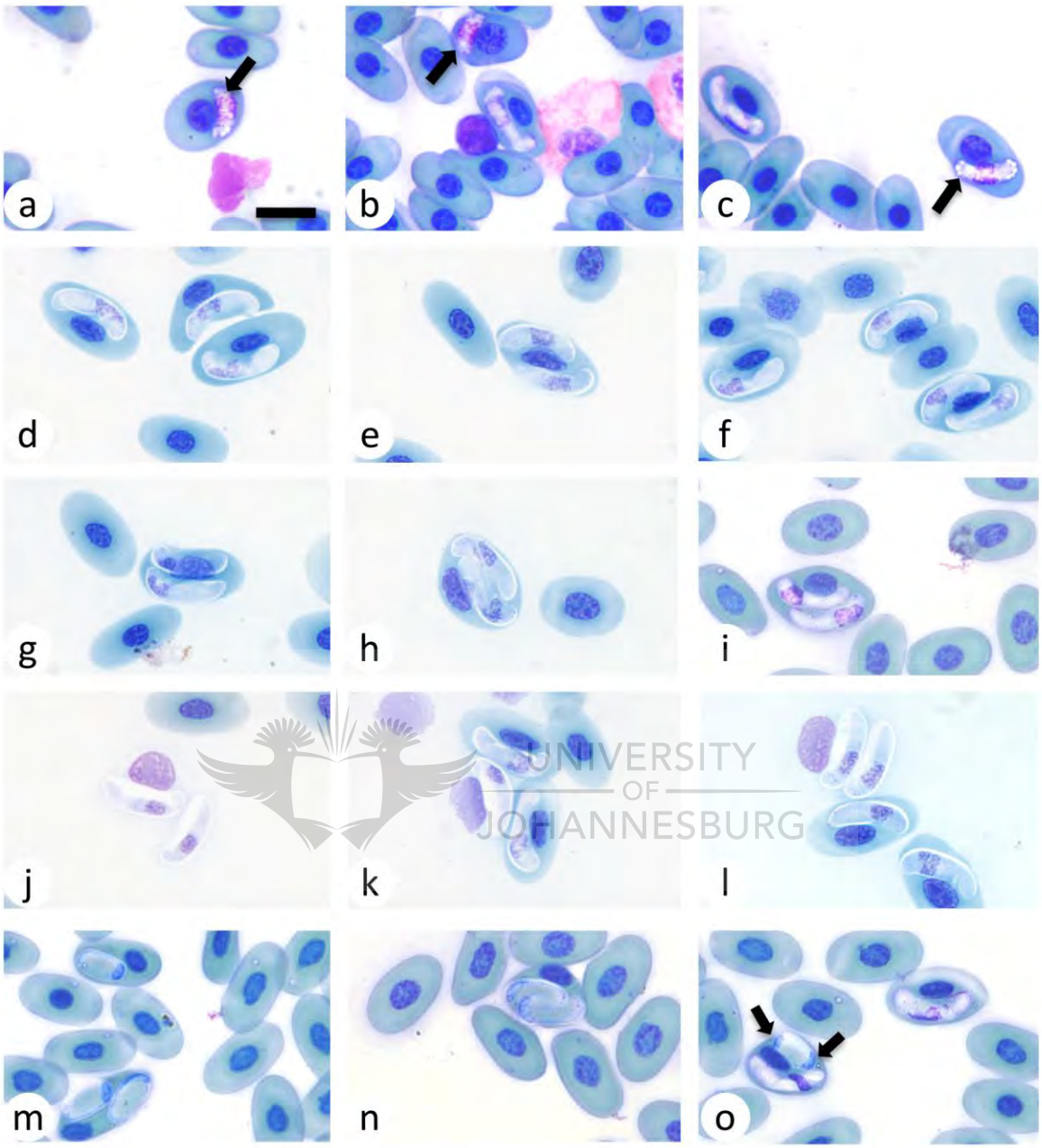


Figure 5.1. Light micrographs of Giemsa stained stages of *Haemogregarina fitzsimonsi* Dias, 1953 and *Haemogregarina parvula* Dias, 1953, recorded during this study. **(a - l)** *H. fitzsimonsi*. **(a - c)** rarely found stages (arrows), recorded only once from a single *Kinixys belliana belliana* Gray, 1830 from KwaZulu-Natal (KZN), possibly trophozoites or merozoites, note the structure of the nucleus and the vacuolation at the poles, **(b – c [centre; left respectively])** immature gamonts, long and slender, recurved at both poles **(d)** mature erythrocytes infected with single mature gamonts, **(e - i)** double infections of mature gamonts within mature erythrocytes, commonly occurring at high parasitaemias, **(j - l)** extracellular mature gamonts. **(m - o)** *H. parvula*, mature gamonts, infecting mature erythrocytes singly and doubly, **(o)** in this case occurring concurrently with *H. fitzsimonsi,* in the same erythrocyte. The two nuclei on opposite poles (arrows) would suggest the presence of two superimposed *H. parvula* gamonts. Scale bar: 10µm.

5.1.3. Ultrastructural observations on tortoise peripheral blood stages of *Haemogregarina fitzsimonsi*

(Figure 5.2.)

Light microscopy observations of *H. fitzsimonsi* and *H. parvula* revealed the two to be morphologically different (see above), and since *H. parvula* had a thick capsule (Fig. 5.1 m – o) which was stain-resistant at this level, it was difficult to make further comparisons. However, transmission electron microscopy (TEM) helped distinguish them to some extent. Although fixation was not ideal for *H. fitzsimonsi* (Fig. 5.2 a) the species was shown to be similar in form to its appearance by light microscopy, lacking an encasing resistant wall and without an extensive parasitophorous vacuole (Fig. 5.2 a – b). Both these features are, however, characteristic of *H. parvula* (Fig. 6.2 a - b). Subpellicular tubules were visible in *H. fitzsimonsi* (Fig. 5.2 a) as well as the nucleus, micronemes, a rhoptry-like body, and lipid-like material (Fig. 5.2 a - b), while the inner layer of the pellicle appeared electron-dense (Fig. 5.2 a - b).

5.1.4. Remarks

Haemogregarina fitzsimonsi mature gamonts were the dominant stages found in the peripheral blood of most tortoises examined, other stages being found very rarely. It was therefore difficult to locate TEM specimens of immature stages. Ultrastructurally, the gamont of *H. fitzsimonsi* was similar or indistinguishable from other examples of apicomplexan haemogregarines such as *Hepatozoon boigae* (Mackerras, 1961) infecting the snake *Boiga irregularis* (Bechstein, 1802) (see Jakes *et al*., 2003) and a *Haemogregarina* sp. infecting the gecko *Ptyodactylus hasselquistii* (Donndorff, 1798) (see Hussein, 2006). As in the above genera, *H. fitzsimonsi* did not appear to have an encapsulated gamont as is evident in the genus *Hemolivia* (Petit, Landau, Baccam and Lainson, 1990) (see Boulard *et al*., 2001; Paperna and Smallridge, 2001). The ultrastructure of the gamont stages of *Hemolivia* will be discussed in further detail in the following chapter.

5.2. Description of tick tissue stages

Ticks found to be infected with sporocyst and sporozoite stages included mainly adult male stages of both *Amblyomma sylvaticum*, and *Amblyomma marmoreum* collected from *H. fitzsimonsi*-infected and apparently non-infected tortoises, including *C. angulata* from the

WC and *S. pardalis* from the Northern Cape (NC). Adult male ticks compared to adult females, were found to be the most dominant stage infesting tortoises and thus provided most of the samples. In total, 83 sets of ticks were collected from tortoises, including 46 sets of *A. sylvaticum*, 35 sets of *A. marmoreum*, and 2 of *Ornithodorus compactus* Walton, 1962 (Appendix 1a), most ticks having been collected during summer (October – February) with only a few in spring (September). Sets included either all three stages (larval, nymphal and adult stages), or a combination of two stages, or a single stage.

Ticks were dissected and impressions made of their intact and dissected viscera, as outlined in Chapter 3. Intact visceral impressions revealed sporocyst stages from a number of adult *A. sylvaticum* ticks from infected *C. angulata* tortoises, while dissection of adult ticks from apparently uninfected *C. angulata* tortoises revealed further sporocyst stages (Table 5.2). Of the 18/46 (39%) sets of *A. sylvaticum* collected from *H. fitzsimonsi* infected tortoises, 3/18 (17%) revealed sporocysts (Table 5.2). Two male *A. sylvaticum* collected from two *C. angulata*, lacking obvious peripheral blood infections, from De Mond (WC) were also infected. An *A. marmoreum* (1/35, 3%) was taken from a *H. fitzsimonsi* infected tortoise, but squashes and impressions revealed no sporocysts. However, sporocysts were observed in two male *A. marmoreum* (2/35, 6%) collected from a *S. pardalis* from Britstown (NC) that showed no peripheral blood infection with *H. fitzsimonsi*. The two *O. compactus* were taken from uninfected tortoises and revealed no sporocysts.

Unlike adult ticks, squashes of larvae and nymphs taken from infected tortoises, and squashes taken at random from these stages from uninfected tortoises, showed no sporocysts. Furthermore, even though extreme care was taken during dissection, no oocysts were observed in any tick stage, only large groups of sporocyts and some free sporozoites, suggesting that the oocysts had ruptured during preparation. Further details of the sporocysts and sporozoites appear below:

5.2.1. Light microscopy of stages found within the tick haemocoel

(Figure 5.3)

Sporocysts: These were found in impressions of intact adult tick viscera, usually free from host tissue; they were narrowly to broadly oval, and measured 27.9 ± 1.9 (25.8 - 29.6) μm long and 11.2 ± 0.06 (9.1 - 13.3) μm wide (n = 10); single sporocysts were observed with an

apparently double-layered, thick non-staining capsule (Fig. 5.3 a - b). Thus, in comparison with adjacent, digesting host tortoise erythrocytes (seen on the occasion when the gut was accidently ruptured) (eg. Fig. 5.3 c), measuring 17.9 ± 1.2 (15.9 - 19.6) μm long and 10.2 ± 1.1 (8.3 - 11.5) μm wide, sporocysts were longer in length. Sporocysts were also seen in clusters, staining more readily, and possibly released recently from oocysts naturally, or during dissection when fragile oocysts may have been ruptured (Fig. 5.1 a). There appeared to be at least 16 - 18 (n = 10) sporozoites within each sporocyst, estimated by counting sporozoite nuclei. Outlines of sporozoites were visible in some sporocysts (Fig. 5.3 b).

Sporozoites: In impressions of intact tick viscera, freed from host tissue, elongate and slender sporozoites free of sporocysts were observed measuring 13.6 ± 0.3 (13.2 - 14.1) μm long and 1.9 ± 0.2 (1.8 - 2.3) μm wide (n = 8). These were slightly curved, with pale blue stained cytoplasm and a small blue stained, almost centrally located, rounded nucleus measuring 1.5 ± 0.2 (1.2 - 1.8) μm long and 1.9 ± 0.1 (1.8 - 2) μm wide (n = 22). Purple stained structures were evident on each side of the nucleus (Fig. 5.3 d), perhaps corresponding to the crystalline bodies observed in other apicomplexan sporozoites such as those of *Cryptosporidium parvum* (see Tetley *et al*., 1998).

5.2.2. Remarks

The proposed vector of all chelonian haemogregarines, the leech (see Siddall, 1995), was not collected from any tortoises during the current study. Only tick stages were observed and on dissection they revealed parasite stages such as those described above, although oocysts were not seen. More importantly, these stages were not collected from the tick intestinal epithelium, but from the haemocoel, free within the tick body cavity. Sporogony within the haemocoel of an invertebrate vector is typical of the development of *Hepatozoon* species, in contrast to *Haemogregarina* where sporocysts are not formed, and *Hemolivia* species where sporogony occurs within the intestinal epithelium (Davies and Johnston, 2000). However, apart from their location, sporogonic stages in the current study were morphologically and morphometrically similar to those of *Hemolivia mauritanica* (Sergent and Sergent, 1904) described by both Michel (1973) and Paperna (2006) from ticks of the species *Hyalomma aegyptium* (Linnaeus, 1758). In the current research sporocysts were 27.9 x 11.2 μm, and sporozoites 13.6 x 1.9 μm (section 5.2.1.), compared to Michel's (1973) sporocysts of 25 - 30 x 10 μm and sporozoites of 12 x 2 μm, and Paperna's (2006) live sporocysts of 25 - 28 x 15

µm and sporozoites of 15 x 12 x 5 µm. Sporocysts observed within this research appeared either broadly or narrowly oval within the same slide (Fig. 5.3 b). Such variation in size and shape is not uncommon for sporocysts as can be seen by the sporocyst descriptions of a number of *Hepatozoon* species (see Telford, 2009). This in mind, and the similarity in size and shape of sporocysts from both *A. marmoreum* (Fig. 5.3 b) and *A. sylvaticum* (Fig. 5.3 c) suggests strongly that these stages in both tick species are likely of the same species. Another feature was the sporocyst walls/capsules in the present study, which as in *Hm. mauritanica*, appeared double-layered (see Paperna, 2006), perhaps rendering the wall less penetrable to Giemsa staining.

Since the ticks had fed on tortoise blood, in several cases containing *H. fitzsimonsi,* these results suggest that the sporocysts were those of this haemogregarine and that it should perhaps be assigned to the genus *Hepatozoon* rather than *Haemogregarina*, as at present (Dias, 1953). However, to substantiate this opinion, molecular data were required (see below). No ticks were collected from *H. parvula* or *Haemogregarina* sp A. infected tortoises (Appendix 1a) from KZN and therefore it was most unlikely that the sporocyst stages found in the ticks in other regions of South Africa belonged to these apicomplexans. Only WC (*C. angulata*) and NC (a single *S. pardalis*) tortoises and their ticks were used in describing sporocysts/sporozoites and in subsequent molecular analysis since no other blood infections besides *H. fitzsimonsi* had been observed in the tortoises in these two regions (see Appendix 1 a, b).

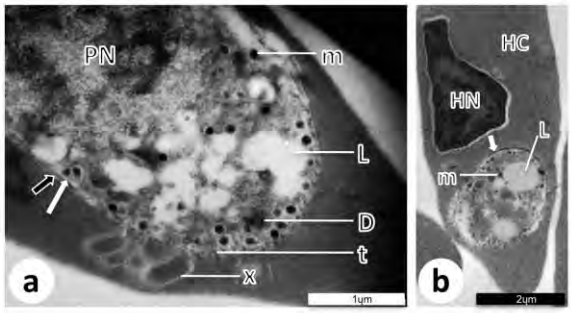


Figure 5.2. Transmission electron micrographs of intraerythrocytic mature gamonts of *Haemogregrina fitzsimonsi* Dias, 1953. **(a)** gamont showing outer (black arrow) and inner layer of pellicle (white arrow), (PN) parasite nucleus, possible leakage of parasite cytoplasm during fixation (x), micronemes (m), lipid-like material (L), electron dense body, possibly a rhoptry (D), and one of several subpellicular tubules (t). **(b)** cross section through a gamont, again showing the pellicle (white arrow), host erythrocyte nucleus (HN) and host cell cytoplasm (HC), lipid-like material (L) and micronemes (m). There is no evidence of a capsule or a parasitophorus vacuole in these sections. Scale bars: 1µm and 2µm respectively.

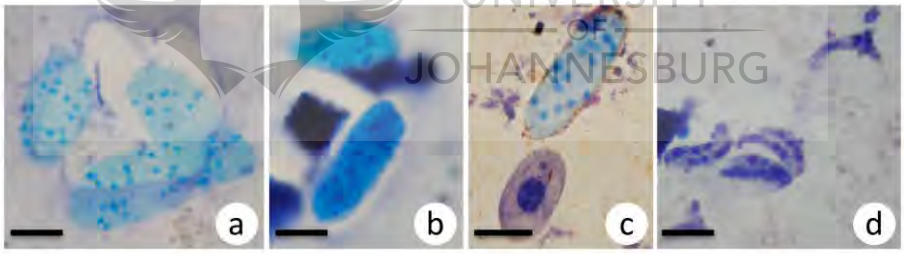


Figure 5.3. Sporocyst and sporozoite stages of possibly *Haemogregarina fitzsimonsi* Dias, 1953 observed within dissected ticks, *Amblyomma marmoreum* Koch, 1844 (b) and *Amblyomma sylvaticum* (de Geer, 1778) (a, c, d). **(a - b)** clustered sporocysts, possibly recently released on dissection from an oocyst, note the thick capsule impressions in **(a)** and the individual sporozoites (and their nuclei), still within the sporocysts, in **(a - b)**, **(c)** showing size of sporocyst in relation to an ingested tortoise erythrocyte, **(d)** sporozoites from a ruptured sporocyst. Scale bar: 10µm.

5.3. Molecular analysis of peripheral blood stages of *Haemogregarina fitzsimonsi*

As reported above, tortoise peripheral blood stages and those found within the ticks collected from infected and uninfected tortoises, greatly support the possibility of *Haemogregarina fitzsimonsi* belonging to the genus *Hepatozoon*. With this in mind, three different sets of previously published apicomplexan specific primers were used in an attempt to amplify the 18S ribosomal RNA (rRNA) gene in order to detect infection and identify parasite species from total DNA extracted from tortoise blood and sporocysts/ticks (Fig. 5.4). The 18S rRNA has formed the basis of the molecular taxonomy and phylogenetics of the haematozoan parasites thus allowing for later sequence comparisons with data published on GenBank. Firstly, primer set HEMO1: 5'-TAT TGG TTT TAA GAA CTA ATT TTA TGA TTG-3', the forward primer, and HEMO2: 5'-CTT CTC CTT CCT TTA AGT GAT AAG GTT CAC-3', the reverse primer, designed by Perkins and Keller (2001), was selected. HEMO1 is specific to haemogregarines and HEMO2 specific to apicomplexan parasites. Secondly, primer set HEPF300: 5'-GTT TCT GAC CTA TCA GCT TTC GAC G-3', the forward primer, and HEPR900: 5'-C AAA TCT AAG AAT TTC ACC TCT GAC-3', the reverse primer, designed by Ujvari *et al*. (2004), was selected specifically for *Hepatozoon* species. Thirdly, another primer set specific to *Hepatozoon* was selected, 4558: 5'-GCT AAT ACA TGA GCA AAA TCT CAA-3', the forward primer, and 2773: 5'-CGG AAT TAA CCA GAC AAA T-3', the reverse primer, designed by Mathew *et al*. (2000).

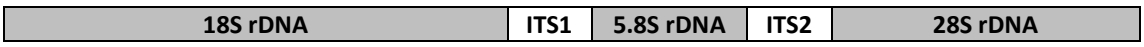


Figure 5.4. Diagrammatic representation, adapted from Barta (1997), showing the regions of the nuclear ribosomal RNA genes used most commonly for phylogenetic analyses. Less evolutionarily variable regions are shaded and those that are more variable are unshaded. In this study the ribosomal 18S and ITS2 regions were selected for amplification of haemogregarine and tick DNA respectively. Included in amplification of tick DNA was the mitochondrial 16S region (not shown here).

5.3.1. Molecular analysis of *Haemogregarina fitzsimonsi* blood slides

Total DNA from two Giemsa stained slides (originally absolute methanol-fixed) from two *C. angulata* from De Mond (WC) with *H. fitzsimonsi* parasitaemias of 3.3% and 0.8% respectively, was extracted using a DNeasy Animal Tissue Kit (using the spin column

protocol) (QIAGEN Ltd., UK) as described in Chapter 3. From the whole DNA extract parasite 18S rRNA was amplified using PCR with HEMO1 and HEMO2, HEPF300 and HEPR900, as well as 4558F and 2773R primers. This was performed using AB Applied Biosystems Veriti 96 well thermal cycler (see Chapter 3, section 3.7.1). PCR products were run on agarose gels and visualised under UV light (see Chapter 3), and firstly HEMO1 and HEMO2, revealed a strong band of ~900 bp for extracts from the slide with the higher parasitaemia and a fainter band of ~900 bp for the other (Fig. 5.5 A). The use of HEPF300 and HEPR900 primers and 4558F and 2773R primers revealed similar results (Figs. 5.5 B, C). HEP primers produced a strong band for the higher parasitaemia slide and a faint band for the slide with the lower parasitaemia, both of which were ~600 bp (Fig. 5.5 B). The last primer set (4558F and 2773R) also produced ~600 bp bands for the Giemsa stained slides, the higher parasitaemia slide again producing the stronger band (Fig. 5.5 C). Results are considered to indicate that apicomplexan/*Hepatozoon* DNA from the Giemsa-stained slides had been amplified by all the above primer sets.

PCR products yielding the best bands were dispatched for sequencing at the Natural History Museum, London (see Chapter 3) and then compared with published *Hepatozoon* sequences on GenBank. HEMO 1 and 2 amplified regions of approximately 900 bp, and HEP primers and 4558F/2773R primers, approximately 600 bp, which matched published sequences. These placed *Haemogregarina fitzsimonsi* a close match within the genus *Hepatozoon* (Fig. 5.7 A, B). Both Maximum Likelihood (ML) and Neighbour Joining (NJ) trees were constructed (see Chapter 3), but the ML trees provided greater phylogenetic resolution as indicated by the higher bootstrap values (≥50) and one *H. fitzsimonsi* sample (sample 10) gave the cleanest sequence and was used to construct such trees, although the sequence was representative of all samples of this organism analysed. The ML tree placed *H. fitzsimonsi* within one of the three major clades, in which other species of reptile *Hepatozoon* clustered (Figure 5.7 A and Table 5.1). Thus, *H. fitzsimonsi* was identified as a *Hepatozoon* lying close to other reptilian *Hepatozoon* species phylogenetically, or to *Hepatozoon* species of ticks derived from reptiles (Table 5.1). This was contrasted in the poorly resolved neighbour Joining (NJ) tree, as the analysis placed the parasite within the same clade as *Hepatozoon* species infecting Palaearctic voles and foxes. This was considered to be an erroneous result as the NJ tree was constructed with low bootstrap values (≤50) which decreased the certainty of nodal support.

Table 5.1. Tabulation of *Hepatozoon* species appearing within the Maximum Likelihood (ML) and Neighbour-Joining (NJ) trees (18S ribosomal DNA sequences). Included below are the three major clades (as seen in Fig. 5.7 A, B), the GenBank (NCBI) accession numbers and *Hepatozoon* species definition, with the corresponding host information, possible invertebrate vector, origin and corresponding biogeographical region (BIOGEO): Afrotropical (A), Australian (AUS), Neotropical (NEO), Oriental (O), Palaearctic (P). Authorities of hosts taken from ITIS (Integrated Taxonomic Information System), Zipcode Zoo as well as Reptile database.

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
OUTGROUP	AB182492	*Plasmodium ovale* gene (OUTGROUP)	*Homo sapiens sapiens* Linnaeus, 1758 (Human)	Mosquito: *Anopheles* spp. (Roberts and Janovy, 2000)	Indonesia: Flores	O
1	GQ377216	*Hepatozoon felis* isolate Korea-1	*Felis catus* Linnaeus, 1758 (Domestic cat)	Unknown (see Taylor *et al*., 2007)	Korea	O
1	AY620232	*Hepatozoon felis* isolate Spain-1	*Felis catus* (Domestic cat)	Unknown (see Taylor *et al*., 2007)	Spain	O
1	GQ926902	*Hepatozoon* sp. ex *Prionailurus bengalensis*	*Prionailurus planiceps* (Vigors and Horsfield, 1827) (Flat-headed cat)	Unknown (see Taylor *et al*., 2007)	South East Asia	O
1	FJ595127	*Hepatozoon* sp. JM-1	*Martes melampus melampus* (Wagner, 1840) (Japanese Marten)	Not reported, possibly tick (Kubo *et al*., 2009)	Japan	O
1	EU686690	*Hepatozoon* sp. pine marten/PM421/Scotland	*Martes martes* (Linnaeus, 1758) (Pine Marten)	Not reported, possibly tick (Simpson *et al*., 2005)	Scotland	P
1	EF222257	*Hepatozoon* sp. European pine marten	*Martes martes* (European pine marten)	Not reported, possibly tick (Criado-Fornelio *et al*., 2009)	Spain: Burgos	P

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
1	EU041717	*Hepatozoon ursi* isolate Gifu 1	*Ursus thibetanus japonicus* Schlegel, 1857 (Japanese black bear)	Tick: *Haemaphysalis japonica* and *Haemaphysalis flava* (Kubo *et al*., 2008)	Japan	O
1	FJ876444	*Hepatozoon* sp. MRA-2009-1	*Leopardus tigrinus* (Schreber, 1775) (Oncilla)	Unknown (see Taylor *et al*., 2007)	Brazil: DF, Brasilia	NEO
1	EU146062	*Hepatozoon americanum*	*Canis lupus lupus* Linnaeus, 1758 (Domestic dog)	Tick: *Amblyomma maculatum* (Taylor *et al*., 2007)	U.S.A.: Oklahoma	NEO
1	AY461377	*Hepatozoon* sp. - Curupira 2	*Dusicyon thous* (Hamilton Smith, 1839) (Lobinho)	Tick spp. (Criado-Fornelio *et al*., 2006)	Brazil	NEO
1	AY471615	*Hepatozoon canis* isolate Curupira 4	*Pseudalopex gymnocercus* (Fischer, 1814) (Pampas fox)	Tick: *Rhipicephalus sanguineus* (Taylor *et al*., 2007)	Brazil	NEO
1	FJ876448	*Hepatozoon* sp. MRA-2009-5	*Speothos venaticus* (Lund, 1842) (Bush dog)	Unknown (André *et al*., 2010)	Brazil: MT, Cuiaba	NEO
2	GQ926901	*Hepatozoon* sp. ex *Prionailurus planiceps*	*Prionailurus planiceps* (flat-headed cat)	Unknown (see Taylor *et al*., 2007)	Thailand	O

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	HQ734805	*Hepatozoon* sp. E16119cp	*Chalcides polylepis* Boulenger, 1890 (Many-scaled cylindrical skink)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Douar Caid	A
3	HQ734797	*Hepatozoon* sp. 129ea	*Eumeces algeriensis* Peters, 1864 (Algerian orange-tailed skink)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: El Guelb	A
3	HQ734796	*Hepatozoon* sp. 127ea	*Eumeces algeriensis* (Algerian orange-tailed skink)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: close to Ouazzane	A
3	HQ734792	*Hepatozoon* sp. 164pv	*Podarcis vaucheri* (Boulenger, 1905) (Andalusian wall lizard)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Lake Tislit	A
3	HQ734791	*Hepatozoon* sp. 133sp	*Scelarcis perspicillata perspicillata* (Duméril and Bibron, 1839) (Moroccan rock lizard)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Debdou	A
3	HQ734803	*Hepatozoon* sp. pb7413pv	*Podarcis vaucheri* (Andalusian wall lizard)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Lake Tislit	A

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	HQ734798	*Hepatozoon* sp. 317am	*Atlantolacerta andreanskyi* (Werner, 1929) (Atlas dwarf lizard)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Oukaimeden	A
3	EU908289	*Hepatozoon* sp. LA81	*Lacerta agilis* Linnaeus, 1758 (Sand lizard)	Tick: *Ixodes ricinus*? (see Majláthová *et al*., 2010)	Poland	P
3	EU430231	*Hepatozoon* sp. 770a	*Amblyomma fimbriatum* Koch, 1844 from *Varanus panoptes* Storr, 1980 (Yellow-spotted monitor)	Tick: *Amblyomma fimbriatum, Amblyomma limbatum*, *Amblyomma moreliae* (see Vilcins *et al*., 2009a)	Australia	AUS
3	EU430236	*Hepatozoon* sp. 786b	*Amblyomma fimbriatum* from *Liasis fuscus* Peters, 1873 (Brown water python)	Tick: *A. fimbriatum, A. limbatum*, *A. moreliae* (Vilcins *et al*., 2009a)	Australia	AUS
3	FJ719813	*Hepatozoon* sp. DG1	*Dromiciops gliroides* Thomas, 1894 (Monito del monte: marsupial)	Tick: *Ixodes neuquenensis* Ringuelet, 1947 (Marín-Vial *et al*., 2007)	Chile	NEO

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	EU430240	*Hepatozoon* sp. 512	*Ixodes tasmani* Neumann, 1899 from *Sarcophilus harrisii* (Boitard, 1841) (Tasmanian devil)	Tick: *Ixodes tasmani* (Vilcins *et al*., 2009b)	Australia	AUS
3	GU344682	*Hepatozoon* sp. turkey vulture/Oklahoma/2009	*Cathartes aura* Linnaeus, 1758 (Turkey vulture)	Not reported (Velguth *et al*., unpublished)	U.S.A.: Oklahoma	NEO
3	-	*Hepatozoon fitzsimonsi* (sample 10)	*Chersina angulata* (Schweigger, 1812) (Angulate tortoise)	Tick: *Amblyomma marmoreum* Koch, 1844, *Amblyomma sylvaticum* (de Geer, 1778) (Horak *et al*., 2006a)		A
3	AF176837	*Hepatozoon catesbianae*	*Rana catesbeiana* Shaw, 1802 (American bullfrog)	Not reported (Mathew *et al.,* 2000) Direct between bullfrogs, mosquitoes: *Culex territans* (Desser *et al*., 1995)	U.S.A.	NEO
3	HM585204	*Hepatozoon* sp. CCS-2010 isolate V9	*Varanus salvator komaini* Nutaphand, 1987 (Black water monitor)	Not reported （Salakij *et al*., unpublished a)	Thailand	O
3	AY252109	*Hepatozoon* sp. ex *Varanus scalaris*	*Varanus scalaris* Mertens, 1941 (Spotted tree monitor)	Not reported (Ujvari *et al*., 2004)	Australia	AUS

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	HQ734807	*Hepatozoon* sp. Ipa1tt	*Timon pater tangitana* (Lataste, 1880) (Timon pater: wall lizard)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Foum Kheneg	A
3	AY252106	*Hepatozoon* sp. ex *Varanus panoptes*	*Varanus panoptes* Böhme, 1988 (Argus monitor)	Tick: *A. fimbriatum*, *A. moreliae* (see Vilcins *et al*., 2009a)	Australia	AUS
3	AY252105	*Hepatozoon* sp. ex *Liasis fuscus*	*Liasis fuscus* (Brown water python)	Tick: *A. fimbriatum*, *A. limbatum*, *A. moreliae* (see Vilcins *et al*., 2009a)	Australia	AUS
3	HM585212	*Hepatozoon* sp. CCS-2010 isolate V66	*Varanus salvator salvator* (Laurenti, 1768) (Malayan water monitor)	Not reported (Salakij *et al*., unpublished b)	Thailand	O
3	AF297085	*Hepatozoon* sp. *Boiga*	*Boiga irregularis* (Bechstein, 1802) (Brown tree snake)	Not reported (Ujvari *et al*., 2004)	Australia: southeastern Queensland	AUS
3	EU430233	*Hepatozoon* sp. 797	*Amblyomma moreliae* (Koch, 1867) from *Liasis fuscus* (Brown water python)	Tick: *A. fimbriatum*, *A. limbatum*, *A. moreliae* (see Vilcins *et al*., 2009a)	Australia	AUS

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	AY252111	*Hepatozoon* sp. *Stegonotus cucullatus* isolate 2	*Stegonotus cucullatus* (Duméril, Duméril and Bibron, 1854) (Slaty grey snake)	Not reported (Ujvari *et al*., 2004)	Australia	AUS
3	HQ734787	*Hepatozoon* sp. db486tm	*Tarentola mauritanica* (Linnaeus, 1758) (Moorish gecko)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Algeria: Djebel Guezoul	A
3	HQ734789	*Hepatozoon* sp. db1606qm	*Quedenfeldtia moerens* (Chabanaud, 1916) (Gecko)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Gorges near Guelmin	A
3	HQ292771	*Hepatozoon* sp. 1SP	*Mabuya wrightii* Boulenger, 1887 (Gecko)	Unknown: bugs, flies, or mosquitoes? (Harris *et al*., 2011)	Seychelles: Saint Pierre	A
3	HQ292773	*Hepatozoon* sp. 35SH	*Lycognathophis seychellensis* (Schlegel, 1837) (Seychelles wolf snake)	Unknown: bugs, flies, or mosquitoes? (Harris *et al*., 2011)	Seychelles: Silhouette	A
3	EF157822	*Hepatozoon ayorgbor*	Lab host: *Lamprophis fuliginosus* Boie, 1827 (Brown house snake) Host: *Python regius* (Shaw, 1802) (Ball python)	Mosquito: *Culex quinquefasciatus* (Sloboda *et al*., 2007)	Ghana	A

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	EF620027	*Hepatozoon* sp. WfM	*Peromyscus leucopus* (Rafinesque, 1818) (White footed mouse)	Unknown, but suggested to be a tick *Amblyomma maculatum* (see Johnson *et al*., 2007)	U.S.A.: Oklahoma	NEO
3	HQ734790	*Hepatozoon* sp. pty01po	*Ptyodactylus oudrii* Lataste, 1880 (Oudri's fan-footed gecko)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Algeria: El Kantara	A
3	HQ734806	*Hepatozoon* sp. 3126tm	*Tarentola mauritanica* (Moorish gecko)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Figuig	A
3	HQ734809	*Hepatozoon* sp. q39qm	*Quedenfeldtia moerens* (Gecko)	Unknown: ticks, mites, or mosquitoes? (Maia *et al*., 2011)	Morocco: Ida ou-buzia	A
3	FJ719815	*Hepatozoon* sp. AO12	*Abrothrix olivaceus* (Waterhouse, 1837) (Olive grass mouse)	Tick: *Ixodes neuquenensis* Ringuelet, 1947 (Merino *et al*., 2009)	Chile	NEO
3	EF222259	*Hepatozoon* sp. Squirrel 1	*Sciurus vulgaris* Linnaeus, 1758 (Eurasian red squirrel)	Not reported (Criado-Fornelio *et al*., 2006), possibly tick *Ixodes ricinus* (see Peter *et al*., 2005)	Spain	P

Table 5.1. continued

CLADE	ACCESSION	DEFINITION	HOST	INVERTEBRATE VECTOR	ORIGIN	BIOGEO
3	AY600626	*Hepatozoon* sp. BV1	*Clethrionomys glareolus* (Schreber, 1780) (Bank vole)	Unknown: suggested to be a tick (see Criado-Fornelio *et al*., 2006)	Spain	P
3	HM212627	*Hepatozoon* sp. Fox-Hep3	*Vulpes vulpes* Linnaeus, 1758 (Fox)	Not reported, possibly tick (see Dezdek *et al*., 2010)	Croatia	P

5.4. Molecular analysis of sporocyst stages from tick viscera

Amplification of haemogregarine (sporocyst) DNA was attempted using the same primer sets as described above for the blood film analysis. Tick specimens included originally absolute methanol-fixed Giemsa stained impression slides from *A. sylvaticum*, an unfixed impression slide from *A. marmoreum* (in which an infection was not visible on dissection), along with dissected fresh specimens of both species, subsequently fixed in 70% molecular grade ETOH (Table 5.2 below).

Table 5.2. Tick specimens used for molecular analysis, collected from *Haemogregarina fitzsimonsi* infected and uninfected tortoises, revealing sporocysts on dissection. The sample site and province, tick species and sex, sporocyst numbers, and corresponding peripheral blood infection with parasitaemia (%) of the tortoise hosts are detailed.

Site (province)	Tick species (sex)	Fixative	Sporocyst numbers	Corresponding peripheral blood infection (parasitaemia)
De Mond (WC)	*A. sylvaticum* (F)	Methanol-fixed Giemsa stained impression slide	High (>30)	*H. fitzsimonsi* infected (0.8%)
De Mond (WC)	*A. sylvaticum* (M)	Methanol fixed Giemsa stained impression slide	Low (<30)	Un-infected
De Mond (WC)	*A. sylvaticum* (89)(M)	Fresh (then 70% molecular grade ETOH)	High (>30)	Un-infected
De Mond (WC)	*A. sylvaticum* (83)(F)	Fresh (mol. ETOH)	High (>30)	*H. fitzsimonsi* infected (0.4%)
Britstown (NC)	*A. marmoreum* (M)	Fresh (mol. ETOH)	High (>30)	Un-infected
Britstown (NC)	*A. marmoreum* (M)	Fresh (mol. ETOH)	Low (<30)	Un-infected

5.4.1. *Amblyomma marmoreum*

Following the use of HEMO1 and HEMO2 primers on DNA extracts of this tick species, as well as 4558F and 2733R primers, no bands appeared when PCR products were run on agarose gels (Fig. 5.5 A, C – both lane 5). Conversely, there was a strong band of ~600 bp from a seemingly uninfected impression slide of *A. marmoreum* following the use of HEPF300 and HEPR900 primers (Fig. 5.5 B). A follow up attempt using the same HEP primers on dissected

specimens fixed in molecular ETOH, which were identified as being infected, produced a very faint band of ~600 bp (Fig. 5.6 C - lane 7).

Unfortunately, the sequenced products were unsatisfactory in all cases and therefore could not be compared readily with published *Hepatozoon* sequences, although the fact that the HEPF300 and HEPR900 primers especially when first used (Fig. 5.5 B – lane 5) gave a strong band on agarose gel suggested that parasite DNA was present in this tick.

5.4.2. *Amblyomma sylvaticum*

In a similar manner to *A. marmoreum*, the use of HEMO1 and HEMO2 primers on *A. sylvaticum* DNA extracts, as well as 4558F and 2733R primers, produced no bands when PCR products were run on agarose gels (Fig. 5.5 A, C – both lanes 1 and 2). However, unlike *A. marmoreum*, no results were obtained using the HEPF300 and HEPR900 primers either (Fig. 5.5 B). A follow up attempt using HEP primers on dissected specimens of *A. sylvaticum* fixed in 70% ETOH, revealed a faint band (Fig. 5.6 A). A further attempt using fresh dissected material, identified as being infected and subsequently fixed in molecular ETOH, produced a strong band with an adult female *A. sylvaticum* (83) (Fig. 5.6 C - lane 9).

The sequenced products were again unsatisfactory and therefore could not be compared with published *Hepatozoon* sequences. However, the fact that use of HEPF300 and HEPR900 primers resulted in bands of ~600bp from both *A. marmoreum* and *A. sylvaticum* suggested that both species of tick may be vectors for *H. fitzsimonsi*. It seems likely that *H. fitzsimonsi* is not intermediate tortoise host specific (see Chapter 4, section 4.1), perhaps because it is not vector specific either.

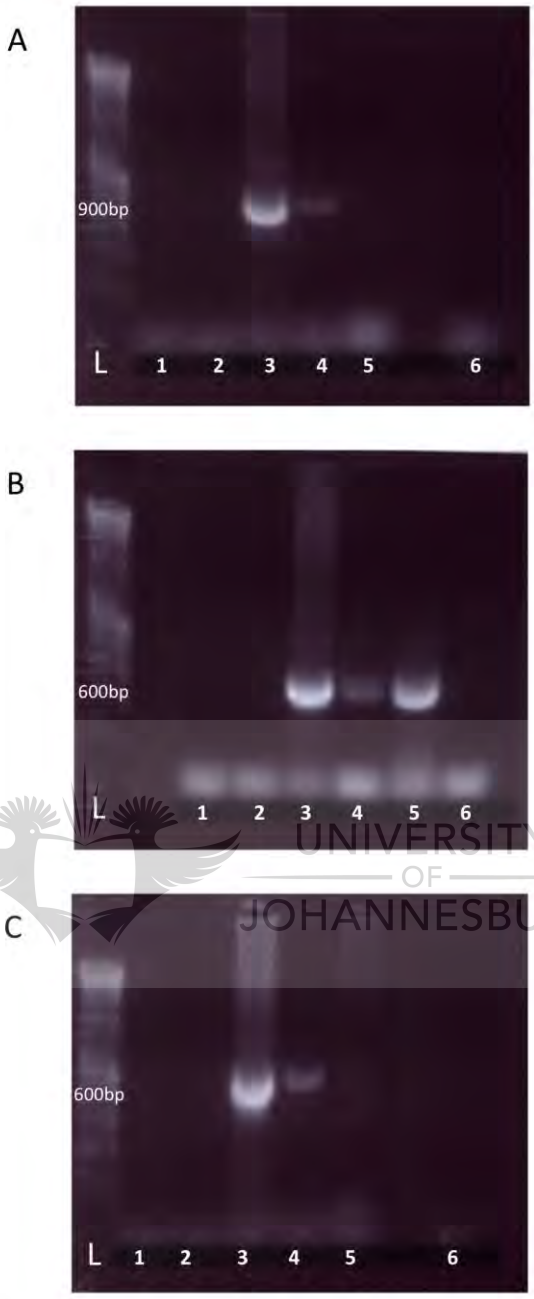


Figure 5.5. Amplification of haemogregarine DNA using **(A)** HEMO1 and HEMO2 primers. Marker Ladder (L). Lanes 1 – 2: Material derived from methanol fixed, Giemsa stained impression slides from *Amblyomma sylvaticum* (de Geer, 1778) containing sporocysts, no bands, 3 – 4: from methanol fixed, Giemsa stained, *Haemogregarina fitzsimonsi* Dias, 1953 infected blood slides, a strong band produced with 3.3% parasitaemia, fainter with 0.8%, 5: from an impression slide from *Amblyomma marmoreum* Koch, 1844 (unfixed), no band, 6: negative control **(B)** HEPF300 and HEPR900 primers. Marker Ladder (L). Lanes 1 – 2: Material derived from impression slides from *A. sylvaticum* fixed in 10% NBF containing sporocysts, no bands, 3 – 4: from methanol fixed, Giemsa stained *H. fitzsimonsi* infected blood slides, stronger bands than in (A), 5: from impression slide from *A. marmoreum* (unfixed), strong band, 6: negative control **(C)** 4558F and 2773R primers. Marker Ladder (L). Lanes 1 – 2: Material derived from impression slides from *A. sylvaticum* fixed in 10% NBF containing sporocysts, no bands, 3 – 4: from methanol fixed, Giemsa stained *H. fitzsimonsi* infected blood slides, bands such as in (A), 5: from an impression slide from *A. marmoreum* (unfixed), no band, 6: negative control.

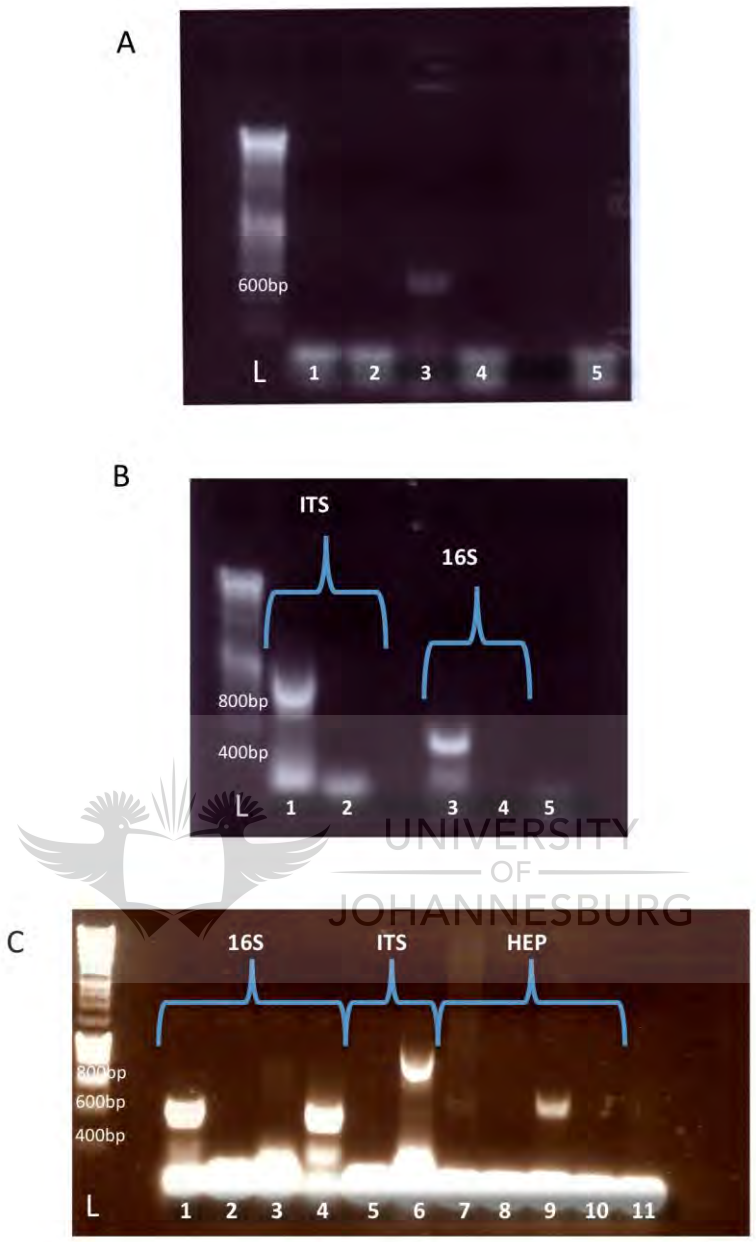


Figure 5.6. Amplification of haemogregarine (A, C) and tick DNA (B - C) using **(A)** HEPF300 and HEP900 primers. Marker Ladder (L). Lanes 1 – 4: Material derived from dissected viscera from *Amblyomma sylvaticum* fixed in 70% ETOH, faint band in (3), 5: negative control, **(B)** tick primers, ITS2F and ITS2R and 16SF and 16SR. Marker Ladder (L). Lanes 1 – 2: Material derived from dissected *Amblyomma marmoreum* and *A. sylvaticum*, amplified with ITS primers respectively, band in lane 1, 3 – 4: from dissected *A. marmoreum* and *A. sylvaticum*, amplified with 16S primers respectively, band in lane 3, 5: negative control, **(C)** tick primers 16S and ITS and *Hepatozoon* primers HEP. Marker Ladder (L). Lanes 1 – 4: derived by amplifying with 16S primers material from dissected *A. marmoreum* and *A. sylvaticum*, strong bands with *A. marmoreum* (lane 1) and *A. sylvaticum* (89) (lane 4), 5 – 6: amplified with ITS2, material derived from dissected *A. marmoreum* and *A. sylvaticum*, strong band with *A. sylvaticum* (89) (lane 6), 7 – 9: HEP primers with dissected *A. marmoreum* and *A. sylvaticum,* very faint band with *A. marmoreum* (lane 7) and a stronger band with *A. sylvaticum* (83) (lane 9), 10: amplified with HEP primers and derived from methanol fixed, Giemsa stained blood slide containing 0.004% *Haemogregarina* sp. A, no band, 11: negative control.

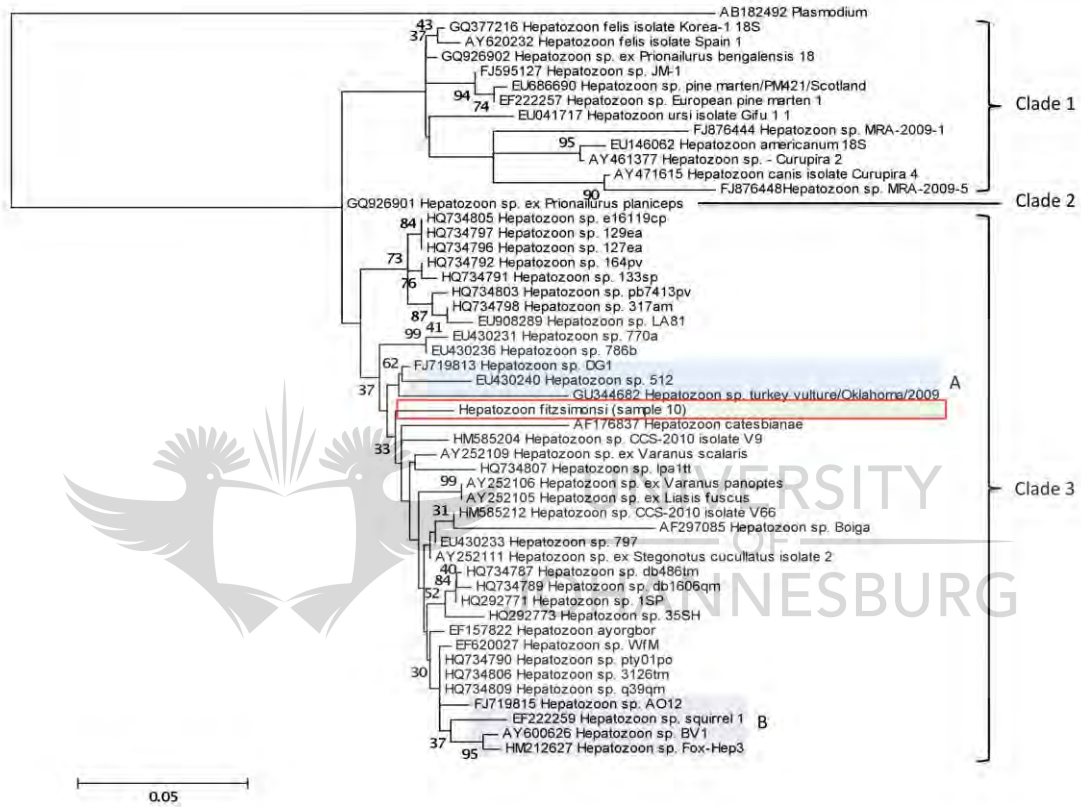


Figure 5.7 A. Maximum Likelihood (ML) analysis phylogram based on 18S ribosomal RNA gene sequences of *Hepatozoon* species listed in Table 5.1, using the Tamura 3-parameter + Gamma model of molecular evolution. Numbers beside and above branches are the ML bootstrap values. *Haemogregarina fitzsimonsi* Dias, 1953 clusters within the major Clade 3, which includes mostly *Hepatozoon* species that infect reptiles. The outgroup is represented by *Plasmodium ovale*.

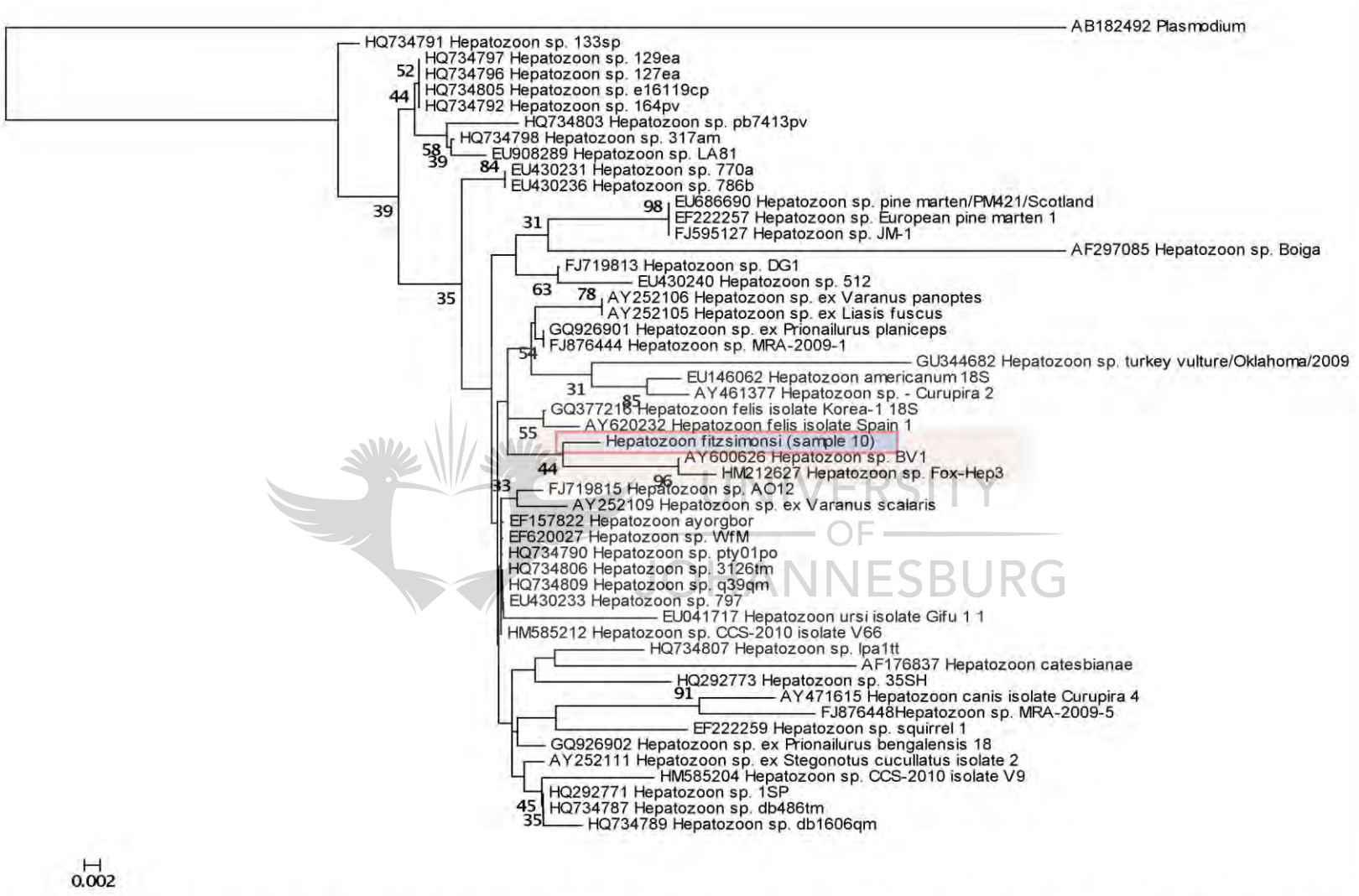


Figure 5.7 B Neighbour Joining (NJ) analysis phylogram based on 18S ribosomal RNA gene sequences of *Hepatozoon* species listed in Table 5.1, using the maximum composite likelihood. Bootstrap values are shown beside and above branches. *Haemogregarina fitzsimonsi* Dias, 1953 can be seen within the same clade as species of *Hepatozoon* infecting species of Palaeartic vole and fox. The outgroup is represented by *Plasmodium ovale*.

5.5. Molecular analysis of tick species, *Amblyomma marmoreum* and *A. sylvaticum* using ITS2 and mitochondrial 16S primer sets

Since little is known about the phylogenetic relationships of African tortoise ticks, and because both may be vectors of *H. fitzsimonsi*, the molecular sequences of these ticks were also analysed. Since the ITS1 and ITS2 regions (Fig. 5.4) are highly variable due to the commonality of insertions, deletions and point mutations (Barta, 1997), and they may be effective for discerning between morphologically similar species (see Fukunaga *et al*., 2000), this marker was included within these analyses. Even though the 16S gene is conservative there are regions which are variable, allowing for differentiation of vertebrate species (Vences *et al*., 2005), and within ticks has proven a good marker for determining relationships between closely related tick species (Mangold *et al*., 1998). The 16S gene was thus included in the current analyses, for comparison with the ITS2 region (the ITS1 region was not employed). The primers for ITS2 later proved far more effective than those for 16S because a suitable useable alignment for 16S that would have provided repeatable phylogenetic results could not to be constructed and thus ITS2 sequences aided in determining the relatedness of *A. marmoreum* and *A. sylvaticum* to other *Amblyomma* species (see below).

5.5.1. *Amblyomma marmoreum*

Both ITS2 and 16S primer sets and the PCR conditions outlined in Chapter 3, amplified tick DNA of ~800 bp and ~600 bp lengths respectively, extracted from an unfixed impression slide of *A. marmoreum* (Fig. 5.6 B). A further attempt with both primer sets using fresh material subsequently fixed in molecular grade ETOH amplified DNA only with the 16S primer set (Fig. 5.6 C – lane 1). PCR products yielding the best bands were dispatched for sequencing at the Natural History Museum, but only 1TS2 sequences proved suitable for drawing phylogenetic trees.

When ITS2 region PCR products were sequenced, and the sequences were compared against others from *Amblyomma* species, phylogenetic analyses placed this tick species within a clade of the genus *Amblyomma*, as predicted. The species was seen within the ML tree (Fig.

5.8 A) to be closely related to *A. sylvaticum* (see below), both lying close to *Amblyomma humerale* infesting species of mammal from Brazil (Marrelli *et al*., 2007) (Table 5.3). The NJ tree (Fig. 5.8 B) also indicated relatedness to *Amblyomma rotundatum* infesting species of lizard (Labruna *et al*., 2005a).

5.5.2. *Amblyomma sylvaticum*

The first attempt at extracting DNA sequences from dissected 70% ETOH fixed tick legs and bodies did not produce any results using primer sets ITS2 or 16S (Fig. 5.6 B). A second attempt with ITS2 and 16S primer sets using fresh, molecular ETOH fixed, whole ticks revealed results of both ~800bp and ~600bp respectively for the two primer sets when PCR products were run on agarose gels (Fig. 5.6 C – lanes 4 and 6 respectively). Again, however, only ITS2 products produced sequences suitable for creating phylogenetic trees, since 16S trees would not resolve.

As above, *A. sylvaticum* lay close to *A. marmoreum* in both ML and NJ trees, both with a 100% confidence limit as shown by the bootstrap values (Fig. 5.8 A, B).

Table 5.3. Tabulation of tick species appearing within the Maximum Likelihood (ML) and Neighbour Joining (NJ) trees (Figs. 5.8 A, B respectively) (ITS2 regions). Included below are the clades (as seen in Fig. 5.8 A, B), the GenBank (NCBI) accession numbers and tick species, with the corresponding vertebrate host order and origin. Tick authorities are based on those from Barker and Murrell (2004).

CLADE	ACCESSION	TICK SPECIES	HOST ORDER	ORIGIN
OUTGROUP	AF271274	*Dermacentor nitens* Neumann, 1897	Mammalia (Taylor *et al*., 2007)	U.S.A.
1	AF469611	*Amblyomma areolatus* [syn. *A. aureolatum* (Pallas, 1772)]	Reptilia (lizard) (Labruna *et al*., 2005a)	Brazil: Sao Paulo
1	AY887110	*Amblyomma coelebs* Neumann, 1899	Mammalia (Marrelli *et al*., 2007)	Brazil: Teodoro Sampaio, Sao Paulo
1	AY887116	*Amblyomma dubitatum* Neumann, 1899	Mammalia (Marrelli *et al*., 2007)	Brazil: Rosana, Sao Paulo
1	DQ006841	*Amblyomma rotundatum* Koch, 1844	Reptilia (lizard) (Labruna *et al*., 2005a)	Brazil
1	AY887111	*Amblyomma humerale* Koch, 1844	Mammalia (Marrelli *et al*., 2007)	Brazil: Monte Negro, RO
1	-	*Amblyomma sylvaticum* (de Geer, 1778)	Reptilia (Horak *et al*., 2006a)	South Africa
1	-	*Amblyomma marmoreum* Koch, 1844	Mammalia, Reptilia (Horak *et al*., 2006a)	South Africa
2	AF548539	*Amblyomma americanum* (Linnaeus, 1758)	Mammalia, Aves (Taylor et al., 2007) (Reichard *et al*., 2005)	USA: eastern Oklahoma
2	AY887115	*Amblyomma oblongoguttatum* Koch, 1844	Mammalia (Labruna *et al*., 2000)	Brazil: Monte Negro, RO
2	AF469605	*Amblyomma cajennense* (Fabricius, 1787)	Mammalia (Taylor *et al*., 2007)	Brazil
2	AY887118	*Amblyomma parvum* Aragão, 1908	Mammalia, Aves (Olegário *et al*., 2011)	Brazil: Bataguassu, MS
2	AY995180	*Amblyomma pseudoconcolor* Aragão, 1908	Mammalia (Marrelli et al., 2007) (Chacon *et al*., 2004)	Brazil: Bataguassu, MS
2	AY887114	*Amblyomma triste* Koch, 1844	Mammalia (Venzal *et al*., 2008)	Brazil: Bataguassu, MS

Table 5.3. continued

CLADE	ACCESSION	TICK SPECIES	HOST ORDER	ORIGIN
3	AF199112	*Amblyomma vikirri* Keirans, Bull and Duffield, 1996	Reptilia (skink) (Keirans *et al*., 1996)	Australia
3	AY887117	*Amblyomma varium* Koch, 1844	Mammalia (Onofrio *et al*., 2008)	Brazil: Sorocaba, Sao Paulo
3	AY887119	*Amblyomma ovale* Koch, 1844	Mammalia (Martins *et al*., 2012)	Brazil: Araraquara, Sao Paulo
3	AY887120	*Amblyomma longirostre* (Koch, 1844)	Mammalia (Barros-Battesti *et al*., 2005)	Brazil: Sao Paulo
3	AY887121	*Amblyomma naponense* (Packard, 1869)	Mammalia (Labruna *et al*., 2002)	Brazil: Monte Negro, RO
3	AY619576	*Amblyomma incisum* Neumann, 1906	Mammalia (Szabó *et al*., 2009)	Brazil
3	AY619572	*Amblyomma latepunctatum* Neumann, 1906	Mammalia (Labruna *et al*., 2005b)	Brazil
3	AY619575	*Amblyomma scalpturatum* Neumann, 1906	Mammalia (Labruna *et al*., 2005b)	Brazil

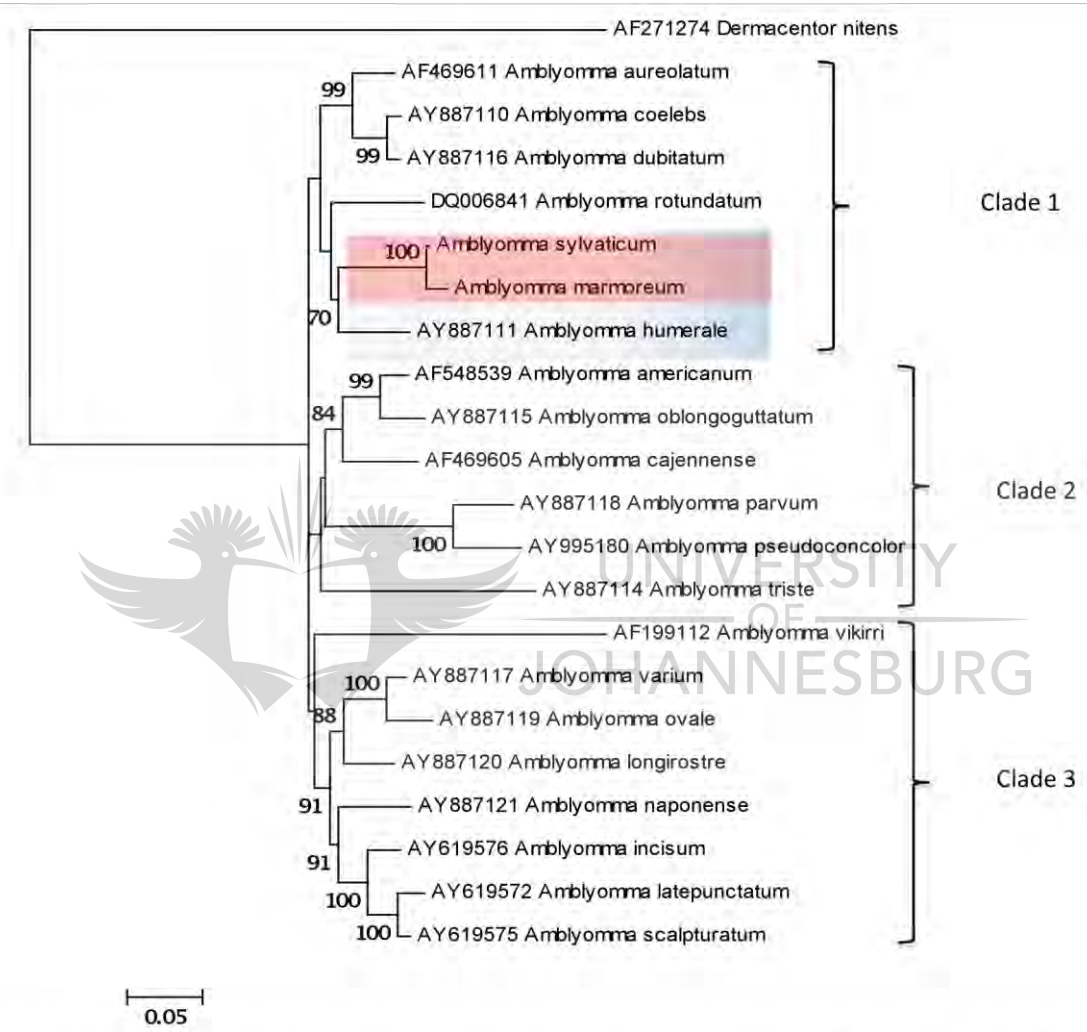


Figure 5.8 A. Maximum likelihood (ML) analysis phylogram based on ITS2 (internal transcriber region) sequences of ticks listed in Table 5.3, using the GTR + G general time reversal model + Gamma distribution model of molecular evolution. Numbers beside and above branches are the ML bootstrap values. *Amblyomma marmoreum* and *Amblyomma sylvaticum* group within the same clade as the Neotropical *Amblyomma humerale*. The outgroup is represented by *Dermacentor nitens*.

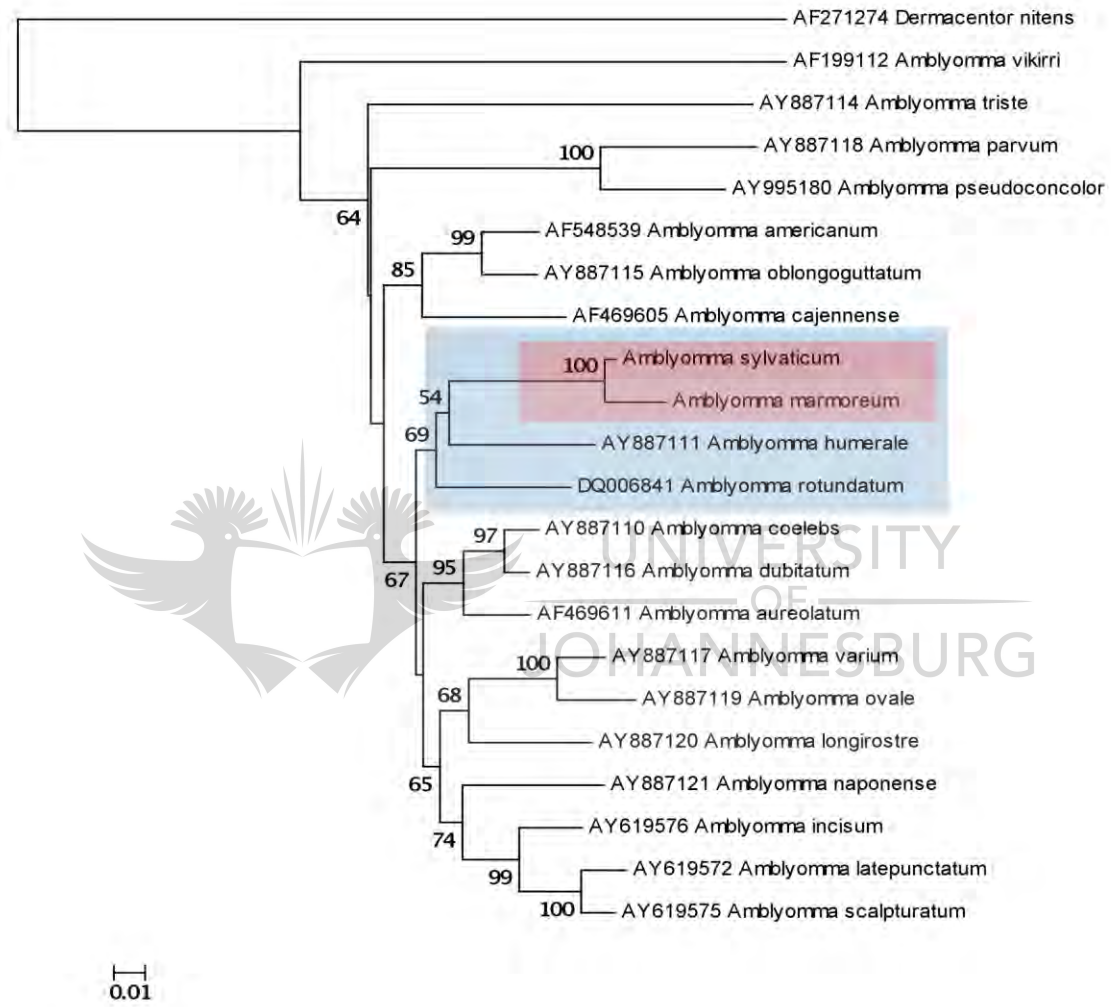


Figure 5.8 B. Neighbour Joining (NJ) analysis phylogram based on ITS2 (internal transcriber region) sequences of ticks listed in Table 5.3, using the Jukes Canter model. Bootstrap values are shown beside and above branches. *Amblyomma marmoreum* and *Amblyomma sylvaticum* group into a clade with *Amblyomma humerale* and *Amblyomma rotundatum*. The outgroup is represented by *Dermacentor nitens*.

5.6. Taxonomic summary

***Haemogregarina* (sensu lato) *fitzsimonsi* Dias, 1953, now *Hepatozoon fitzsimonsi* (Dias, 1953) (see Discussion, below)**

Vertebrate type host: *Kinixys belliana belliana* Gray, 1831.

Vertebrate hosts from this study and Cook *et al*. (2009a): *Chersina angulata* (Schweigger, 1812), *Kinixys belliana belliana*, *K. lobatsiana* (Power, 1927) *K. natalensis* Hewitt, 1935 and *Stigmochelys pardalis* (Bell, 1828) (Testudinidae, Cryptodira).

Type locality: Maputo, Mozambique.

Localities in this study and Cook *et al*. (2009a): Gauteng (National Zoological Gardens, Pretoria; Johannesburg Zoological Gardens, Johannesburg; private collections, Johannesburg); Kwazulu-Natal (Crocodile Creek, Crocodile Valley, Flag Animal Farm; Mkuze Nature Reserve); North West (Rustenburg, rural markets); Western Cape (Hondeklip Bay, Velddrif; Paternoster; West Coast conservancy; De Hoop Nature Reserve; De Mond Nature Reserve; Butterfly World, Paarl).

Site of infection: Peripheral blood.

Vector: likely ticks of both *Amblyomma marmoreum* and *Amblyomma sylvaticum* species.

Deposition of voucher specimens: Protozoan collection of the South African Museum, Cape Town, South Africa (blood film of *C. angulata* with mature gamonts, SAM A25092; blood film of *K. b. belliana* with mature gamonts, SAM A25093; blood film of *K. lobatsiana* with mature gamonts, SAM A25094; blood film of *K. natalensis* with mature gamonts, SAM A25095; blood film of *S. pardalis* with trophozoites, probable meronts, merozoites, immature gamonts and mature gamonts, SAM A25096).

5.7. Discussion

Haemogregarina fitzsimonsi Dias, 1953 during Siddall's (1995) cladistic partial revision of the haemogregarine complex, was placed along with haemogregarines of terrapins, thus becoming *Haemogregarina* (sensu stricto). However, a leech vector would likely transmit such haemogregarines, this process probably occurring during blood feeding. A good example of a haemogregarine infecting a terrapin is *Haemogregarina stepanowi* Danilewsky, 1885. Its type host, *Emys orbicularis* (Linnaeus, 1758), when bitten by an infected leech, *Placobdella catenigera* Blanchard, 1893, presents with haemogregarine division stages in the peripheral blood. Stages include secondary erythrocytic meronts, along with gamonts (Telford, 2009). Division stages are thus

a common occurrence within the peripheral blood. However, *H. fitzsimonsi* rarely has such stages and the probability of land tortoises being fed on by an infected leech vector is very low, given the terrain most infected tortoises inhabit. Previously, these observations led to the conclusion that *H. fitzsimonsi* probably did not belong to the genus *Haemogregarina* (see Cook, 2008; Cook *et al*., 2009a).

A terrestrial tortoise haemogregarine, transmitted by a tick vector, and which is more comparable to *H. fitzsimonsi*, is *Hemolivia mauritanica* (Sergent and Sergent, 1904). In this latter species, division stages, such as meronts, were not commonly reported within the peripheral erythrocytes of the tortoise type host, *Testudo graeca* (Linnaeus, 1758) (see Ŝiroký *et al*., 2007), as with *H. fitzsimonsi*. It was therefore suggested previously that *H. fitzsimonsi* could be a species of *Hemolivia* (see Cook *et al*., 2009a). However, current ultrastructural studies of *H. fitzsimonsi* gamonts showed that they were very different from mature gamonts of *Hemolivia mariae* Smallridge and Paperna, 1997 and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1990 which have an encasing wall (see Paperna and Smallridge, 2001; Boulard *et al*., 2001, respectively).

Hepatozoon, a common haemogregarine genus infecting reptiles (see Telford, 2009), was an alternative. A characteristic of the genus is that it lacks, or forms few division stages in the peripheral blood of the intermediate host and may form oocysts and sporocysts in the definitive host haemocoel (see Davies and Johnston, 2000). Both these features appeared to apply to *H. fitzsimonsi*. Sporocysts and sporozoites were found in the haemocoel of ticks collected from *H. fitzsimonsi* infected and uninfected tortoises and it seemed likely that these stages were the sporogonic stages of *H. fitzsimonsi*. Since oocysts were not observed in the current study, it was difficult to differentiate *H. fitzsimonsi* from stages of *Hemolivia*, in which oocysts usually are characteristically stellate in form (Landau and Paperna, 1997; Paperna, 2006), or of *Haemogregarina* species which have oocysts containing eight or more naked sporozoites (Davies and Johnston, 2000; Telford, 2009). However, the sporozoites of *H. fitzsimonsi* are likely covered by a thick, non-staining sporocyst wall and are thus very different from those of *Haemogregarina* spp., which lack sporocysts (see Davies and Johnston, 2000)

The decision to attempt amplification of *H. fitzsimonsi* DNA using HEMO1/HEMO2, HEPF300/HEPR900 and 4558F/2773R primers was determined by the above information, because this might establish once and for all, the genus to which *H. fitzsimonsi* belongs. Genetic analyses proved successful and 18S *H. fitzsimonsi* sequences clearly matched with those of other *Hepatozoon* species.

In correlating Fig. 5.7 A to Table 5.1, Clades 1 and 2, unlike Clade 3 (the group into which *H. fitzsimonsi* falls), do not group with *Hepatozoon* species infecting reptiles. Clades 1 and 2 comprise mammalian *Hepatozoon* species respectively, which are mostly tick transmitted, whether directly or indirectly through predation of prey animals. *Hepatozoon* species of mammals have been found infecting mostly rodents and carnivores (Kubo *et al*., 2008). Clades 1 and 2 consist of only mammalian carnivorous animals (Table 5.1). Also none of the infected host animals grouped in Clades 1 and 2 originate from Africa or Australia.

Clade 3 (Fig. 5.7 A) (Table 5.1), on the other hand, groups together mostly reptilian *Hepatozoon* species (including *H. fitzsimonsi*), with 27/36 (75%) reptiles, 7/36 (19%) mammals, a bird at 1/36 (3%) and an amphibian at 1/36 (3%). In Clade 3, where known, most *Hepatozoon* species have a tick or mosquito invertebrate vector (Table 5.1) and the mammal examples are likely to be transmitted by tick vectors.

Reptile hosts of those *Hepatozoon* species grouped in Clade 3 originate from the continents of Africa and Australia as well as the islands of the Seychelles (Table 5.1). African hosts in Table 5.1 include species of gecko (HQ734787, HQ734789, HQ734790, HQ734806, HQ734809), lizard (HQ734792, HQ734791, HQ734803, HQ734798, HQ734807) and skink (HQ734805, HQ734797, HQ734796), the *Hepatozoon* of which are likely transmitted by ticks, mites or mosquitoes (Maia *et al*., 2011). The hosts from the Seychelles include a species of gecko (HQ292771) and snake (HQ292773), and the vectors of these *Hepatozoon* species are suggested to be species of bug, fly or mosquito (Harris *et al*., 2011). The *Hepatozoon* species of Australian monitor lizards (EU430231, AY252109, AY252106) and snakes (EU430236, AY252105, EU430233, AY252111) also grouped in Clade 3 are transmitted by ticks of the genus *Amblyomma* (Table 5.1).

The African tortoise parasite, *H. fitzsimonsi*, is also likely transmitted by species of *Amblyomma* (see below). However, the above Australian intermediate host species are all predatory (Reptile Database, 2012), feeding on prey animals or insects, while the South African tortoises infected with *H. fitzsimonsi* are largely herbivorous, though not always so (Chapter 2, section 2.2.3). Terrestrial tortoises (Testudinidae) have never, according to the fossil record, inhabited Australia or Melanesia (Auffenberg, 1974). Such tortoises are thought to have dispersed throughout the African continent only ~60myr. bp. (Parham *et al*., 2006), long after the separation of Gondwana and the possible divergence of tick vectors and *Hepatozoon*. The evolution of *Hepatozoon* species within reptiles (snakes and lizards) and *Amblyomma* ticks from Australia, and African tortoises and the same tick genus, may thus be examples of convergent evolution. *Hepatozoon* spp. are therefore suggested to

have invaded tortoise hosts opportunistically in Africa long after its evolution in ticks and with tick divergence across continents of what was Gondwana.

The possible old phyletic age of the members of *Hepatozoon* is well demonstrated by species found infecting the American 'living fossil' marsupial *Dromiciops gliroides* Thomas, 1894 (Monito del monte) transmitted by the tick vector *Ixodes neuquenensis* Ringuelet, 1947 (Marín-Vial *et al*., 2006) (Table 5.1). Unlike the current research (see Fig. 5.7 A and Table 5.1), a phylogenetic study by Merino *et al*., (2009), grouped *Hepatozoon* species from American marsupial 'Monito del monte' and Australian marsupials into different clades. It was therefore suggested that *Hepatozoon* may have existed in ticks and marsupials before the separation of the continents making up Gondwana (Merino *et al*., 2009), South America from Antarctica and Australia ~>100myr. bp. (see Hedges, 2010), and finally Antarctica and Australia, 60 – 70myr. bp (Springer *et al*., 1998). This again, is long before tortoises are thought to have invaded the African continent. Also, the lineages of *Hepatozoon* infecting 'Monito del monte' appear to be host-specific, suggesting that these *Hepatozoon* lineages may be ancient (Merino *et al*., 2009).

In the past there has been contention regarding the host-specificity of *Hepatozoon* species (see Telford *et al*., 2001; Vilcins *et al*., 2009a). The question thus arises whether it could be that phyletically old lineages of *Hepatozoon*, such as those of 'Monito del monte', are more species-specific than those of a phyletically younger age, such as the *Hepatozoon* identified infecting reptiles and associated ticks of Australia by Vilcins *et al*. (2009a)? This may be a possible trend worth investigating in the future.

The tortoise species' *H. fitzsimonsi* might be expected to group in the ML tree more closely with *Hepatozoon* species of African reptiles, rather than between *Hepatozoon* of the American turkey vulture (GU344682) and bullfrog (AF176837) (Table 5.1). Chelonian *Hepatozoon* species may be only distantly related to those of other reptile lineages, possibly as a result of the phyletic age of chelonians. As mentioned in Chapter 2, the oldest known member of the order Testudines (Batsch, 1788) is dated to ~200myr. bp, with terrestrial tortoise (Testudinidae Gray, 1825) fossils collected from 175 – 161myr. bp. (Danilov and Parham, 2008). Ticks, on the other hand, are proposed to have originated 120myr. bp. (Murrell *et al*., 2001). The molecular clock for apicomplexans indicates that vertebrates were not their original hosts (Escalante and Ayala, 1995) (see Chapter 2, section 2.3.3.), and may have been, in this case, ticks. Therefore, the current relationship between the tick vector and the vertebrate tortoise host may have evolved at the most 120myr. bp. However, as tortoises appeared in Africa only ~60myr. bp., the *Hepatozoon* species infecting these animals may have been

the result of an opportunistic invasion by the parasite which had already evolved in species of *Amblyomma* and diverged across continents more than 100myr. bp. as explained above regarding 'Monito del monte'. *Amblyomma marmoreum* is known to infest mammals as well as other species of reptiles (Horak *et al*., 2006a) and therefore this species or a similar one may have served as the origin of African tortoise *Hepatozoon* infections. The infection may subsequently have been transmitted to tortoise-specific tick species such as *A. sylvaticum*. It would be of interest, in future, to sequence haematozoans of other species of *Amblyomma* from South American tortoises. Nonetheless, as stated in Chapter 2, reptiles show both a well-adapted host-parasite relationship with their haematozoans (Jacobson, 2007), as well as a great diversity of haematozoans (Telford, 2009). This implies again that the relationship between parasites such as *Hepatozoon* and their hosts is likely older than more recent.

In the NJ tree (Fig. 5.7 B), *H. fitzsimonsi* grouped together with species infecting the Palaearctic fox (HM212627) and vole (AY600626). Besides this dubious result and the lack of congruence, congruency dominant in the ML tree, the NJ tree also presented with lower bootstrap values. The differences in apparent relatedness between the two trees may be explained by the difference in the models used to construct the trees. Models used to construct the topology of NJ trees calculate the pairwise genetic difference between sequences and are thus based on summarised changes based on genetic distance. This is an indirect method compared to the models used in construction of the ML tree, which use nucleotide differences in the sequence data (Bos and Posada, 2005).

Additionally, only a single ribosomal gene (18S) was employed for *H. fitzsimonsi* in both these instances (for ML and NJ tree construction), and essentially only a fragment of that gene. Put simply, evolutionary relationships among the above *Hepatozoon* species have been inferred or hypothesized based on the relationship between random copies of fragments of a small subunit gene (Bos and Posada, 2005). Even though the 18S rRNA gene has been extensively used for phylogenetic studies, problems have arisen. The 18S gene cannot always resolve all taxonomic nodes and therefore its efficacy may vary among clades (Meyer *et al*., 2010). In apicomplexan phylogenetic studies, numerous deletions and insertions within the 18S gene may occur, along with an unusual organization of the gene, which may make alignment of sequences difficult (Perkins and Keller, 2001). The limitations of using single gene analysis and the advantages of multi-gene analyses have also been emphasized by Meyer *et al*., (2010) as more reliable in tracing ancestral events or relationships. Therefore, more research is required in future into the use of other genes in

combination, such as other nuclear genes, mitochondrial and plastid genes (Wilson and Williamson, 1997), before the evolutionary relationships of *Hepatozoon* species may be fully understood.

It was anticipated that *H. fitzsimonsi* DNA would be extracted from ticks infected with sporocysts and sequenced as this haemogregarine species. Distinct bands on agarose gels were produced with HEPF300/HEPR900 primers, but unfortunately PCR products failed to yield clean sequences. Since HEP primers produced gel bands with both tick types, it does suggest strongly that *Hepatozoon* DNA was present in these, and *H. fitzsimonsi* may be transmitted by both *A. marmoreum* and *A. sylvaticum*. *Amblyomma sylvaticum* is mostly restricted to the coastal regions of the NC, WC, Eastern Cape (EC), and parts of Namaqualand (NC) (see Fig. 2.6), being for the most part host-specific to *C. angulata* (see Horak *et al*., 2006a). *Amblyomma marmoreum*, conversely, may be found throughout most of South Africa (Fig. 2.6). If *A. marmoreum* is a vector, this would explain the wide geographical range and tortoise host range of *H. fitzsimonsi*. The ML (Fig. 5.8 A) and NJ (Fig. 5.8 B) phylogenetic trees for these ticks, drawn from ITS2 sequences, suggest that these two *Amblyomma* species are closely related, increasing the likelihood that *H. fitzsimonsi* can infect both tick species. Furthermore, Clade 1 in the ML tree, in which the two tick species are grouped, comprises 4/8 (50%) reptile tick species, even though the other two hosts are lizards from South America (AF469611, DQ006841) (Table 5.3). Clade 2 (Table 5.3), includes no reptile hosts and Clade 3 only one, a species of skink from Australia (AF199112).

Clade 1 (Fig. 5.8 A; Table 5.3) of the ML tree groups together *Amblyomma* from South America and South Africa, Clade 2 only species from South America and finally Clade 3 from both South America and Australia. As mentioned before, South America, Africa and Australia were all included along with Antarctica and India into the super-continent Gondwana (Hedges, 2010). Australia and Antarctica broke away from the continental mass comprising both South America and Africa ~100myr. bp. (Hedges, 2010), suggesting divergence of tick species at this time, possibly with their *Hepatozoon* parasites (see Merino *et al*., 2009).

The ITS2 region is an intronic section of highly variable rRNA, important during the processing of more conservative genes (such as 5.8S, 18S and 28S), but is not itself a gene (Hunter *et al*., 2007). Thus, 'evolutionary/genetic noise' may be apparent (see Raser and O'Shea, 2005). The ITS regions compared to the genes themselves are therefore subject to more mutations (see Raser and O'Shea,

2005; Hunter *et al*., 2007). ITS2 is thus of better use in population genetics, differentiating amongst very closely related organisms, than in phylogenetic studies (see Caisová *et al*., 2011). Further work could thus employ nucleic ribosomal 18S and 28S gene sequences and/or mitochondrial *cox* 1 or 12S.

It appears that *H. fitzsimonsi* is neither intermediate host nor vector host-specific, perhaps with the ability to infect novel tortoise and tick hosts. Since tortoises are popular in the exotic pet trade, and are not necessarily kept free of ectoparasites in these circumstances, there may be reason for concern for their welfare. In the past, large numbers of reptiles were imported into Florida (U. S. A.) infested with *A. marmoreum* (see Burridge *et al*., 2000; Burridge and Simmons, 2003). A population of *A. marmoreum* established itself in Florida, both in and around a reptile breeding facility (Allan *et al*., 1998; Burridge *et al*., 2000; 2002). Thus, *H. fitzsimonsi* could also spread, conceivably, to Floridian tortoises such as *Gopherus polyphemus* (Daudin, 1802).

The identification of *H. fitzsimonsi* as a species of *Hepatozoon* allows speculation about its life cycle, which may be similar to that of *Hepatozoon* from other reptile species (see Bashtar *et al*., 1984; Telford, 2009). Since no other tortoise *Hepatozoon* species or life cycles have been described to date (see Davies and Johnston, 2000; Telford, 2009), life cycle speculation for *H. fitzsimonsi* relies mostly on those *Hepatozoon* species of snakes and lizards, with a brief comparison to *Hm. mauritanica*. Unlike snakes and lizards which are carnivorous and insectivorous, it is unclear how largely herbivorous tortoises might ingest a tick vector to perpetuate the life cycle. It may be that at resting sites shared by others of its species, a tortoise may feed on surrounding vegetation which harbours infected ticks that have dropped off previous tortoise visitors. The tortoise may therefore ingest infected ticks, itself becoming infected. After ingestion of the infected tick vector, *A. marmoreum* or *A. sylvaticum*, sporozoites may release from sporocysts and invade the intermediate tortoise gut lining, followed by the host blood supply. *Hepatozoon najae* (Laveran, 1902) in the snake host *Naja naja* (Linnaeus, 1758) (syn. *Naja tripudians*) lodges in capillary lining cells, *Hepatozoon aegypti* Bashtar, Boulos and Mehlhorn, 1984 infecting the snake *Spalerosophis diadema* (Schlegel, 1837) lodges in lung capillaries, and *H. najae* and *Hepatozoon kisrae* Paperna, Kremer-Mecabell and Finkelman, 2002 from the agamid host *Laudakia stellio* (Linnaeus, 1758) occur in epithelial cells of the spleen and liver (see Telford, 2009). Sporozoites subsequently likely develop into meronts producing a number of merozoites which erupt from one or more of the above cell types, invading young erythrocytes or other cells. Cyclic merogony may occur within the blood capillary, lung, spleen

and/or liver cells (such as in *Hemolivia mauritanica*, to date the only terrestrial tortoise haemogregarine species for which the life cycle has been elucidated) allowing for perpetuation of the infection within the tortoise host (see Široky *et al*., 2007). In *H. fitzsimonsi*, infected erythrocytes likely enter the peripheral blood in which it is known that the parasite forms a trophozoite stage (see Cook *et al*., 2009a). The trophozoite then matures into either a meront (rarely) or an immature gamont (more commonly) (see Cook *et al*., 2009a). Immature gamonts mature and upon ingestion by the tick vector through a blood meal, exit the digested erythrocyte and then likely penetrate the tick gut wall and enter the haemocoel. In other species such as *H. najae* (see Bashtar *et al*., 1984; Telford, 2009) gamonts likely associate in pairs in a fat body cell of the haemocoel, and this may occur in *H. fitzsimonsi.* Differentiation of micro- and macrogamonts may also occur, allowing for further division of the microgamont into flagellated forms (such as in *H. najae*). Flagellated microgametes may fertilise the macrogamont resulting in a young oocyst. The oocyst may develop within the cells of the haemocoel, as occurs in the mosquito *Culex quinquefasciatus* Say, 1823 (syn. *Culex fatigans*) infected with *Hepatozoon tupinambis* (Laveran and Salimbeni, 1909) of the lizard *Tupinambis teguixin* (Linnaeus, 1758). Oocysts finally develop large numbers of sporocysts, each with with a double-layered, thick wall. These sporocysts are then ready to release sporozoites on ingestion of infected tick by the tortoise host.

Life cycle similarities with that of *Hemolivia mauritanica* are notable. Sporozoites in the present study (derived from ticks) were morphologically and morphometrically similar to those belonging to *Hm. mauritanica*. Infected ticks were mostly adult male, occurring in higher numbers on tortoises than adult females (see Table 5.1), with sporocysts being the dominant stage found within these invertebrates, as in the tick *Hyalomma aegyptium* with *Hm. mauritanica* (Paperna, 2006). It has been proposed that the male ticks are responsible for continuing the infection of *Hm. mauritanica* into the next year (Paperna, 2006), since female ticks die after egg-laying concludes (Taylor *et al*., 2007). It would appear that this suggestion is substantiated in this study, with no infection having been found in engorged larvae or nymphs. Sporocyts were mainly found within adult male ticks (4/6 – 67%), with the exception of 2 engorged adult female ticks (2/6 – 33%) (see Table 5.2).

Except for *Hepatozoon mauritanicum* Sergent and Sergent, 1904 (Levine, 1988), which has since been assigned to the genus *Hemolivia* (see Landau and Paperna, 1997; Davies and Johnston, 2000; Telford, 2009), no other *Hepatozoon* species have been reported from chelonians, especially terrestrial tortoises. This fact makes the findings of the work presented here particularly significant and novel.

CHAPTER 6

MORPHOLOGICAL ANALYSIS AND DESCRIPTION OF HAEMOGREGARINES *HAEMOGREGARINA* (SENSU LATO) *PARVULA* DIAS, 1953 AND AN UNKNOWN SPECIES *HAEMOGREGARINA* SP. A.

During this study, *Haemogregarina parvula* Dias, 1953 has been recorded consistently with *Haemogregarina fitzsimonsi* Dias, 1953, but infecting only *Kinixys belliana belliana* Gray, 1830, tortoises from KwaZulu-Natal (KZN) (see Appendix 1a). The latter haemogregarine, however, occurs widely in the absence of *H. parvula* (see Chapters 4 and 5). *Kinixys belliana belliana* (syn. *Kinixys belliana zuluensis*) is the type host of *H. parvula*, the species having only once been recorded from another host species, a single *Stigmochelys pardalis* (Bell, 1928) (see Cook, 2008; Cook *et al*., 2009a). This particular infection may have been exceptional due to the tortoise's ill health (it was very thin and had been badly burnt) and its inadequate living conditions (over crowding), which it shared with specimens of *H. parvula* infected *K. b. belliana*.

As mentioned previously, KZN displays the greatest diversity of tortoise haematozoans of all four South African provinces examined (see sections 4.1 and 4.2). In addition to *H. fitzsimonsi*, described in the previous chapter, and *H. parvula*, described below, a previously unknown intraleucocytic *Haemogregarina* sp. A. was also identified within the peripheral blood of two *S. pardalis* (refer to Appendix 1a) from this province, as well as a haemoproteid (see Chapter 7).

Cook *et al*. (2009a) reported on the immature and mature intraerythrocytic gamonts of *H. parvula* in two captive *K. b. belliana*. During the current study, *H. parvula* was for the first time discovered in two wild-caught *K. b. belliana* (refer to Appendix 1a). This new discovery is reported here as well as a detailed ultrastructural description of the *H. parvula* gamonts. This chapter also includes a detailed light microscopy description of the intraleucocytic *Haemogregarina* sp. A. from the blood of two *S. pardalis* caught at the same locality as the *H. parvula* infected *K. b. belliana*. To conclude, the chapter also includes a taxonomic summary for both parasite species and a discussion of the results.

6.1. Morphological analysis of *Haemogregarina* (sensu lato) *parvula* Dias, 1953

6.1.1. Light microscopy observations on tortoise peripheral blood stages of *Haemogregarina* (sensu lato) *parvula* Dias, 1953

(Figures 5.1 m - o; 6.1 o – p)

A single stage, the encapsulated mature gamont (Fig. 5.1 m – o; 6.1 o - p), was seen within the peripheral blood erythrocytes of two *K. b. belliana* collected in the Mkuze Nature Reserve, KZN.

Mature gamonts: measuring 11 ± 0.2 (10.7 – 11.3) µm long by 5.8 ± 0.1 (5.4 – 6.2) µm wide (n = 14), with ill-defined perimeters likely due to an exceptionally thick non-staining capsule (see below) and parasitophorous vacuole, were found either singly (Fig. 5.1 m, top; 6.1 p) or in pairs (Fig. 5.1 m, bottom; Fig. 5.1 n) within mature erythrocytes. Gamont nuclei, staining pale to dark blue, measured 2.3 ± 0.2 (1.9 – 2.6) µm long by 4.2 ± 0.2 (3.9 – 4.5) µm wide (n = 14). Cytoplasm stained very pale blue and lacked granules, or vacuolation. As noted above, *H. parvula* was never recorded as a single infection, but on both occasions occurred concurrently with *H. fitzsimonsi*. Also, it was only observed infecting *K. b. belliana*. Although *H. fitzsimonsi*-infected *S. pardalis* occupied the same area as the infected *K. b. belliana*, *S. pardalis* was apparently not infected with *H. parvula*. In both *K. b. belliana* specimens, *H. fitzsimonsi* and *H. parvula* gamonts could be seen infecting the same erythrocytes (Fig. 5.1 o; 6.1 o). The *H. parvula* gamont in Fig. 5.1 o (arrows) appears to have nuclei occurring at opposite poles, and thus this may represent two gamonts, the one superimposed on the other, facing in opposite directions.

6.1.2. Ultrastructural observations of tortoise peripheral blood stages of *Haemogregarina* (sensu lato) *parvula* Dias, 1953

(Figure 6.2)

The mature gamont stages of *H. parvula* seemed to be fixative resistant as well as stain resistant to some extent, providing a variety of parasite morphologies in transmission electron micrographs (Fig. 6.2 a - c). The *H. parvula* gamont, slender in form, appeared to be enveloped by a large parasitophorus vacuole (PV), with a distinct boundary membrane (Fig. 6.2 a - b). The PV contained a granular matrix, the gamont lying within this (Fig. 6.2 a - b). The gamont, whether in its original form or a distorted form, had an encasing electron dense surface layer, capsule, or wall, terminating anteriorly with a nipple-like suture point (Fig. 6.2 a - c). Such a point was apparently also present posteriorly (Fig. 6.2 b). In most parasites observed the suture had detached from the boundary membrane of the PV, but left an impression in it (Fig. 6.2 a), suggesting shrinkage of the gamont during fixation. No pellicle was visible underlying the surface, fixative-resistant wall of the gamont (Fig. 6.2 b). However, an enlarged view of the gamont (Fig. 6.2 b - c) at its apical end (Fig. 6.2 c), revealed organelles such as micronemes, numerous ribosomes, dense bodies, amylopectin granules and possibly lipid vesicles. Rhoptries were not identified with certainty and the gamont nucleus was very faint (Fig. 6.2 a).

6.1.3. Remarks

Gamonts of *Haemogregarina parvula* observed in this study resembled the size of immature gamonts reported by Cook *et al*. (2009a), which measured 10.9 and 12.1 µm long, by 5 and 6.4 µm wide (n = 2). However, gamonts found in the current study did not lack an observable nucleus like the immature gamonts of Dias (1953) and Cook *et al*. (2009a). It was therefore concluded that the current study's gamonts were mature, even though their nuclei were smaller than those reported by Cook *et al*. (2009a) in mature gamonts, which measured 3.2 µm long by 4.4 µm wide in both *K. b. belliana* and *S. pardalis*. Dias' mature gamonts were 9.2 – 13.2 µm long by 5.7 – 5.9 µm wide, thus slightly longer than both this study's gamonts and those reported in Cook *et al*. (2009a). In all three studies the nucleus appeared at one pole, gamonts were broadly oval, with ill-defined perimeters, and enclosed by a non-staining capsule and parasitophorous vacuole. Parasite cytoplasm, which was difficult to see, appeared to stain pale blue and lacked granules or vacuoles. Host erythrocyte nuclei were also displaced. Thus, the similarities between Dias' *H. parvula*, that of Cook *et al*. (2009a) and the haemogregarine reported here, suggest strongly that they are identical.

The concurrent infections of *H. parvula* and *H. fitzsimonsi* are something of an enigma and in both cases the only stages seen in peripheral blood smears were the gamont stages. A characteristic feature of the mature gamonts of *H. parvula*, separating them immediately at the light microscopy and ultrastructural level from those of *H. fitzsimonsi*, are their resistant capsules. Further characteristic features of these capsules, not shared with *H. fitzsimonsi*, are the presence of suture points, seen by transmission electron microscopy (TEM). The function, however, of these particular points is unknown (see Paperna and Smallridge, 2001; Boulard *et al*., 2001), as discussed later.

As very little was visible through the parasitophorous vacuole and capsule of *H. parvula* at a light microscopy level, this caused concern initially that *H. parvula* was a life cycle stage of *H. fitzsimonsi*. Cook *et al*. (2009a) preferred to retain *H. fitzsimonsi* and *H. parvula* as separate species, even though Siddall (1995) regarded *H. parvula* as a junior synonym of *H. fitzsimonsi.* However, TEM has justified the former action of Cook *et al*. (2009a) by clarifying that what lies within the capsule of *H. parvula* is a mature gamont stage, and that this is very different from that of *H. fitzsimonsi* (as discussed later). It is also remarkably close to mature gamonts of *Hemolivia mariae* Smallridge and Paperna, 1997 from lizards and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1990 of toads in

ultrastructure (Paperna and Smallridge, 2001; Boulard *et al*., 2001), suggesting strongly that *H. parvula* belongs to the genus *Hemolivia*, rather than *Haemogregarina*.

6.1.4. Taxonomic summary

***Haemogregarina* (sensu lato) *parvula* Dias, 1953**

Vertebrate type host: *Kinixys belliana belliana* Gray, 1830.

Vertebrate hosts from this study and Cook *et al*. (2009a): *Kinixys belliana belliana*, *Stigmochelys pardalis* (Bell, 1828), (Testudinidae, Cryptodira).

Type locality: Maputo, Mozambique.

Localities in this study and Cook *et al*. (2009a): KwaZulu-Natal (Crocodile Creek, Flag Animal Farm; Mkuze Nature Reserve, South Africa).

Site of infection: Peripheral blood.

Vector: Unknown

Deposition of voucher specimens: Protozoan collection of the South African Museum, Cape Town, South Africa (blood film of *K. b. belliana* with immature and mature intraerythrocytic gamonts, SAM A25097; blood film of *S. pardalis* with mature intraerythrocytic gamonts, SAM A25098).

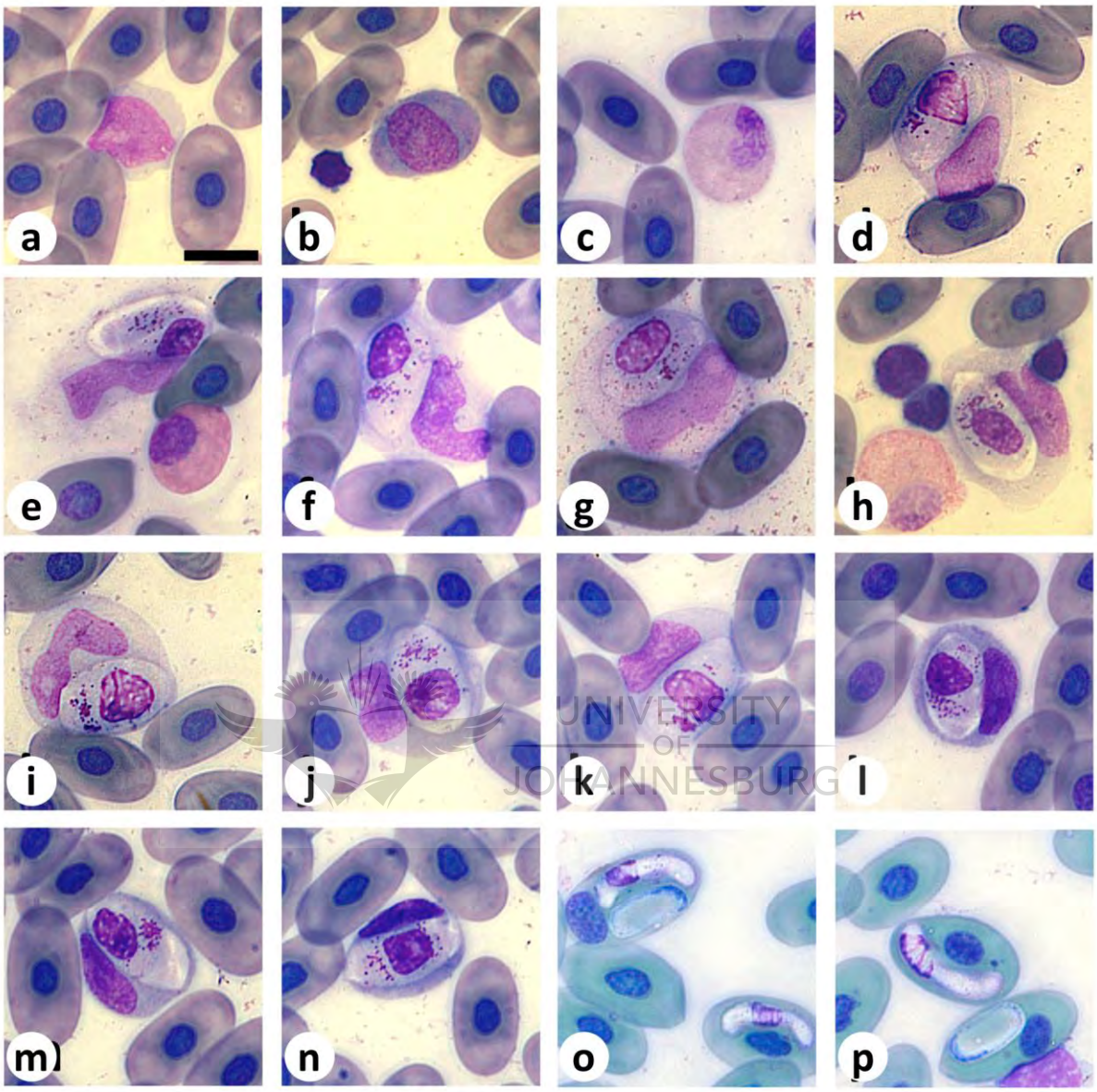


Figure 6.1. Light micrographs of the haemogregarine species recorded infecting South African tortoises, stained with Giemsa stain and some host cells. **(a – c, e, h)** leucocytes found circulating within the peripheral blood. **(a)** monocyte (centre), surrounded by erythrocytes, **(b)** lymphocyte (centre) with a thrombocyte (diagonally left), **(c)** heterophil (centre) **(e, h)** eosinophils (bottom right and left) [cells identified using McArthur *et al*. (2004)], **(d - n)** intraleucocytic haemogregarine recorded infecting two *Stigmochelys pardalis* (Bell, 1828) from Mkuze Nature Reserve, KZN, **(d - k)** appear to be infecting monocytes, **(l - n)** appear to be infecting lymphocytes, **(o - p)** concurrent infection of *Haemogregarina fitzsimonsi* Dias, 1953 (slender form) and *Haemogregarina parvula* Dias, 1953 (globular, encapsulated form). Note the difference in size between haemogregarine forms. Scale bar: 10µm.

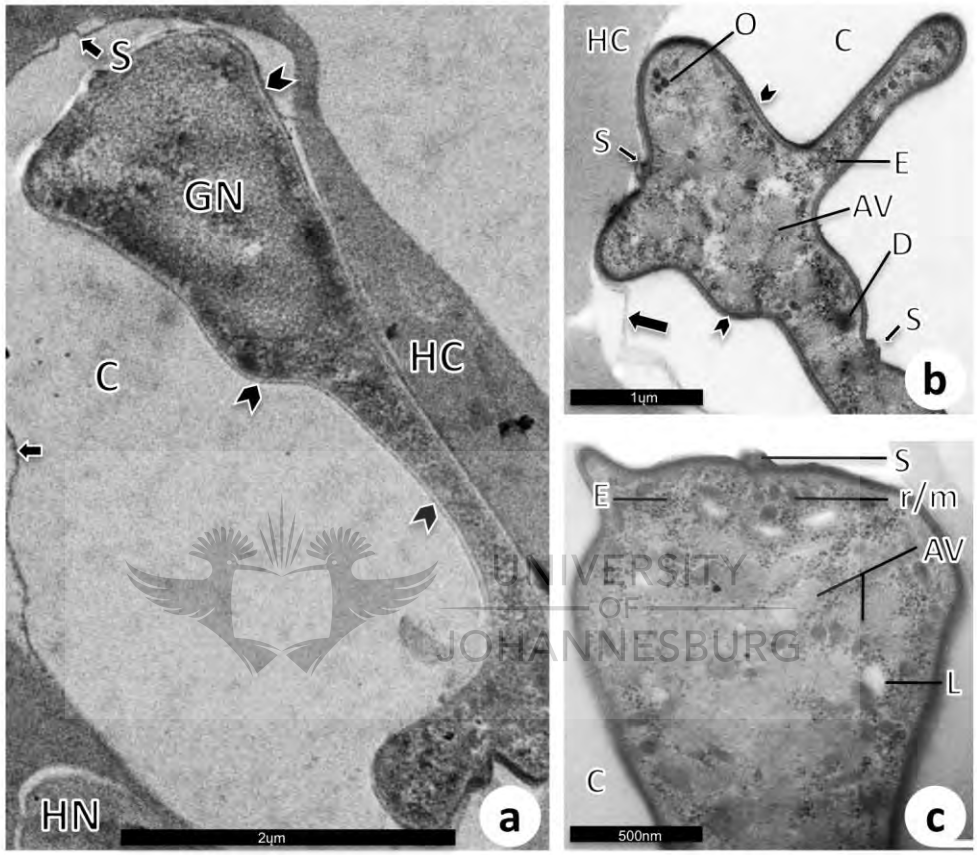


Figure 6.2. Intraerythrocytic mature gamonts of *Haemogregrina parvula* Dias, 1953. **(a)** electron micrograph showing slender form of gamont within a large parasitophorous vacuole, complete with boundary membrane (arrow), top arrow showing suture (S) indent, hard capsule wall (arrow heads) preventing fixation surrounding gamont, (C) granular matrix within the parasitophorous vacuole, (GN) gamont nucleus, (HC) host cytoplasm, (HN) host nucleus. **(b)** cross section through gamont showing both suture sites (S), PV boundary membrane (arrow), hardening capsule wall (arrow heads), (D) electron dense body, (E) ribosomes, (O) osmiophilic body, (AV) either an amylopectin body or vesicle. **(c)** higher power view of gamont with apical zone showing possible (r/m) rhoptries, but more likely micronemes (r/m), and (L) lipid bodies. Scale bars: 2µm, 1µm and 500nm respectively.

6.2. Morphological analysis of the intraleucocytic *Haemogregarina* sp. A.

6.2.1. Light microscopy of tortoise peripheral blood stages of the intraleucocytic *Haemogregarina* sp. A.

(Figure 6.1 a - n)

Only suspected mature gamonts were seen infecting two different types of leucocytes, the monocyte (Fig. 6.1 a) and the lymphocyte (Fig. 6.1 b), from two individuals of *S. pardalis* from Mkuze Nature Reserve, KZN. No other leucocytes, including thrombocytes, heterophils or eosinophils, were found to be infected (Fig. 6.1 b, c, e, h).

Suspected mature gamont: In Giemsa-stained blood films gamonts appeared loosely recurved within monocytes (see Fig. 6.1 e), but mostly they appeared globular within these cells (Fig. 6.1 d, f - k), or within lymphocytes (Fig. 6.1 l - m), perhaps because they were tightly recurved within a constraining parasitophorus vacuole. In some gamonts one pole (anterior?) was slightly rounder than the other, which was more tapered (posterior?) (Fig. 6.1 f, k). Gamonts measured 17.6 ± 0.9 (15.9 - 19.2) µm long, and 9.7 ± 0.8 (8 - 10.6) µm wide, with a surface area of 141.8 ± 12.5 (112.9 - 151.2) μm^2. Anterior to mid-nucleus measurements were 3.6 ± 1.5 (1.4 - 6.3) µm, while posterior to mid-nucleus measurements were 7.2 ± 1.6 (4.8 - 10.3) µm. Nuclear lengths and widths were 7.5 ± 1 (6 - 9) µm and 6.5 ± 1.2 (5.3 - 8.5) µm respectively. The nucleus was deep stained purple, oval, rounded or square in outline and either dense or foamy in appearance. Deep red stained granular pigments formed clusters in the grey-blue stained, occasionally vacuolated, cytoplasm in the vicinity of the nucleus (note Fig. 6.1 m - n), or at some distance from it.

Blood from the tortoises infected with *Haemogregarina* sp. A. was also prepared for TEM and for molecular analysis (see Chapter 5, Fig. 5.6 C, lane 10). However owing to the very low parasitaemia (0.004%), no specimens were found within semi-thin sections and, unlike *H. fitzsimonsi*, it was not possible to extract and amplify sufficient DNA from the blood slides containing the organism, to observe bands on gels, and allow its sequencing.

6.2.2. Remarks

Intraleucocytic haemogregarines from chelonians are rare, none apparently having been described previously from terrestrial tortoises (Levine, 1988; Telford, 2009). *Haemogregarina pseudemydis*

Acholonu, 1974 appears to be the only such species recorded and this from a number of Neotropical terrapin species. These include: *Trachemys scripta elegans* (Wied, 1838) (syn. *Pseukemys scripta elegans*); *Trachemys decorata* (Barbour and Carr, 1940) (syn. *Pseudemys decorate*); *Pseudemys floridana* (Le Conte, 1830) (syn. *Pseudemys floridana hoyi*); *Trachemys stejnegeri* (Schmidt, 1928) (syn. *Pseudemys stejneri*); *Chelydra serpentina* (Linnaeus, 1758) (syn. *Chelydra serpentine serpentine*); *Graptemys pseudogeographica* (Gray, 1831) (syn. *Graptemys kohni*); *Kinosternon subrubrum hippocrepis* Gray, 1855 (syn. *Kinosternon subrubrum hippocrepis*); *Terrapene carolina carolina* (Linnaeus 1758) (syn. *Terrepene carolina carolina*); *Terrapene carolina triunguis* (Agassiz 1857) (*Terrepene carolina triunguis*); and *Apalone spinifera* (Le Sueur, 1827) (syn. *Trionyz spinifer*) (see Acholonu, 1974; Levine, 1988).

Acholonu (1974) described intraerythrocytic and possible extracellular (?) trophozoite, meront and merozoite, and intraerythrocytic gamont stages of *H. pseudemydis*. Trophozoite and intracellular gamont stages were only ever recorded infecting erythrocytes, meront stages were often seen free within the blood usually adhering to erythrocytes. Rarely, merozoite stages were seen developing within leucocytes, however, these were the only recorded stages infecting leucocytes.

In *H. pseudemydis* developing trophozoite stages, u-shaped in form, measured 31.7 x 5.9 µm and developed trophozoites, looped with arms fused, 13.6 x 4.2 µm. Shorter, bean-shaped gamonts measured 14 x 6 µm with a nucleus of 5.1 x 3.2 µm, whilst longer more slender forms (extracellular trophozoites or gamonts?) measured 17.5 x 3.7 µm with a nucleus of 8.4 µm long. Meronts of *H. pseudemydis* were spherical to oval in shape and measured on average 10.9 x 9.7 µm. It is interesting that the younger trophozoite stages of *H. pseudemydis* are, uncharacteristically for haemogregarines (see Telford, 2009), much larger than the developed trophozoite, gamont and meront stages. Both *Haemogregarina macrochelysi* Telford, Norton, Moler and Jensen, 2009 and *Haemogregarina stepanowi* Danilewsky, 1885, well described haemogregarines of terrapins (see Telford, 2009), have similar u-shaped intraerythrocytic forms, but these are identified as gamont stages. Also, the size of these above gamonts resemble that of Acholonu's trophozoite stages, measuring (29 – 35) x (3 - 4.5) µm and (29 - 37) x (3 - 5.5) µm respectively (see Table 2.1, Chapter 2).

Haemogregarina sp. A. compared to *H. pseudemydis* is much larger than the meront and gamont stages, *H*. sp. A measuring 17.6 x 9.7 µm with a nucleus of 7.5 x 6.5 µm and therefore more resembling the larger trophozoite stage. However, as mentioned above, the trophozoite stage of *H.*

pseudemydis was observed infecting only erythrocytes and only merozoite stages occurred within leucocytes.

The most characteristic features of the present *H*. sp. A are its large size, especially its width (up to 10.6 µm), and occurrence within leucocytes. The presumed gamont was seen in some instances to be recurved within the PV, suggesting it to be longer/larger than what for now can be measured. Unfortunately further stages of this parasite have not been observed, which would have been particularly informative, especially if extracellular stages of *H.* sp. A. had been observed for comparison to the extracellular stages of *H. pseudemydis*.

6.2.3. Taxonomic summary

***Haemogregarina* sp. A.**

Vertebrate type host: *Stigmochelys pardalis* (Bell, 1828).
Vertebrate hosts from this study: only *Stigmochelys pardalis* (Testudinidae, Cryptodira).
Type locality: Mkuze Nature Reserve, KwaZulu-Natal, South Africa.
Localities in this study: only Mkuze Nature Reserve, KwaZulu-Natal, South Africa.
Site of infection: Peripheral blood.
Vector: Unknown

6.3. Discussion

During the present study only mature gamont stages were found for both *H. fitzsimonsi* and *H. parvula*, with the exception of possibly trophozoite/merozoite stages of *H. fitzsimonsi* within one *K. b. belliana* from Mkuze (Fig. 5.1 a – c). It was suggested, based on morphological and life cycle traits, and then proved by molecular means in the previous chapter, that *H. fitzsimonsi* belongs to the genus *Hepatozoon*. *Haemogregarina parvula* is more difficult to assess than *H. fitzsimonsi* at a light microscopy level due to its thick non-staining capsule and large parasitophorous vacuole. Thus, even if the parasite is dividing, a characteristic feature of the genus *Haemogregarina,* it might be difficult to observe. The gamonts, however, do share a close resemblance to the gamont stages of another tortoise haematozoan, *Hemolivia mauritanica* (Sergent and Sergent, 1904) (see Široký *et al*., 2007; Cook *et al*. 2009a). Both *Hm. mauritanica* and *H. parvula* share an encysted, stain resistant gamont

with a polar nucleus. The possibility that *H. parvula* is a member of the genus *Hemolivia* and its similarity to *Hm. mauritanica* was discussed by Cook *et al*. (2009a). These authors noted that along with the above characteristics, the gamonts of *Hm. mauritanica* are close in size to those of *H. parvula*. *Hemolivia mauritanica* gamonts observed within the blood of *Testudo graeca* Linnaeus, 1758 and *Testudo marginata* Schoepff, 1792, measured 12 – 15 by 6 µm (see Sergent and Sergent 1904), and 10 – 14 by 4 – 7 µm (Široký *et al*. 2005) respectively. The similarity in morphology and size between the encapsulated gamonts of *H. parvula* and *Hemolivia mauritanica* are therefore striking.

However, proving that *H. parvula* is a member of the genus *Hemolivia* and determining its relationship with *Hm. mauritanica*, will require either detailed life cycle studies like those of Michel (1973), Landau and Paperna (1997) and Široký *et al*. (2004, 2005, 2007), or genetic characterization like that described in the previous chapter for *H. fitzsimonsi*. *Hemolivia mauritanica* remains the only species of the genus *Hemolivia* recorded from terrestrial tortoises, and transferred by a tick vector, *Hyalomma aegyptium* Linnaeus, 1758 (see Široký *et al*. 2007). During his study, Dias (1953) reported the tick *Amblyomma nuttalli* Dönitz, 1909 to parasitize *K. b. belliana*. This tick species has also been recorded infesting *K. b. belliana* from KZN (Horak *et al*., 2006a). It is therefore proposed that *A. nuttalli* may be the vector for *H. parvula*. A necessary requirement for the future would be to collect a larger sample number of *K. b. belliana* in order to obtain specimens of this tick species for histological and molecular characterization.

Transmission electron microscopy observations on *H. fitzsimonsi* (Chapter 5; Fig. 5.2) and *H. parvula* (Fig. 6.2) have shown the two species to be morphologically different and has offered some insight into the morphology of *H. parvula* inside the capsule. Comparing *H. parvula* with ultrastructural studies of *Hemolivia mariae* Smallridge and Paperna, 1997 and *Hemolivia stellata* Petit, Landau, Baccam and Lainson, 1990, indicates a striking resemblance between the mature gamonts of all three species (Paperna and Smallridge, 2001; Boulard *et al*., 2001 respectively). Characteristically all three are long and slender terminating at both ends in a pole complete with a suture. Also typically, all three species are encased by a very thick stain and fixative resistant capsule, enclosed in a parasitophorous vacuole. The thick capsule wall thus resulted in some shrinking of the parasite and a loss of cytoplasmic detail. The above morphological and ultrastructural characteristics and similarities to known *Hemolivia* species of *H. parvula*, suggest strongly that this parasite may be a species of *Hemolivia*, rather than *Haemogregarina*.

The intraleucocytic species, *Haemogregarina* sp. A., is temporarily placed within the genus *Haemogregarina* based on Siddall's (1995) phylogenetic assignment of all chelonian haemogregarines into the genus *Haemogregarina* (sensu stricto), transmitted solely by a leech vector. The parasite is clearly not encased like *H. parvula,* but since it is intraleucocytic and apparently lacks division stages in peripheral blood, it aligns better with the genus *Hepatozoon* (see Davies and Johnston, 2000). Furthermore, even though leeches theoretically might be found on tortoises in Mkuze as a result of its subtropical environment, it is more likely that this haemogregarine species is transmitted by a tick vector, like *H. fitzsimonsi*. As mentioned previously in Chapter 5, it is common to see intraerythrocytic division stages of chelonian *Haemogregarina* within the peripheral blood (see Telford, 2009). Also, gamonts of *Haemogregarina* predominantly infect the erythrocytes (Davies and Johnston, 2000), whilst *Hepatozoon* species infect cells of both the red and white blood cell series (Levine, 1988; Davies and Johnston, 2000). Chapter 5 proved *H. fitzsimonsi* to be a *Hepatozoon*, however, *H.* sp. A is far larger than *H. fitzsimonsi* (and *H. parvula*).

The intraleucocytic haemogregarine also has a granular appearance unlike *H. fitzsimonsi*, but could be a stage of this parasite as they have been recorded occurring concurrently. If this was the case, however, it begs the question as to why it was not recorded by Cook *et al.* (2009a), who found *H. fitzsimonsi* in 35 different individual tortoises from the provinces of Gauteng, KwaZulu-Natal, North West and Western Cape, or within the current study's other 37 tortoises infected with *H. fitzsimonsi* (see Chapter 5). Findings of only what are assumed to be large, mature gamont stages of *Haemogregarina* sp. A in monocytes and lymphocytes, and an apparent lack of merogony within peripheral blood films, typical of the genus *Hepatozoon* (see Davies and Johnston, 2000), suggest that this parasite may be a new species of *Hepatozoon*, but clearly further studies on this unusual haemogregarine are needed to clarify this.

CHAPTER 7

MORPHOLOGICAL ANALYSIS AND DESCRIPTION OF HAEMOPROTEIDS *HAEMOPROTEUS TESTUDINALIS* (LAVERAN, 1905) AND A NEW SPECIES *HAEMOPROTEUS NATALENSIS* COOK, SMIT AND DAVIES, 2010, BOTH AS PUBLISHED IN COOK *ET AL*. (2010)

As recorded in Chapter 4, immature and mature macro- and microgametocyte stages of *Haemoproteus testudinalis* (Laveran, 1905), were found infecting 2/6 (33%) of *Kinixys lobatsiana* (Power, 1927) from the Johannesburg Zoological Gardens, Gauteng Province (GP) during the present study. The infection had been reported previously in both *K. lobatsiana* and *Stigmochelys pardalis* (Bell, 1828) from the North West Province and the National Zoological Gardens, Pretoria (GP) as *Haemoproteus balazuci* Dias, 1953 (see Cook, 2008; Cook *et al*., 2010a), but below, and in the publication by Cook *et al*. (2010a), a strong argument is/was presented that this organism is *Hp. testudinalis.*

In addition to *H. testudinalis*, *Haemoproteus* sp. A, described as *Haemoproteus natalensis* Cook, Smit and Davies, 2010 by Cook *et al*. (2010a), was found infecting 1/4 (25%) of *Kinixys natalensis* Hewitt, 1935 from KwaZulu-Natal (KZN) (see Chapter 4). Cook (2008) reported briefly only two blood stages of this parasite, possible immature and mature gametocyte stages. However, the tortoise harbouring the infection was sampled on two separate occasions, the second revealing additional peripheral blood stages. These additional stages aided in differentiating both immature developmental stages, and mature micro- and macrogametocytes and these are recorded here as well as in the publication, Cook *et al*. (2010a). The stages presented were considered unique enough to allow for differentiation of this species from other tortoise haemoproteids (see Telford, 2009; Cook *et al*., 2010a).

Members of the genus *Haemoproteus* Kruse, 1890 belong to the family Haemoproteidae Doflein, 1916. The genus is characterised by the presence of pigment, haemozoin and, unlike the closely related malarial genus *Plasmodium* Marchiafava and Celli, 1885, its members lack dividing stages in peripheral blood host cells, which may be of the red or white cell series (Lainson and Naiff, 1998). Approximately 450 'malarial' species within 12 genera have been described worldwide, with *Haemoproteus* species included within this group (Perkins and Schall, 2002). Only 12 species of haemoproteids have been reported globally from chelonians (Chapter 2, Table 2.2), five of these from African tortoises. However, until recently, only *Hp. testudinalis* was known from South African tortoises (Laveran, 1905; Cook *et al*., 2010a).

As indicated above, *Hp. testudinalis* was first described by Laveran (1905) from a tortoise (*S. pardalis*) likely captured in the Cape. Probably the same species was later recorded as *Hp. balazuci* by Dias (1953), infecting *Kinixys belliana belliana* Gray, 1830 (syn. *Kinixys belliana zuluensis*) from Mozambique. Additional sampling of South African tortoises (Cook, 2008), and morphological

comparisons in this thesis (see below) and in Cook *et al*. (2010a) revealed that *Hp. testudinalis* and *Hp. balazuci* were likely the same, *Hp. balazuci* thus becoming a junior synonym to *Hp. testudinalis*.

Thus, Chapter 7 aims to provide detailed morphological descriptions of a re-described species and a new species, *Hp. testudinalis* and *Hp. natalensis* respectively, comparing both species to other known tortoise haemoproteids of Africa. The chapter discusses the features that sustain synonymy of *Hp. balazuci* with *Hp. testudinalis*, as well as those which support the new species status of *Hp. natalensis*. In accordance with the regulations for PhD students at the University of Johannesburg (at least one submitted, or published paper, is required for the fulfilment of the degree), the content of this chapter together with some material from an earlier MSc thesis (Cook, 2008) was published in the *Journal of Parasitology* in 2010 (see Cook *et al*. 2010). Cook *et al*. (2010a) thus contains mostly material derived from the current study, but also some data from Cook (2008).

7.1. Re-description of *Haemoproteus testudinalis* (Laveran, 1905) and re-assignment of *Hp. balazuci* Dias, 1953 as published in Cook *et al*. (2010a).

(Figure 7.1)

During this study and that of Cook (2008), peripheral blood forms of *Hp. testudinalis*, including immature stages (Fig. 7.1 a - d) and mature stages of both macro- (Fig. 7.1 e, f) and microgametocytes (Fig. 7.1 g, h), were recorded from two captive *K. lobatsiana* housed at the Johannesburg Zoological Gardens (GP) during winter (June) 2009. The morphology and dimensions of parasites recorded in Giemsa stained peripheral blood films in the present study conform to those described by Cook *et al*. (2010a), as described below:

Immature stages: These were oval, or ring-like, and found rarely, measuring up to 6.0 µm across; they usually occurred singly in host cells. They generally lacked staining overall, but their periphery was sometimes purple-stained; they also lacked pigment granules (Fig. 7.1 a, b). Such stages were not observed during the initial sampling and were thus not described by Cook (2008).

Immature macrogametocytes and microgametocytes: These were rounded (~9.0 µm in diameter) to elongate, measuring 9.5 ± 0.7 (8.0 - 10.8) µm long by 6.5 ± 0.8 (5.3 - 7.9) µm wide; their circumference was 25.9 ± 2.2 (22.5 - 30.3) µm and surface area 50 ± 9.0 (36.7 - 66.8) μm^2 (n = 16); they occurred singly, or in pairs, within erythrocytes, often lying terminally. Immature

macrogametocytes were bluish-stained, with foamy cytoplasm, and had several, mainly peripheral, pigment granules; their nuclei were not often discernible (Fig. 7.1 b) (they were only observed during the current study). Immature microgametocytes stained pale bluish-pink, they had peripheral or scattered pigment granules, pink-stained nuclei, and lay in various positions peripherally within erythrocytes (Figs. 7.1 c, d) (they were observed during the current study and by Cook, 2008 respectively).

Mature macrogametocytes: These were laterally or terminally halteridial, 27.6 ± 3.1 (23.6 - 34.0) µm long by 6.8 ± 0.9 (5.2 - 7.6) µm wide, and had a circumference of 59.5 ± 7.7 (46.3 - 72.0) µm, and a surface area of 155.8 ± 19.1 (130.3 - 190.0) μm^2 (n = 11) (Fig. 7.1 e, f, as seen by Cook, 2008). The cytoplasm was pale blue, foamy or sponge-like with the periphery tending to stain deeper blue; 1 to 8 vacuoles occurred in the cytoplasm, scattered or in clusters, and darkly stained, often bacillus-shaped haemozoin granules (~27), were also scattered randomly (n = 11). A nucleus was not always evident, but stained pale-pink when visible, lying peripherally on outward margin of parasite body (Fig. 7.1 e, f). No parasitophorus vacuole was observed.

Mature microgametocytes: These were terminally or laterally microhalteridial to halteridial, 17.6 ± 3.4 (10.4 – 22.6) µm long by 6.4 ± 1.3 (4.9 – 9.5) µm wide, with a circumference of 40.9 ± 5.1 (33.8 – 51.1) µm, and a surface area of 84.4 ± 12.7 (70.5 – 105.9) μm^2 (n = 10) (Fig. 7.1 g, h, as seen by Cook, 2008). The cytoplasm stained pale pinkish-purple, with the perimeter staining pale purple; 1 or 2 vacuoles, and darkly stained haemozoin granules (~15), generally smaller than in macrogametocytes, lay scattered around the parasite body (n = 13). The nucleus was often pink stained, lying on the outward facing perimeter of parasite body (Fig. 7.1 g, h). No parasitophorous vacuole was evident.

Host cells infected with macro- or microgametocytes resembled mature erythrocytes, although some host cells were more rounded than others; the nuclei of several host cells with microgametocytes stained lighter and were more granular than in uninfected erythrocytes. Additionally, host cells containing both types of gametocytes frequently had an intracytoplasmic, dark-blue-stained, spherical granule, of no fixed location (Fig. 7.1 g, as seen by Cook, 2008).

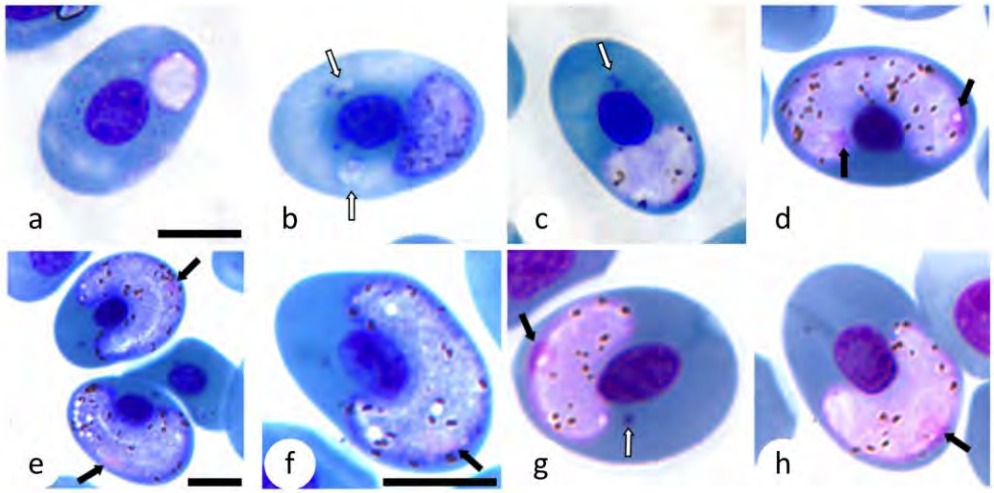


Figure 7.1. Light micrographs of *Haemoproteus testudinalis* (Laveran, 1905) from the peripheral blood of *Kinixys lobatsiana*, and stained with Giemsa, as published in Cook *et al*. (2010). Images a - c are derived from the current study, whereas d - h originate from the MSc thesis, Cook (2008). **(a)** Immature ring stage, **(b)** immature macrogametocyte sharing erythrocyte with two young ring stages (white arrows), **(c)** immature microgametocyte sharing erythrocyte with a young ring stage (white arrow), **(d)** Overlapping paired immature microgametocytes sharing erythrocyte. Note nuclei (black arrows) and pigment granules. **(e - f)** Mature macrogametocytes displaying sponge-like cytoplasm, vacuoles, and prominent bacillus-shaped granules. Nuclei are pale-stained (black arrows). **(g - h) M**ature microgametocytes, lacking a sponge-like cytoplasm, having few vacuoles, and with generally smaller granules but more strongly stained nuclei (black arrows). A spherical granule within the erythrocyte cytoplasm in g (white arrow). Scale bars: 10µm.

7.1.1. Taxonomic summary

***Haemoproteus testudinalis* (Laveran, 1905) (syn. *Haemoproteus balazuci* Dias, 1953)**

Vertebrate type host: *Stigmochelys pardalis* (Bell, 1828) (syn. *Geochelone pardalis*, *Testudo pardalis*) (Testudinidae, Cryptodira).
Vertebrate hosts from this study and that of Dias, 1953 and Cook *et al*., 2010a: *Kinixys belliana belliana* (Dias, 1953) (syn. *Haemoproteus balazuci*), *Kinixys lobatsiana* (Cook, 2008; Cook *et al*., 2010a), *Stigmochelys pardalis* (Cook, 2008; Cook *et al*., 2010a) (Testudinidae, Cryptodira).
Type locality: Cape, South Africa.
Localities in this study and Cook *et al*. (2010a): Johannesburg Zoological Gardens, Johannesburg, Gauteng (GP); National Zoological Gardens, Pretoria (GP) and North West Province (Cook, 2008; Cook *et al*., 2010a), South Africa.
Site of infection: Peripheral blood.
Vector: Unknown.
Deposition of voucher specimens: Protozoan collection of the South African Museum, Cape Town, South Africa (Cat. no. A25099, A25100).

7.1.2. Remarks

Dias (1953) provided a detailed description of *Haemoproteus balazuci* from Mozambican tortoises. Comparisons of the haemoproteid infecting *K. lobatsiana* from Johannesburg Zoological Gardens (GP) in the current study (see Appendix 1b), that from *K. lobatsiana* from the North West Province (NWP) (Cook, 2008), and a haemoproteid from *S. pardalis* from the NWP (Cook, 2008; Cook *et al*., 2010a), with Dias' (1953) *Hp. balazuci* from *K. b. belliana* in Mozambique, indicate their closeness in size, overall appearance, and position within host erythrocytes.

Dias (1953) did not report the circumference or surface area his gametocytes, but macrogametocytes of *Hp. balazuci* were 19.1 - 24.8 μm long by 6.6 - 7.5 μm wide and microgametocytes were 11.9 - 18.8 μm long by 5.9 - 8.9 μm wide. Thus, *Hp. balazuci* macrogametocytes are at the lower end of the length range of the haemoproteids found by the present author in South African *K. lobatsiana* and *S. pardalis* (23.6 - 34.0 μm), but close in width (5.2 - 7.6 μm), while *Hp. balazuci* microgametocytes overlap in both length and width with the South African specimens (10.4 - 22.6 by 4.9 - 9.5 μm). Staining reveals some difference, with

macrogametocytes of *Hp. balazuci* being violet in color, whereas those of the present haemoproteid stain blue. Both species, however, have sponge-like cytoplasm, are vacuolated, have numerous, large pigment granules, and are commonly terminally or laterally halteridial (Dias, 1953; Cook *et al*., 2010a). Microgametocytes are also similar; they are close in shape and position, both stain pink, cytoplasm is uniform rather than foamy, vacuoles are few or lacking, and pigment granules are small and less numerous than in macrogametocytes. The haemoproteid observed by the present author in *K. lobatsiana* and *S. pardalis* from South Africa, therefore, is clearly similar to *Hp. balazuci* from *K. b. belliana* in Mozambique (Dias, 1953).

However, complicating the problem of identifying the haemoproteid in the current study and in Cook (2008) (as discussed in Cook *et al*., 2010a) as *Hp. balazuci* are earlier reports of *Hp. testudinalis* (Laveran, 1905) Wenyon, 1915, in *S. pardalis*, from South Africa (Laveran, 1905). Laveran (1905) described *Hp. testudinalis* from a single *S. pardalis* from the Cape, although the exact origin of the host was not detailed. For *Hp. testudinalis*, immature gametocytes were round and 10.0 – 12.0 µm across, while mature, halteridial stages were 20.0 µm long by 7.0 – 8.0 µm wide, assuming polar or lateral positions within the erythrocytes (Laveran, 1905). In addition, Laveran (1905) illustrated dark, elongate, pigment granules within the cytoplasm of *Hp. testudinalis*; he observed parasites of two types, one staining bluer than the other, and noted that host cells changed little in volume during parasite maturation. Furthermore, no division stages were detected. *Haemoproteus testudinalis* is thus also similar in overall size, position, and appearance to the present haemoproteid and they apparently share a host, *S. pardalis*.

If the type host originated from the Cape, it might be expected that *Hp. testudinalis* would be observed during the current study in *S. pardalis* from the Western Cape Province (WC) (n = 30) and the Northern Cape Province (NC) (n = 1). However, it was not. Laveran's observations were made more than a century earlier than in the current study, and therefore the parasite fauna of the area may have undergone significant changes during this timescale. Also, the season in which Laveran (1905) recorded *Hp. testudinalis* is unknown and thus cannot be easily compared with observations the current study. Interestingly, *Hp. testudinalis,* now regarded as synonymous with *Hp. balazuci* (see below), was not observed in *K. b. belliana* from Kwazulu-Natal (KZN) in the current study, or by Cook (2008) (both observations published in Cook *et al*., 2010a), although *Hp. balazuci* was originally described from this host in Mozambique (Dias, 1953).

Reports of *Haemoproteus roumei* (Bouet, 1909) Wenyon 1915, in *K. b. belliana* from the Ivory Coast, West Africa (Bouet, 1909) also further complicate the issue. *Haemoproteus roumei* was originally identified as *Plasmodium roumei* by Bouet (1909), and then as *Haemocystidium roumei* (1911). It has rounded or elongated macrogametocytes 12.6 µm in diameter, or 16.2 µm long by 10.8 µm wide, and spherical or oval microgametocytes 9.0 µm in diameter, or 14.4 µm long by 3.6 µm wide (Bouet, 1909). It thus overlaps in size with *Hp. testudinalis*, though it tends to be rounder and does not attain such length, and it occurs in the same host as *Hp. balazuci*, namely, *K. b. belliana*. França (1911) noted in the same host tortoise (*K. b. belliana*) that macrogametocytes had vacuolated cytoplasm and black pigment granules and lacked a visible nucleus; he also observed pale-stained microgametocytes with small pigment granules and lacking vacuoles. These features also resemble those of *Hp. testudinalis*. The most recent record of *Hp. roumei* was a short report by Joyeux (1913), who found it in 19 of 34 *K. b. belliana* from French Guinea, Africa, although it was also listed by Rousselot (1953), Bray (1964), Lainson and Naiff (1998), and others.

Clearly, the three known land tortoise *Haemoproteus* species from Africa, as well as the species described here (reported by Cook *et al*., 2010a), share features in size and appearance, as well as sharing some hosts. It was thus recommended that *Hp. testudinalis*, *Hp. balazuci*, and the haemoproteid from this study (also noted in Cook, 2008), all from southern Africa, should be considered identical and, because the first-named species predates the second, they should all be identified as *Haemoproteus testudinalis* (Laveran, 1905) Wenyon, 1915 (syn. *Haemoproteus balazuci* Dias, 1953) (see Cook *et al*., 2010a). It is also suspected that *Hp. roumei*, from West Africa, may also align with *Hp. testudinalis*, but at present it is still retained as a separate species until further examples of it can be traced and re-examined.

7.2. Description of *Haemoproteus natalensis* Cook, Smit and Davies, 2010 as published in Cook *et al*. (2010a).

(Figure 7.2)

Haemoproteus natalensis, a species new to science, was recorded infecting the erythrocytes of a single captive *K. natalensis* from KZN. It was not observed in any other South African tortoise species sampled in KZN or anywhere else in the country. Details of its peripheral blood stages, as seen by light microscopy, appear below. All images were taken from the second sampling occasion (spring, September 2006) of this tortoise host, the first observations being recorded in Cook (2008). Thus, the unique stages from the second sampling do not appear in Cook (2008). Unfortunately, additional

samples of this parasite from other *K. natalensis* have not been located as yet and the parasite was thus described from the available material.

Immature stages: These were rounded (Fig. 7.2 b - c) to amoeboid (Figs 7.2 a, c - f), 6.8 ± 1.4 (4.1 - 9.1) µm long by 4.1 ± 0.9 (2.9 - 6.1) µm wide, with a circumference of 18.7 ± 3.5 (12.3 - 24.2) µm, and a surface area of 22.5 ± 7.5 (8.7 - 38.6) μm^2 (n = 17); 1 to 2 such stages occurred within a single erythrocyte and the stages of growth present apparently allowed immature macro- and microgametocytes to be distinguished (Fig. 7.2 c - f). The cytoplasm of amoeboid forms probably destined to become macrogametocytes stained pale pinkish-blue with a pinkish-purple perimeter, and lacked vacuoles, but had a few, mostly marginal granules, occasionally occurring in clusters (Fig. 7.2 b, d). The cytoplasm of amoeboid stages likely to become microgametocytes, was pink-stained with deep pink margins, lacked vacuoles and largely lacked granules (Fig. 7.2 a, c, e, f).

Mature macrogametocytes: These were elongated, halteridial and 31 ± 3.7 (24.9 - 35.5) µm long by 7.1 ± 1.8 (4.2 - 9.3) µm wide, with a circumference of 63.5 ± 5.3 (55.7 - 70.3) µm and a surface area of 142.5 ± 16.5 (109.2 - 171.2) μm^2 (n = 13); they were also circumnuclear, 40 ± 4.1 (36.7 - 47.3) µm long and 6.9 ± 1.0 (5.8 - 9.3) µm wide, with a circumference of 84 ± 10 (74.2 - 98.8) µm and a surface area of 221 ± 65.1 (160.3 - 305.0) μm^2 (n = 6) (Fig. 7.2 g). Cytoplasm stained deep blue with prominent vacuoles (~12) and clusters of coarse brownish pigment granules were present (>20). The nucleus was rarely clearly defined and no parasitophorous vacuole was evident.

Mature microgametocytes: These were elongated, halteridial and 10.1 ± 3.0 (6.5 - 15.0) µm long by 7.4 ± 0.8 (5.9 - 8.0) µm wide, with a circumference of 30.3 ± 4.9 (26.1 - 39.4) µm and a surface area of 58.9 ± 10.0 (45.5 - 72.3) μm^2 (n = 6); they were also circumnuclear, 20.4 ± 2.7 (16.9 – 25.8) µm long and 7.3 ± 1.0 (5.3 - 10.1) µm wide, with a circumference of 47.1 ± 4.9 (39.1 - 58.4) µm and a surface area of 87.3 ± 13.5 (69.2 - 117.2) μm^2 (n = 19) (Fig. 7.2 c, e, h). Cytoplasm stained deep pink-purple with a few prominent vacuoles (~8) and clusters of small, brownish-red pigment granules (>20). No nucleus or parasitophorous vacuole was observed in these stages.

Mature gametocytes (Fig. 7.2 c, e, g - h), did not appear to alter the shape or size of their host cells greatly, inducing only some lengthening and narrowing of these cells.

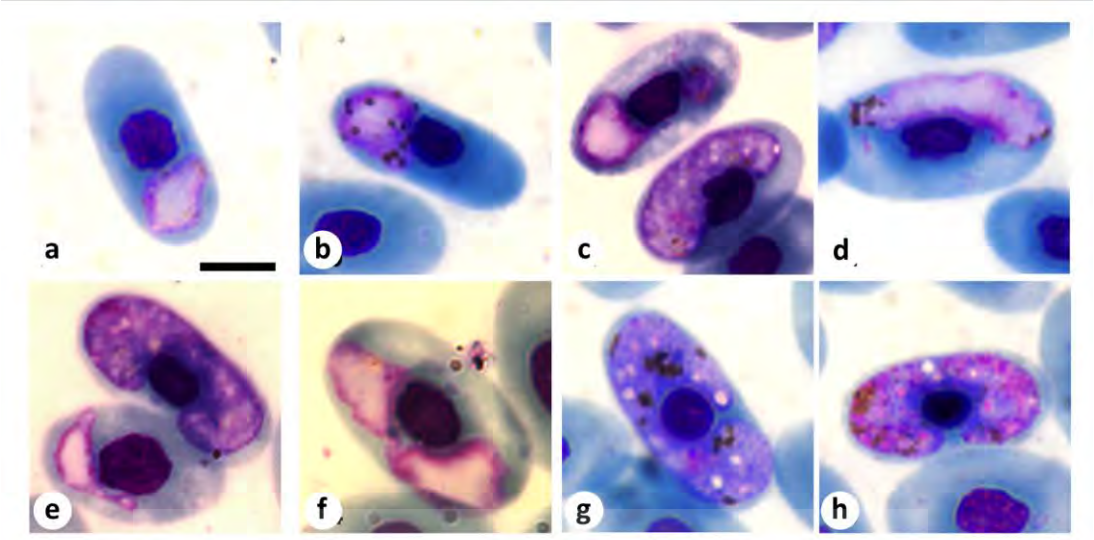


Figure 7.2. Light micrographs of *Haemoproteus natalensis* n. sp. from the peripheral blood of a captive *Kinixys natalensis*, and stained with Giemsa, as published in Cook *et al.* (2010). **(a – b)** Immature amoeboid or rounded stages, the latter containing rounded pigment granules, **(c)** paired immature stages (upper host cell) and developing halteridial microgametocyte in adjacent erythrocyte, **(d)** large, pale, amoeboid stage, of possibly a developing macrogametocyte, **(e)** maturing microgametocyte in host cell adjacent to immature amoeboid stage, **(f)** two immature pink-staining amoeboid stages, of possibly developing microgametocytes, **(g – h)** mature circumnuclear macrogametocyte (note the coarse granules) and microgametocyte, respectively. Scale bar: 10µm.

7.2.1. Taxonomic summary

***Haemoproteus natalensis* Cook, Smit and Davies (2010)**

Vertebrate type host: *Kinixys natalensis* Hewitt, 1935 (Testudinidae, Cryptodira).

Type locality: Ballito, KwaZulu-Natal (KZN), South Africa.

Site of infection: Peripheral blood.

Vector: Unknown.

Prevalence: 1/4 (25%) *K. natalensis* collected (3 captive, 1 wild).

Parasitaemia: ~15% of erythrocytes (captive *K. natalensis*).

Etymology: The species is named for the type host.

Deposition of voucher specimens: Protozoan collection of the South African Museum, Cape Town, South Africa (Cat. no. A25101).

7.2.2. Remarks

Prevalence of this parasite was very low, in 1/4 (25%) of *K. natalensis*, as reported in Cook *et al*. (2010a). However, its description was possible based on the high parasitaemia (~15% of erythrocytes) found in the infected tortoise when it was sampled for a second time. Morphologically, *Hp. natalensis* is much larger than *Hp. testudinalis* (syn. *Hp. balazuci*) and *Hp. roumei* (Bouet, 1909) (see above and Cook *et al*., 2010a). The amoeboid features of the immature stages and the circumnuclear form of the mature macrogametocyte are not typical of most chelonian haemoproteids (see Telford, 2009), but more typical of such species in birds (Valkiūnas *et al*., 2007). Also, during general tortoise sampling from 2006 – 2008 (Cook, 2008), as reported in Cook *et al*. (2010a) and the current study (spring and summer, September - November 2009), *Hp. natalensis* has only been recorded infecting a captive *K. natalensis* in sub-tropical Ballito, KZN. This is unlike *Hp. testudinalis* which has been observed infecting *K. lobatsiana* (Cook *et al*., 2010a) and *S. pardalis* (Laveran, 1905; Cook *et al*., 2010a) from South Africa, in particular, from the relatively arid North West and Gauteng Provinces (Cook *et al*., 2010a) and in *K. b. belliana* from sub-tropical Mozambique (Dias, 1953). So far, *Hp. roumei* has been recorded from *K. b. belliana* only in West Africa (Bouet, 1909; França, 1911; Joyeux, 1913).

Haemoproteus natalensis in comparison with both *Hp. testudinalis* and *Hp. roumei* appears to be rather host species-specific as well as locality specific and the above observations suggest strongly

that *Hp. natalensis* is a different species from the others known from African terrestrial tortoises (as published in Cook *et al.,* 2010a).

7.3. Discussion

The hosts of *Hp. testudinalis* now apprear to include two new species of tortoises, *K. lobatsiana* (this study and Cook *et al*., 2010a) and *K. b. belliana* (see Dias, 1953), as well as the type host *S. pardalis* (Laveran, 1905; Cook *et al*., 2010a). The geographic range of the haemoproteid also appears to have been extended from possibly the South African Cape (Laveran, 1905), to the North West Province (Cook, 2008; Cook *et al*., 2010a) and Gauteng Province (Cook *et al*., 2010a and this study) and Mozambique (Dias, 1953). However, recent molecular analyses of haemosporidian DNA sequences suggest that morphologically similar species, distributed over a large geographical area, may form genetic lineages that are more localized (Martinsen *et al*., 2006). This implies that even though *Hp. testudinalis* and *Hp. balazuci* are seemingly morphologically identical, they may still prove in future to be different genetically. It will be interesting to determine whether this, in fact, proves to be the case.

In contrast, the distinct morphological differences in *Hp. natalensis* in comparison to other tortoise haemoproteids found in Africa, suggest a possible early divergence of a parasite species that is now very restricted, as a result of its particular ecological requirements (intermediate and definitive hosts), to a very specific area (KZN). In birds, species of *Haemoproteus* and *Leucocytozoon* may show considerable association to a single resident bird fauna/population, suggesting that these genera are species and locality specific, and thus introductions of such parasite genera into new populations from one biogeographical zone to another, are likely slow processes from an evolutionary perspective (Hellgren *et al*., 2007). Such events may determine that *Hp. natalensis* is now to be found only in a particular area of KZN; unfortunately however, the tortoise in which the infection was recorded was a captive individual, and its exact origin is unknown. This poses a considerable problem in acquiring more samples and applying molecular techniques, such as those detailed in Chapter 5 for *Haemogregarina fitzsimonsi*. It is known that morphology alone is not particularly good at elucidating phylogenetic and evolutionary relationships among species (Perkins and Schall, 2002). It is therefore important in future to locate wild specimens with *Hp. natalensis* so that this species may be afforded the chance to be genetically and phylogenetically assessed.

At present, only the 'morphological similarity' concept can describe these parasite species. Indeed, a new haemoproteid species had not been reported from South African tortoises since the study of Laveran (1905), until *Hp. natalensis* was observed and described in the current research (as in Cook *et al*., 2010a). Molecular knowledge and techniques will need to be greatly advanced before it is possible to extract DNA efficiently from type specimens without their destruction, if further wild specimens cannot be obtained. For the time being, *Hp. balazuci* from Mozambique has been synonymised with the apparently widespread South African *Hp. testudinalis* based on the great morphological similarities, and *Hp. natalensis* has been described on its distinctive morphology and its host and locality specificity. Furthermore, of great importance is the need to discover the vector and determine the transmission of the above haemoproteid species. It is suggested, based on haemoproteid species accounts detailed by Telford (2009), that the vectors might be species of dipteran biting flies. Specimens of these insects will thus need to be looked out for and collected in future for histological examination.

CHAPTER 8

HAEMOGREGARINA FITZSIMONSI DIAS, 1953 AS A FUTURE BIO-INDICATOR FOR TORTOISE POPULATION STRESS/'HEALTH', BIOLOGICAL TAGGING USING SOUTH AFRICAN TORTOISE HAEMATOZOANS AND THE FEASIBILITY OF METAL-FINGERPRINTING OF WILD TORTOISES AS A FUTURE CONSERVATION MEASURE FOR RETURNING CAPTIVE INDIVIDUALS TO THE WILD

Parasites may reflect the immunological status of their hosts, since poor host body condition and lowered immunity may predispose to infection. During periods of stress and lowered immunological condition, the parasite response may be manifest either as an increase, or decrease, in prevalence and parasitaemia (Beldomenico and Begon, 2009).

The first main aim of this section of the present study was to assess whether there is a significant relationship between tortoise body condition, and parasite prevalence and parasitaemia, with the commonly recorded apicomplexan, *Haemogregarina fitzsimonsi* Dias, 1953. The chapter will include a discussion on the future use of such relationships, especially their potential employment in tortoise population 'health' studies. The haemogregarine, along with the other haematozoan species infecting South African tortoises, will also be evaluated as future biological tags. The second major aim was to present the results of the metal analysis of three tortoise tissue types (bone, nail and scute) and of the soil from different wild tortoise collection sites. The relationships of these tissues to each other, as well as to the soil samples will be presented and tested statistically, and the future use of these tissues in determining origin of tortoises assessed and discussed.

8.1. General observations, comparing tortoise body condition with *Haemogregarina fitzsimonsi* prevalence and parasitaemia

As each tortoise genus and species examined had different shell shapes, the body condition index (BCI) for each had to be assessed separately according to genus. In addition, since species within some genera had very similar shell dimensions, such as species of *Kinixys* Bell, 1827 and species of *Psammobates* Fitzinger, 1835, average BCI was calculated (according to the method of Loehr *et al*., 2006, see Chapter 3) collectively for the species within their respective genera.

Homopus Duméril and Bibron, 1835 and *Psammobates* species were not parasitized by *H. fitzsimonsi*, and thus these genera/species were omitted from further bio-indicator research (see Appendix 1a). *Kinixys belliana belliana* was found to have the highest prevalence of *H. fitzsimonsi*, but the number of specimens collected was small (3 individuals) (Table 8.1). Also, the other two *Kinixys* species were difficult to collect and seemingly lacked *H.*

fitzsimonsi. For example, no wild *Kinixys lobatsiana* were found during the study, and neither this species nor *Kinixys natalensis* were parasitized with *H. fitzsimonsi* during the present research, even though previous studies (Cook, 2008; Cook *et al*., 2009a) reported such infections. However, tortoises *Chersina angulata* and *Stigmochelys pardalis* were readily collected and showed *H. fitzsimonsi* prevalences of 26% and 10% respectively (Table 8.1). *Chersina angulata* and *S. pardalis* were also the most commonly collected host species overall between sites (Table 8.1), making these tortoises suitable for further bio-indicator investigation.

Table 8.1. Tortoise species and the corresponding numbers of specimens examined for *Haemogregarina fitzsimonsi* Dias, 1953; tortoise body condition index (BCI) and standard deviation (STDEV); average parasitaemia (% of erythrocytes) and range, and prevalence (%) of *H. fitzsimonsi* infecting each species; number of sites of total (%) from which each tortoise species was collected.

Tortoise species (number)	Tortoise BCI±STDEV	Parasitaemia *H. fitzsimonsi* (%)	Prevalence *H. fitzsimonsi* (%)	Number of sites (%)
Chersina angulata (97)	1.2±0.1 (0.8 – 1.5)	1.6±7.7 (0 – 60)	25/97 (26%)	9/17 (53%)
Homopus areolatus (26)	0.9±0.1 (0.8 – 1.2)	0	0/26 (0%)	
Kinixys belliana bellliana (3)		5.1±5 (0 – 10)	2/3 (67%)	1/17 (6%)
Kinixys lobatsiana (6)	1.4±0.4 (0.9 – 2.1)		0/6 (0%)	
Kinixys natalensis (2)		0	0/2 (0%)	
Psammobates tentorius trimeni (3)	1.6±0.3 (1.2 – 2)	0	0/3 (0%)	2/17 (12%)
Psammobates oculiferus (10)			0/10 (0%)	1/17 (6%)
Stigmochelys pardalis (121)	1.2±0.3 (0.1 – 3.2)	0.6±4.4 (0 – 46)	12/121 (10%)	8/17 (47%)
Testudo graeca (2)	Alien species, thus not considered	0	0/2 (0%)	1/17 (6%)

Both *C. angulata* and *S. pardalis* had an average BCI of 1.2 (Table 8.1). BCI values of <1.2 in these species therefore implied low body condition with the possibility of immunosuppression, increased chance for infection and perhaps increased parasitaemia (based on Beldomenico and Begon, 2009). At the same time, BCI value of ≥1.2 implied the opposite. Parasitaemia with *H. fitzsimonsi* for an average BCI of <1.2 in *C. angulata* (n = 34) was higher at 1.3% compared to 0.8% at an average BCI of ≥1.2 (n = 63). However, statistical testing of the relationship between individual BCIs and the corresponding parasitaemias suggested no correlation existed (P = 0.6, R^2 = 0.0003) (Fig. 8.1 A). Similarly, an average BCI of <1.2 for *S. pardalis* (n = 63) also apparently presented a higher parasitaemia at 1.2%

compared to 0.003% at an average BCI of ≥1.2 (n = 58). However, even though the relationship between individual BCIs and their corresponding parasitaemias was found to be significant according to the Student's T-test (P = <0.001) for this host tortoise, the linear regression relationship was insignificant (R^2 = 0.02) (Fig. 8.1 B).

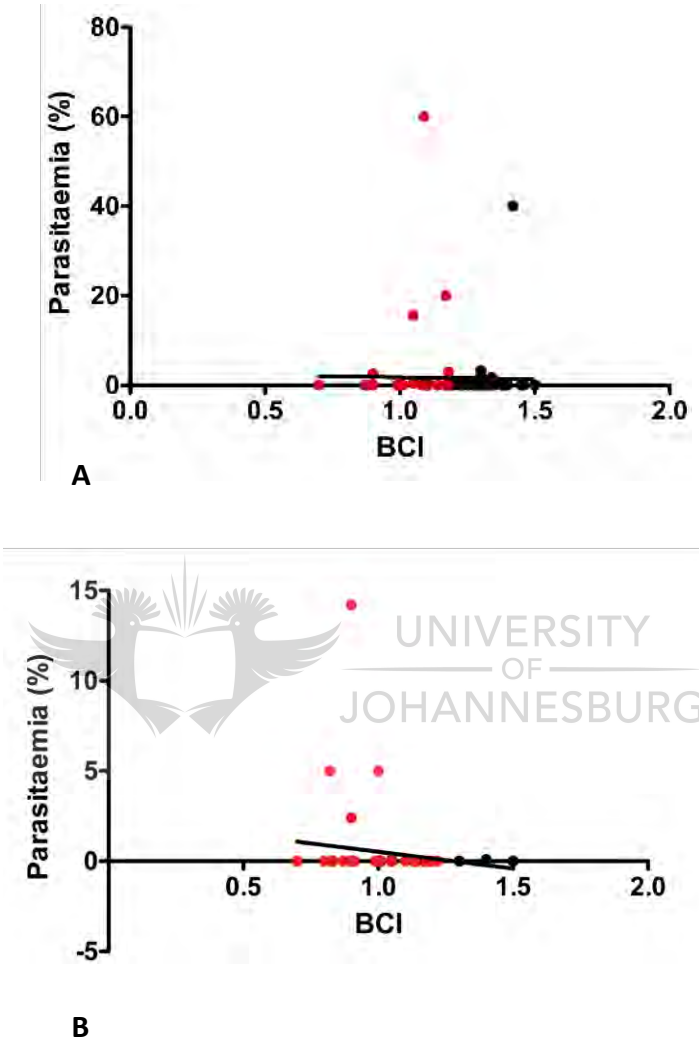


Figure 8.1 Individual BCIs for **(A)** *C. angulata* (n = 97) and **(B)** *S. pardalis* (n = 121) versus their recorded parasitaemias (%) with *H. fitzsimonsi*. Plotted points in red represent individual BCIs <1.2 and their corresponding parasitaemias.

Collective results for both *C. angulata* and *S. pardalis* at an average BCI of <1.2 similarly showed a higher parasitaemia at 1.8% compared to 0.4% at an average BCI of ≥1.2. However, once again, individual BCIs versus their corresponding parasitaemias showed a

significant relationship with the T-test (P = 0.01), but this relationship was not significant using linear regression (R^2 = 0.008) (Fig. 8.2) (n = 218).

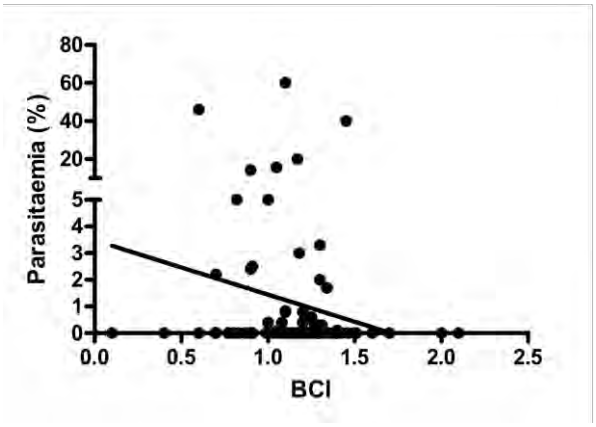


Figure 8.2 Individual BCIs for *C. angulata* and *S. pardalis* collectively (n = 218) versus their recorded parasitaemias (%) with *H. fitzsimonsi*.

Interestingly, in the collective data, no infection (in other words, a parasitaemia of 0%) was observed between BCIs of 0.09 – 0.5 and 1.5 – 2.5, infection being most prevalent between BCIs of 0.5 – 1, and highest parasitaemias between BCIs of 1 - 1.5 (see Fig. 8.2 above).

Additionally, individual BCIs versus parasitaemias within the range of 0.5 – 1.5 were found to be insignificant in both the T-test and linear regression analyses (P = 0.11; R^2 = 0.01) (Fig. 8.3).

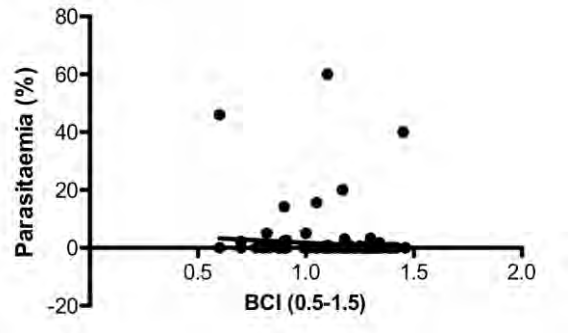


Figure 8.3 Individual BCIs (0.5 – 1.5) for combined *C. angulata* and *S. pardalis* data versus corresponding parasitaemias (%) of *H. fitzsimonsi*.

The prevalence and parasitaemia of *H. fitzsimonsi* was higher for anthropogenically impacted areas compared to nature reserves and conservancies (un-impacted) (see Chapter 4). *Chersina angulata* was collected from five impacted areas including Arniston (WC), De Beers (NC), Gouritzmond (WC), Paarl (WC) and Paternoster (WC), and four reserve areas including De Hoop (WC), De Mond (WC), Namaqualand (NC) and the West Coast (WC) (Table 8.2). But again, even though reserve tortoises (n = 28) and impacted area tortoises (n = 69) averaged the same BCIs, the correlation between individual BCIs versus their corresponding parasitaemias was found to be insignificant for both un-impacted areas (P = 0.004, R^2 = 0.3) and impacted areas (P = 0.9, R^2 = 0.0001) (Fig. 8.4). The T-test revealed insignificant differences (P > 0.05) in both prevalence and parasitaemia between the areas.

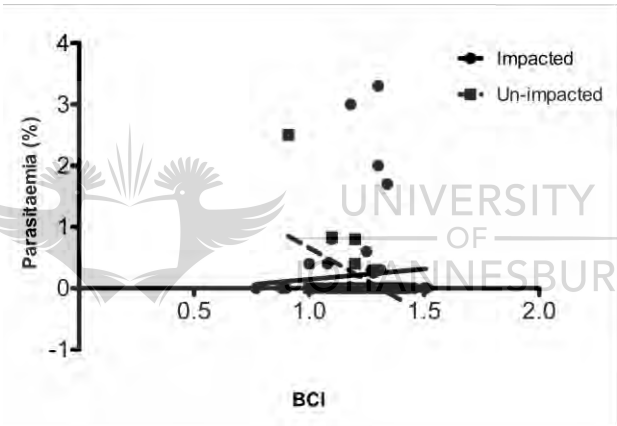


Figure 8.4 Individual BCIs for *C. angulata* versus corresponding parasitaemias (%) of *H. fitzsimonsi* from impacted versus un-impacted areas. The relationship between BCI and parasitaemia was found to be insignificant for both areas. Un-impacted = dashed regression line.

Conversely, specimens of *S. pardalis* (n = 43) in the 'impacted areas' (that is, in close human contact) of Johannesburg Zoological Gardens (GP), Paarl Butterfly World (WC) and in private collections (GP), presented with a higher prevalence of *H. fitzsimonsi*, but lower parasitaemias with this haemogregarine, than *S. paradalis* (n = 78) from the reserves areas of De Hoop (WC), Mkuze (KZN), Pongola (KZN) and Tswalu (NC), even though the BCI was the same for tortoises in both categories (Table 8.2). Individual BCIs versus parasitaemias were not significant for either impacted (P = 1; R^2 = 0.0002) or un-impacted areas (P = 0.8; R^2 =

0.07) (Fig. 8.5). The T-test revealed insignificant differences (P > 0.05) for both prevalence and parasitaemia between the areas.

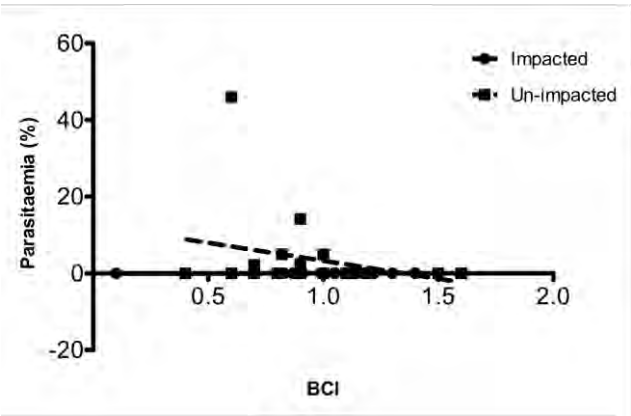


Figure 8.5 Individual BCIs for *S. pardalis* versus their corresponding parasitaemias (%) with *H. fitzsimonsi* from impacted versus un-impacted areas. The relationship between BCI and parasitaemia was found to be insignificant for both impacted and un-impacted areas. Un-impacted = dashed regression line.

Table 8.2 Sites from both human impacted and reserve (un-impacted) areas, detailing and comparing the average (AVG) body condition index (BCI), prevalence (Pr%) and average parasitaemia (P%) of *H. fitzsimonsi* for both *C. angulata* and *S. pardalis* separately. Sites and species combined to provide a total average for BCI, and total prevalence and average parasitaemia, comparing human impacted and reserve (un-impacted) sites (BW = Butterfly World, Paarl).

IMPACTED				**UN-IMPACTED (RESERVES)**			
Species	***Chersina angulata***						
Site	**BCI**	**Pr%**	**P%**	**Site**	**BCI**	**Pr%**	**P%**
Arniston	1.2±0.1 (0.9-1.4)	6/20 (30%)	3.4±13.4 (0-60)	De Hoop	1.2±0.1 (1.04-1.3)	0/4 (0%)	0
De Beers	1.2±0.09 (1.1-1.3)	5/5 (100%)	7.3±9.7 (0.02-20)	De Mond	1.1±0.09 (1-1.2)	3/4 (75%)	0.5±0.4 (0-0.8)
Gouritzmond	1.2±0.1 (1.1-1.3)	0/3 (0%)	0	Namaqualand	1.3±0.1 (1.1-1.4)	1/14 (7%)	0.02±0.08 (0-0.3)
Paarl (BW)	1.2±0.2 (0.8-1.3)	0/22 (0%)	0	West Coast	1.2±0.2 (1-1.4)	1/6 (17%)	0.4±1 (0-2.5)
Paternoster	1.3±0.1 (1.1-1.5)	6/19 (32%)	1.9±8 (0-40)				
TOTAL PR%	**-**	**17/69 (25%)**	**-**		**-**	**5/28 (18%)**	**-**
AVERAGE	**1.2±0.1 (0.9-1.5)**	**-**	**2.5±3 (0-3.4)**		**1.2±0.1 (1.1-1.3)**	**-**	**0.2±0.3 (0-0.8)**
Species	***Stigmochelys pardalis***						
Site	**BCI**	**Pr%**	**P%**	**Site**	**BCI**	**Pr%**	**P%**
JHB Zoo	1.1±0.5 (0.1-2.1)	2/11 (18%)	0.1±0.2 (0-0.7)	De Hoop	1±0.3 (0.8-1.2)	1/2 (50%)	2.5±3.5 (0-5)
Paarl (BW)	1.1±0.3 (0.8-1.4)	3/31 (10%)	0.004±0.02 (0-0.1)	Mkuze	0.9±0.3 (0.4-1.6)	6/13 (46%)	4.4±11.7 (0-46)
Private	1.1±0 (1.1)	0/1 (0%)	0	Pongola	1.3±0.4 (0.8-1.6)	0/3 (0%)	0
				Tswalu	1.3±0.4 (0.7-3)	0/60 (0%)	0
PREVALENCE	**-**	**5/43 (12%)**	**-**		**-**	**7/78 (9%)**	**-**
AVERAGE	**1.1±0 (0.1-1.4)**		**0.03±0.06 (0-0.7)**		**1.1±0.2 (0.9-1.3)**		**1.7±2.1 (0-4.4)**
TOTAL PR%	**-**	**20/112 (18%)**	**-**		**-**	**12/106 (11%)**	**-**
TOTAL AVG	**1.2±0.1 (1.1-1.3)**		**2.1±2.9 (0-7.3)**		**1.1±0.2 (0.9-1.3)**		**1.2±1.7 (0-4.4)**

8.1.1. Discussion

While Chapter 5 determined that tick vectors, possibly species of both *Amblyomma marmoreum* Koch, 1844 and *Amblyomma sylvaticum* (de Geer, 1778), transmit *Haemogregarina fitzsimonsi*, *Psammobates* and *Homopus* tortoise species in Chapter 4 and the current Chapter 8 showed no peripheral blood infections with this haemogregarine. As neither one of the *Amblyomma* tick species was collected from specimens of *Psammobates* species, this suggests at least one reason why this genus is apparently free from *H. fitzsimonsi*. Both species of *Psammobates* examined during this study occurred in the arid regions of the Northern and Western Cape. Vegetation in these areas is sparse compared to subtropical regions, perhaps compelling tortoises to increase their home ranges in order to acquire enough food (see BenDor *et al*., 2009). As a result, as it would take longer for them to navigate such territory, individual tortoise resting sites might be less frequently used, perhaps resulting in fewer chances for ticks to infest such tortoises and transmit the haemogregarine. Heavy infestations of *A. marmoreum* were, however, collected from a wild *Homopus areolatus* (Thunberg, 1787) population near Paarl (WC). It was considered likely that an equivalent infection of *H. fitzsimonsi* would be found in this population, but this was not the case. Noting the geography of the area, it was evident that this tortoise population was situated on a rocky outcrop covered with dense vegetation and between open sheep pastures. *Homopus areolatus* is an elusive species, requiring vegetation and rock crevices for shelter from predators such as birds of prey (Branch, 2008). It is possible that the Paarl population had been isolated for a long period of time, with the present ticks and tortoises never having been infected with *H. fitzsimonsi*.

In contrast to *Psammobates* and *Homopus* species, *Kinixys b. belliana* had the highest prevalence and parasitaemia of *H. fitzsimonsi* of all tortoise species collected and examined in this study (Table 8.1). Unfortunately, however, it could not be included in bio-indicator investigation due to its small sample size. On the other hand, the availability, as well as the frequency, of *H. fitzsimonsi* parasitism in *Chersina angulata* and *S. pardalis* allowed them to be considered for use as bio-indicators.

Initial data sets using these latter two tortoise species seemed promising. At a BCI of 0.5 – 1, prevalence was high (Fig. 8.2) and average parasitaemia was 2.5%. At a BCI of 1 – 1.5 the highest prevalence was displayed, but with lower average parasitaemia of 1%, whereas a BCI

of >1.5 was associated with no infection. This suggested that at a lower body condition, prevalence is high as well as parasitaemia, and when the body condition increases prevalence is still high, but parasitaemia is reduced. It could be postulated that a 'healthy' or non-stressed population has a high prevalence of *H. fitzsimonsi*, transmitted by a prevalent tick population, but parasitaemia is low, suppressed by an uncompromised tortoise immune system. However, statistical comparisons of both tortoise species BCIs and parasitaemias separately, as well as collectively, proved the relationship between body condition and parasitaemia to be insignificant, except sometimes when using the T-test. Whether or not this relationship will improve in significance with more specimen data remains to be seen, but this is doubtful considering the current total sample size (n = 218).

It might also be assumed that reserve areas would provide the host-vector-parasite 'model relationship' to which impacted sites might be compared. However, De Mond (WC) and West Coast (WC) may present the only natural tortoise populations within this study, which fit the above proposal that 'healthy' systems will have tortoises with a high body condition, high prevalence of ticks and *H. fitzsimonsi*, but with low parasitaemias. This was not the case with reserve areas such as Mkuze (KZN) and De Hoop (WC). Such findings may be attributable to environmental impacts such as fire or drought that may possibly have occurred in the past, resulting in food and water stress. This is especially true of De Hoop in which occasional burning is required to replenish plant species that without it become nutritionally deficient (De Hoop authorities pers. comm.).

Additionally, tortoises from some impacted areas, such as Arniston, De Beers and Paternoster, showed a high prevalence and parasitaemia with *H. fitzsimonsi*, but also, conversely to what might be expected, a high body condition (Table 8.2). These areas presented tortoises with both wild and cultivated vegetation in the form of farmland and residential gardens planted with both exotic and indigenous plant species (pers. obs.). Lack of food is therefore not a contributing stress factor and the density of tortoises and ticks may increase the chance of transmission in these areas. Thus, it may be important, to assess different sites separately, so as to assess specific impacts that may be particular to those sites. Very local impacts may thus affect tortoises, ticks and transmission of *H. fitzsimonsi* differently. These findings emphasize that high prevalence and parasitaemia with *H. fitzsimosi* may not always be indicative of tortoise population stress. This is further supported by the fact that no statistically significant differences were found when

comparing prevalence and parasitaemias of *H. fitzsimonsi* for *C. angulata* and *S. pardalis* between impacted and un-impacted areas.

Finally, even though *H. fitzsimonsi* was found infecting a number of tortoise species in the past, it does not apparently infect all species examined in this study, and statistically its appearance and behaviour within its hosts cannot be linked satisfactorily to tortoise body condition, or tortoise environment. Overall then, the use of *H. fitzsimonsi* as a bio-indicator is not particularly promising, since currently it does not appear to lend itself as a cosmopolitan indicator of tortoise population, or of environmental, 'health'.

8.2. A brief overview of haematozoans of South African tortoises for biological tagging

Biological tagging through the use of parasites is a relatively new concept, with increased interest observable over the last decade. Endemic, host-species-specific parasites or endemic parasite-community structures may be used as 'fingerprints' (MacKenzie and Abaunza, 1998; MacKenzie *et al*., 2008; Mele *et al*., 2010), in this case, to identify a tortoise species site of origin, or its population. The question arises whether the haematozoans recorded from different tortoise species and provinces could be used as indicators of tortoise origin.

Of the thirteen species (not including the 4 subspecies) of tortoise occurring within South Africa, eight (62%) have been examined for apicomplexan haematozoans in the current study. Five species of haematozoan have been recorded (see Chapter 4), including a species new to science (see *Haemogregarina* sp. A., Chapter 6).

Three haemogregarine species were observed during the current study (see Chapter 4), the most commonly recorded species, *H. fitzsimonsi*, being detected in 3/8 (38%) species of tortoise (Appendix 1 a, b). Cook (2008) and Cook *et al*. (2009a) recorded the parasite from two additional tortoise species, i.e. wild *K. lobatsiana* and captive *K. natalensis*. *Haemogregarina fitzsimonsi* has therefore been reported infecting 5/8 (63%) of the tortoise species examined from five provinces within South Africa (Table 8.3). The species, therefore, does not appear to be species or locality specific (see Chapter 5) and may be of limited use as a biological tag (but see below). *Haemogregarina parvula*, on the other hand, was found

concurrently with *H. fitzsimonsi* during the current study infecting only wild *K. b. belliana* from Mkuze, KZN. Previously (Cook, 2008), and as published by Cook *et al*. (2009a), it was recorded infecting both captive *K. b. belliana* and a single captive *S. pardalis*, also from KZN and therefore it appears to be relatively host species specific, but certainly locality specific and therefore might be a useful tag. The intraleucocytic *Haemogregarina* sp. A (see Chapter 6), was recorded only from two specimens of wild *S. pardalis*, from a single locality, Mkuze, KZN. This species may be highly host species and locality specific, and therefore, like *H. parvula* might prove useful in tagging, although more infected tortoises would need to be located.

Two haemoproteids were also described, firstly *Haemoproteus natalensis* Cook, Smit and Davies, 2010, from a single tortoise species and locality, suggesting the species to be rare and possibly host species specific; secondly *Haemoproteus testudinalis* (Laveran, 1905) was recorded from two species of tortoise, wild *Kinixys lobatsiana* and *Stigmochelys pardalis* from two localities, one in the North West province (Cook, 2008; Cook *et al*., 2010a) and (during the current study) from one captive *K. lobatsiana* from Johannesburg Zoological Gardens within Gauteng (see Chapter 7) (Table 8.3). Certainly, *Hp. natalensis* might prove useful as a future tag, but again many more infected tortoises would need to be located before firm conclusions could be drawn.

Table 8.3. Haematozoan species recorded during the present study with corresponding host tortoise species parasitized, prevalence among eight tortoise species examined, provinces in which infection was recorded, and provincial prevalence among five provinces examined.

Haematozoan species	Hosts parasitized	Tortoise species prevalence (%)	Provinces	Provincial prevalence (%)
Haemogregarina fitzsimonsi Dias, 1953	*Chersina angulata* *Kinixys belliana belliana* *Kinixys lobatsiana* *Kinixys natalensis* *Stigmochelys pardalis*	5/8 (63)	Gauteng (GP) KwaZulu-Natal (KZN) North West (NWP) Northern Cape (NC) Western Cape (WC)	5/5 (100)
Haemogregarina parvula Dias, 1953	*K. b. belliana* *S. pardalis*	2/8 (25)	KZN	1/5 (20)
Haemogregarina sp. A	*S. pardalis*	1/8 (13)	KZN	1/5 (20)
Haemoproteus natalensis Cook, Smit and Davies, 2010	*Kinixys natalensis*	1/8 (13)	KZN	1/5 (20)
Haemoproteus testudinalis (Laveran, 1905)	*K. lobatsiana* *S. pardalis*	2/8 (25)	GP NWP	2/5 (40)

8.2.1. Discussion

Haemoproteus natalensis and *Haemogregarina* sp. A appear to be the most species and locality specific of all the apicomplexan haematozoans recorded and thus perhaps most useful as prospective biological tags. *Haemoproteus testudinalis* and *H. parvula* also appear relatively host and locality specific, but they have now been identified infecting more than one species of tortoise and from more than one location which perhaps limits their tagging potential. Both of these species were described by Dias (1953) as infecting *K. b. belliana* (*K. b. zuluensis*) from Mozambique, this increasing the host range of *Hp. testudinalis* (described by Dias (1953) as *Haemoproteus balazuci*), to 3/8 (38%) of the tortoise species examined in the current study. The geographic distribution of *H. testudinalis* can also be increased by one province since this organism was first described by Laveran (1905) infecting a *S. pardalis* from possibly the Cape region of South Africa.

Since *H. fitzsimonsi* is neither host species nor locality specific, it is not an ideal biological tag if morphometrics alone is used to identify it. Conversely, if its genetic make-up is examined further in future, a wide host and geographic range may be beneficial for the parasite's use as a biological tag, if local variations in its genetic makeup become evident. Morphologically, examples of *H. fitzsimonsi* from different hosts and localities are indistinguishable (Cook *et al*., 2009a; present study); genetically, however, this may not be the case. Gene sequences, as seen in Chapter 5, may aid in inferring evolutionary relationships. Different genes evolve at different rates. Slow evolving genes are useful for inferring ancestral relationships, whilst fast evolving genes may be used to distinguish species or subspecies (Barta, 1997). Chapter 5 discussed the use of the fast evolving (less constrained) ITS2 gene region, which was employed to infer relationships between species of *Amblyomma*. Even though ITS regions have been used successfully in differentiating strains of certain apicomplexans, their use in studying other apicomplexans has proven unproductive (Barta, 1997). Apicomplexans have both chromosomal and extrachromosomal DNAs. Chromosomal DNAs include the commonly used ribosomal nucleic DNA (rDNA) from which the genes 28S, 18S, 5.8S and transcriber regions ITS1 and ITS2 can be amplified. Extrachromosomal DNAs include both plastid DNA (plDNA) or 35-kb circle DNA, and mitochondrial DNA (mtDNA), the latter containing the *cox* 1 and cytochrome *b* regions. To date, insufficient knowledge exists for effective use of plDNA, and the *cox* 1 and cytochrome *b* proteins are highly conservative (Wilson and

Williamson, 1997). Thus, it appears that the ITS regions may be the way forward in differentiating potential *H. fitzsimonsi* strains for biological tagging purposes.

8.3. Metal-fingerprinting

Multi-element analysis was proposed within the current research as a means of identifying the site of origin of an individual or populations of tortoises by providing a metal-fingerprint (Batista *et al*., 2008). A metal-fingerprint can be established through the analysis of a range of trace and macro-elements in a specific tissue of an individual animal or those of a population. However, to date, a limited amount of research, involving metal analysis, has been done in reptiles (Smith *et al*., 2007). On the other hand, contaminant accumulation in reptiles has been documented in recent years and the mechanisms of contaminant uptake discussed (Smith *et al*., 2007). Ingestion of food as well as geophagy may lead to an accumulation of contaminants within the tissues (Gardner *et al*., 2006) and in a similar way so can naturally occurring metals from the environment be accumulated. As mentioned in Chapter 2, it is those tissues that are long-lived that would be of interest in determining the origin of the individual or population. Three tissues, i.e. bone, scute and nail were selected to ascertain the viability of a metal-fingerprint in differentiating populations of wild tortoises. These data would subsequently be compared to soil metal concentration data to determine if a relationship might exist that may be of use in future.

8.3.1. Overview of tissue collection

Provinces and corresponding sites, as well as tortoise species and number have been tabulated below with the corresponding tissue types collected from each (Table 8.4). A representative of each tissue (bone, scute and nail) were collected, when possible, from three provinces including 14 different sites, from seven species of tortoise (all wild). Generally nail was collected from living specimens, also scute, when occasion allowed, whereas bone and scute were collected from dead specimens. In total, tissue samples were collected from 189 live and 97 dead specimens.

Table 8.4. Province (KZN = KwaZulu-Natal, NC = Northern Cape and WC = Western Cape) from which tortoises were collected along with site, tortoise species, number of each, status (L = live; D = dead) and tissue type collected (B = bone, N = nail and S = scute).

Province	Site	Tortoise Species	Number	Tissue
KZN				
	Mkuze			
		Kinixys belliana belliana	3 (L)	-
		Stigmochelys pardalis	13 (L)	S
	Pongola	*Kinixys natalensis*	1 (L)	-
		S. pardalis	3 (L)	S
SUB-TOTAL			**20 (L)**	**S**
WC				
	Arniston			
		Chersina angulata	20 (L)	N
			2 (D)	S, B
	De Hoop			
		C. angulata	4 (L)	N
		S. pardalis	2 (L)	N
	De Mond			
		C. angulata	4 (L) 2 (D)	N (L) S (D) B (D)
	Elandsberg			
		Psammobates geometricus	2 (D)	B
	Gouritzmond			
		C. angulata	3 (L) 2 (D)	N (L) S (D) B (D)
	Paarl			
		Homopus areolatus	20 (L) 3 (D)	S (D) B (D)
	Paternoster			
		C. angulata	18 (L) 3 (D)	N (L) S (D)
	West Coast			
		C. angulata	6 (L)	-
SUB-TOTAL			**77 (L) + 14 (D) = 91**	**B, N, S**
NC				
	Britstown			
		Psammobates tentorius tentorius	2 (D)	S, B
		S. pardalis	1 (L)	N
	De Beers			
		C. angulata	5 (L)	-

Table 8.4. continued

Province	Site	Tortoise Species	Number	Tissue
NC				
	Namaqualand			
		C. angulata	14 (L) 79 (D)	N (L) S (D) B (D)
		Psammobates tentorius trimeni	2 (L) 1 (D)	S (D) B (D)
		Unidentifiable species	1 (D)	B
	Tswalu			
		Psammobates oculifer	10 (L)	-
		S. pardalis	60 (L)	N (L) S (L)
SUB-TOTAL			**92 (L) + 83 (D) = 175**	**B, N, S**
TOTAL			**189 (L) + 97 (D) = 286**	**B, N, S**

8.3.2. Spatial comparisons of metal concentrations in wild tortoise bone, scute and nail

It was not possible to determine whether there were differences in metal accumulation between species at a particular site. Therefore data from different species at the same site were pooled to undertake spatial analyses. Sites that showed a significant difference between particular metals were focussed upon.

The following metals were significantly different ($P < 0.05$) in bone: Mn, Co, Ni, Zn, Se, Ag, Pb, U, Al and K (Fig. 8.6 A, B, C: a - s). Paarl (WC) and Namaqualand (NC) showed significant differences for the six metals: Mn, Co, Ni, Zn, Se, Ag, Pb and U; Gouritzmond (WC) and Namaqualand (NC) for five metals: Mn, Ag, Pb, U and Al; De Hoop (WC) and Namaqualand (NC) for three metals: Ni, Ag and Pb; Britstown (NC) and Namaqualand (NC) for two metals: Ag and K. Even sites in the same region of the WC (i.e. De Hoop, Gouritzmond and Paarl) recorded significant differences in metal concentrations in bone. De Hoop and Paarl, as well as Gouritzmond and Paarl were significantly different for Pb. Namaqualand (NC) consistently had significantly higher metal concentrations for the metals Mn, Ag, U, Al and K, and had significantly lower metal concentrations for the metals Co, Ni, Zn, Se and Pb, to those sites situated in the southern part of the WC Province.

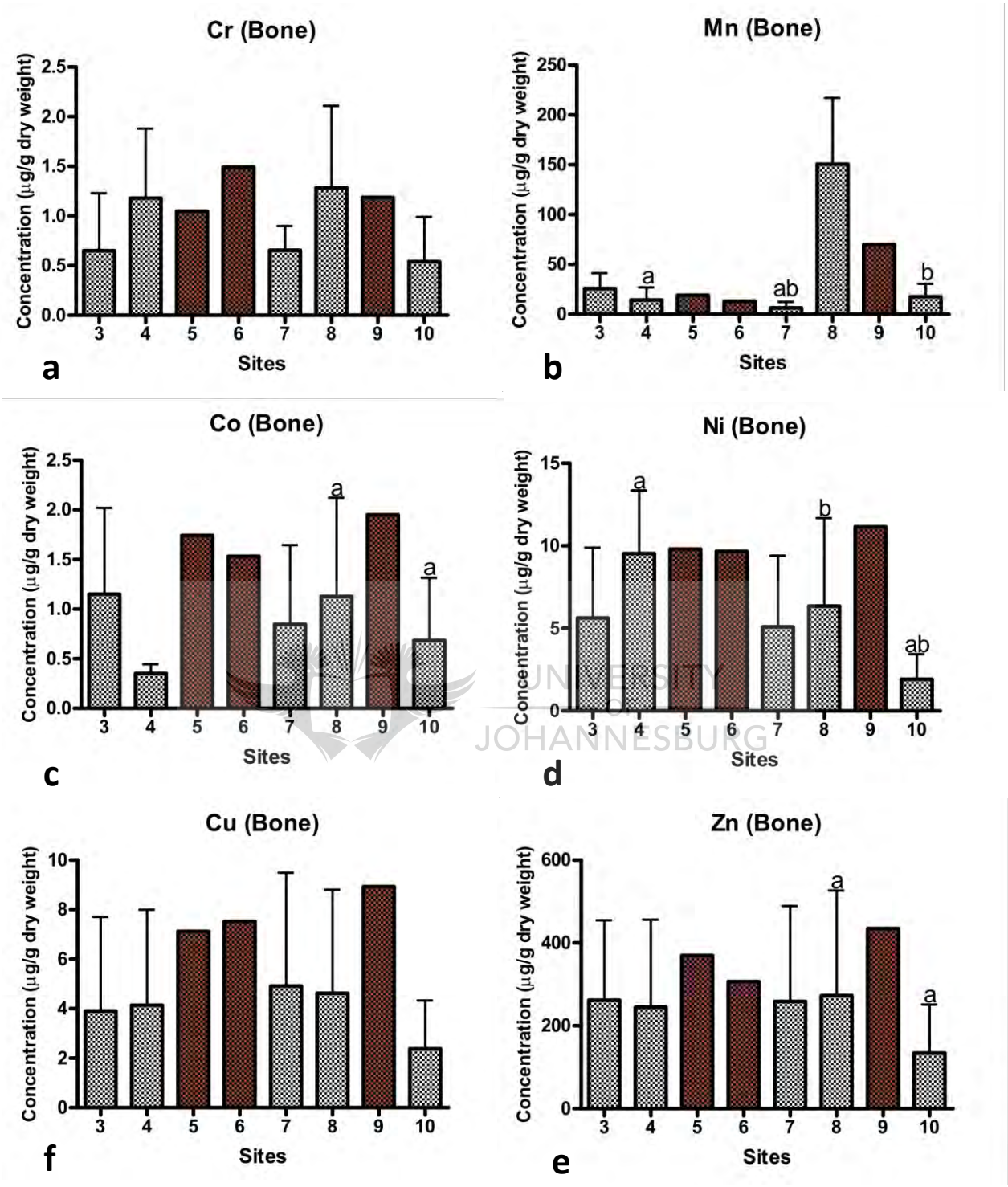


Figure 8.6A. Mean + standard error concentrations (µg/g dry weight) in tortoise bone samples collected from 8/12 (67%) sites used within this study. **(a)** Cr, **(b)** Mn, **(c)** Co, **(d)** Ni, **(e)** Cu and **(f)** Zn. Sites: 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC) and 10=Namaqualand (NC). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

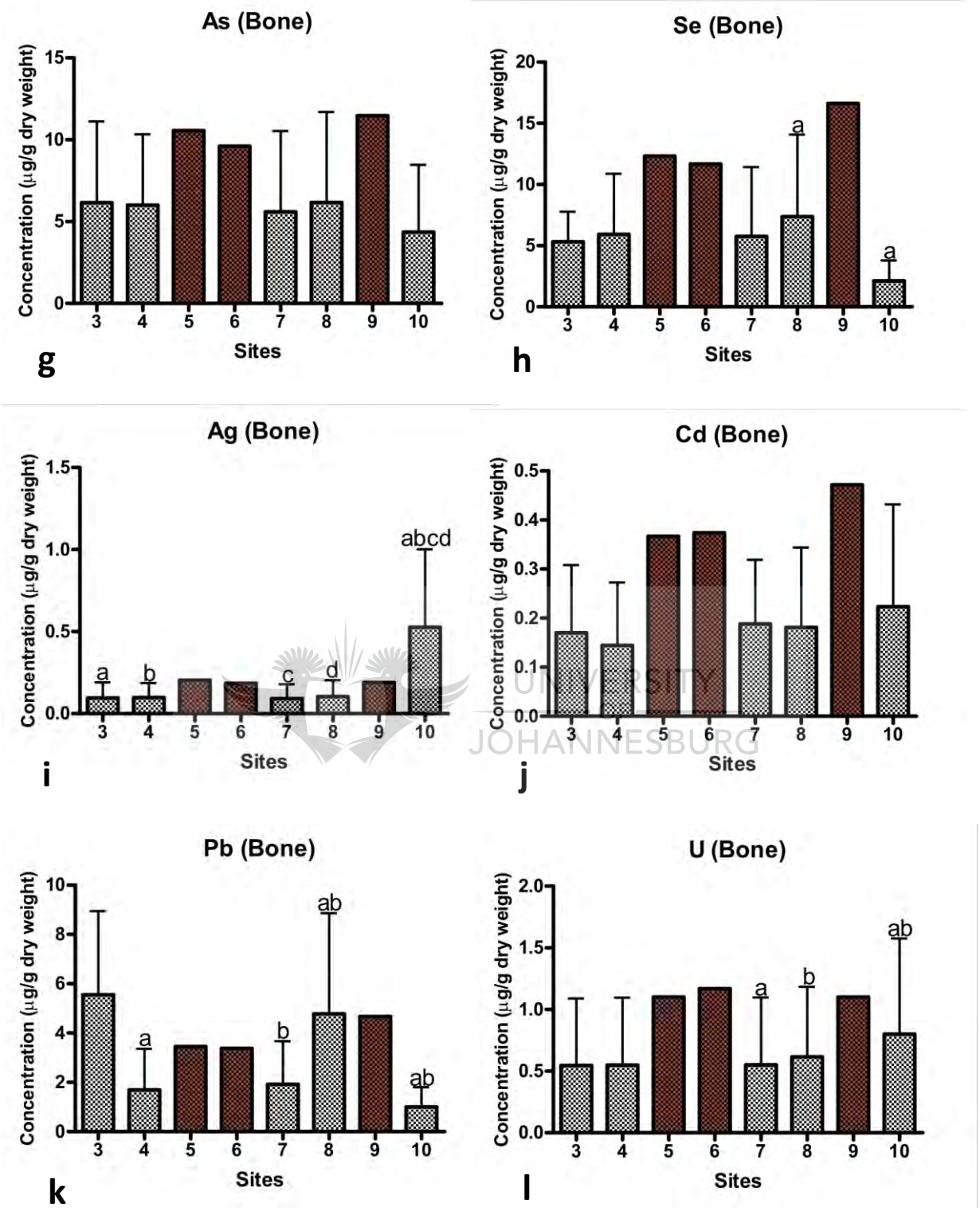


Figure 8.6B. Mean + standard error concentrations (µg/g dry weight) in tortoise bone samples collected from 8/12 (67%) sites used within this study. **(g)** As, **(h)** Se, **(i)** Ag, **(j)** Cd, **(k)** Pb and **(l)** U. Sites: 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC) and 10=Namaqualand (NC). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

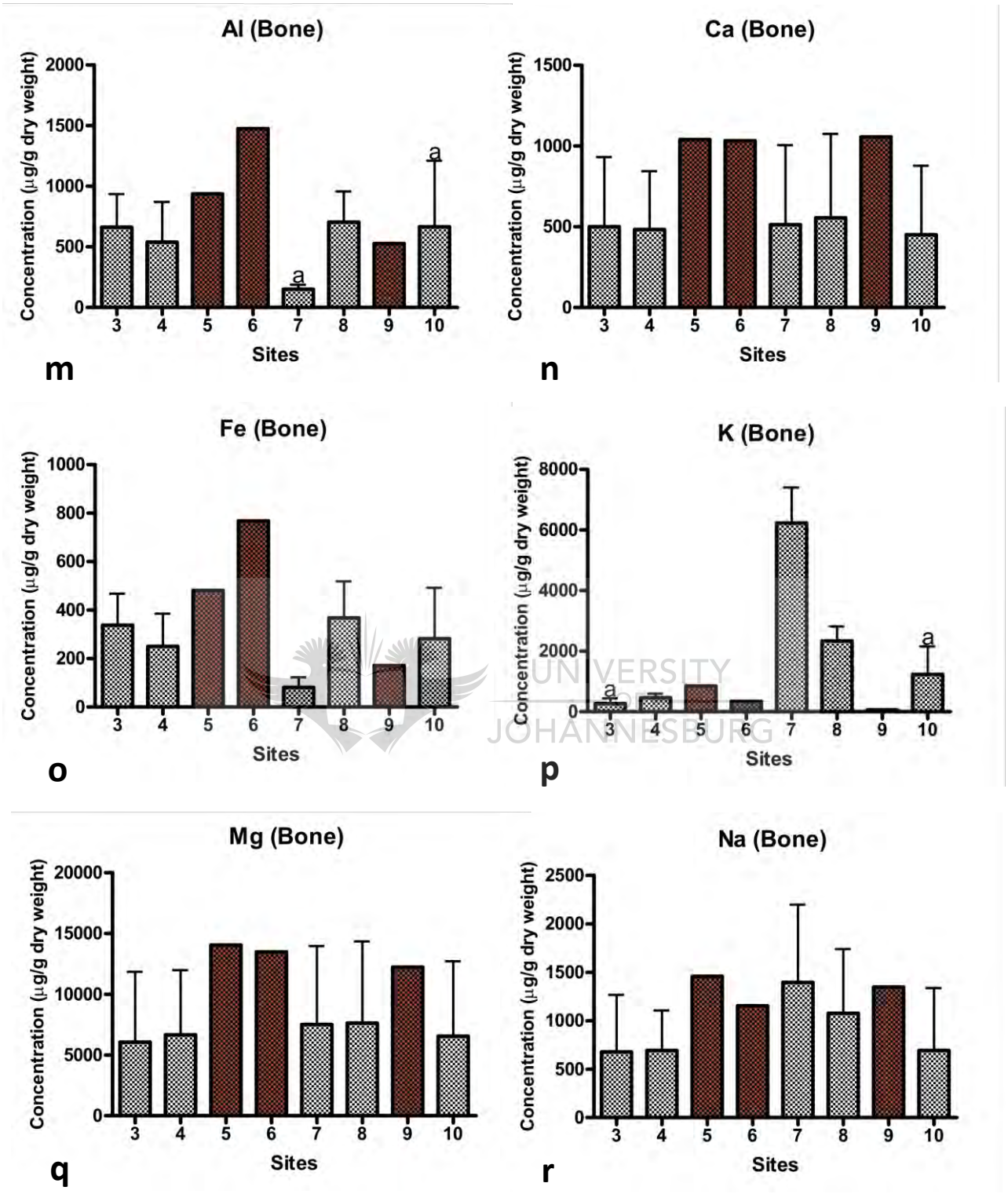


Figure 8.6C. Mean + standard error concentrations (µg/g dry weight) in tortoise bone samples collected from 8/12 (67%) sites used within this study. **(m)** Al, **(n)** Ca, **(o)** Fe, **(p)** K, **(q)** Mg and **(r)** Na. Sites: 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC) and 10=Namaqualand (NC). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

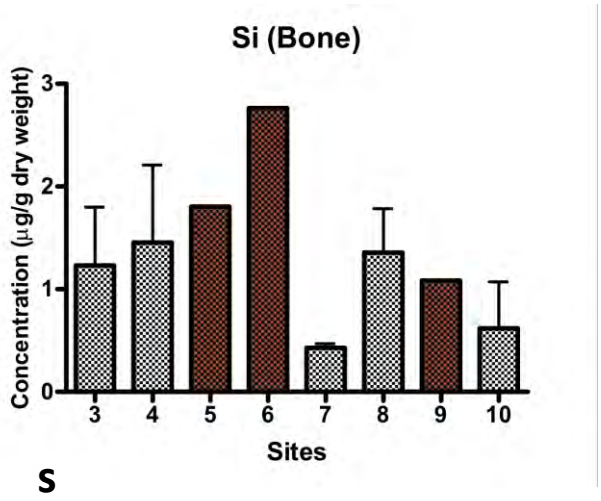


Figure 8.6D. Mean + standard error concentrations (µg/g dry weight) in tortoise bone samples collected from 8/12 (67%) sites used within this study. **(s)** Si. Sites: 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC) and 10=Namaqualand (NC). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

Metal concentrations in scute were significantly different ($P < 0.05$) for the following (Fig. 8.7 A, B, C: a – s): Paarl (WC) and Namaqualand (NC) showed differences for 10 metals: Mn, Co, Cu, Zn, As, Ag, Cd, Pb, U and Al; Gouritzmond (WC) and Namaqualand (NC) for eight metals: Co, Cu, Zn, As, Ag, Cd, Pb, and U; De Hoop (WC) and Namaqualand (NC) for 10 metals: Mn, Co, Cu, Zn, As, Se, Ag, Cd, Pb and U; Britstown (NC) and Namaqualand (NC) for ten metals: Mn, Co, Cu, Zn, As, Se, Ag, Cd, Pb and U; Mkuze/Pongola (KZN) and Namaqualand (NC) for 11 metals: Co, Ni, Cu, Zn, As, Se, Ag, Cd, Pb, U and Fe. Zinc concentrations measured in scute samples from the WC differed significantly between Paarl and Gouritzmond and between Paarl and De Hoop.

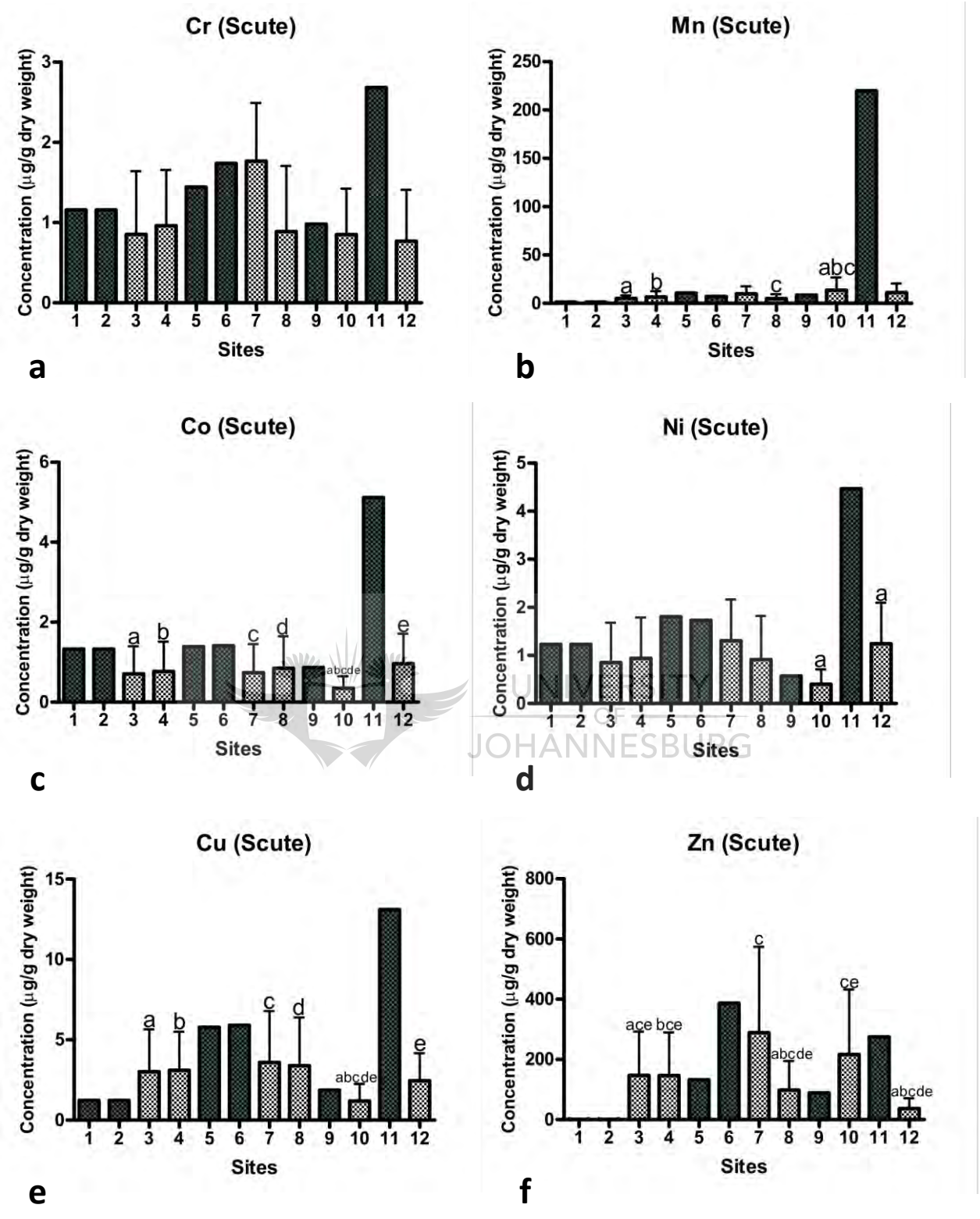


Figure 8.7A. Mean + standard error concentrations (µg/g dry weight) in tortoise scute samples collected from 12/12 (100%) sites used within this study. **(a)** Cr, **(b)** Mn, **(c)** Co, **(d)** Ni, **(e)** Cu and **(f)** Zn. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

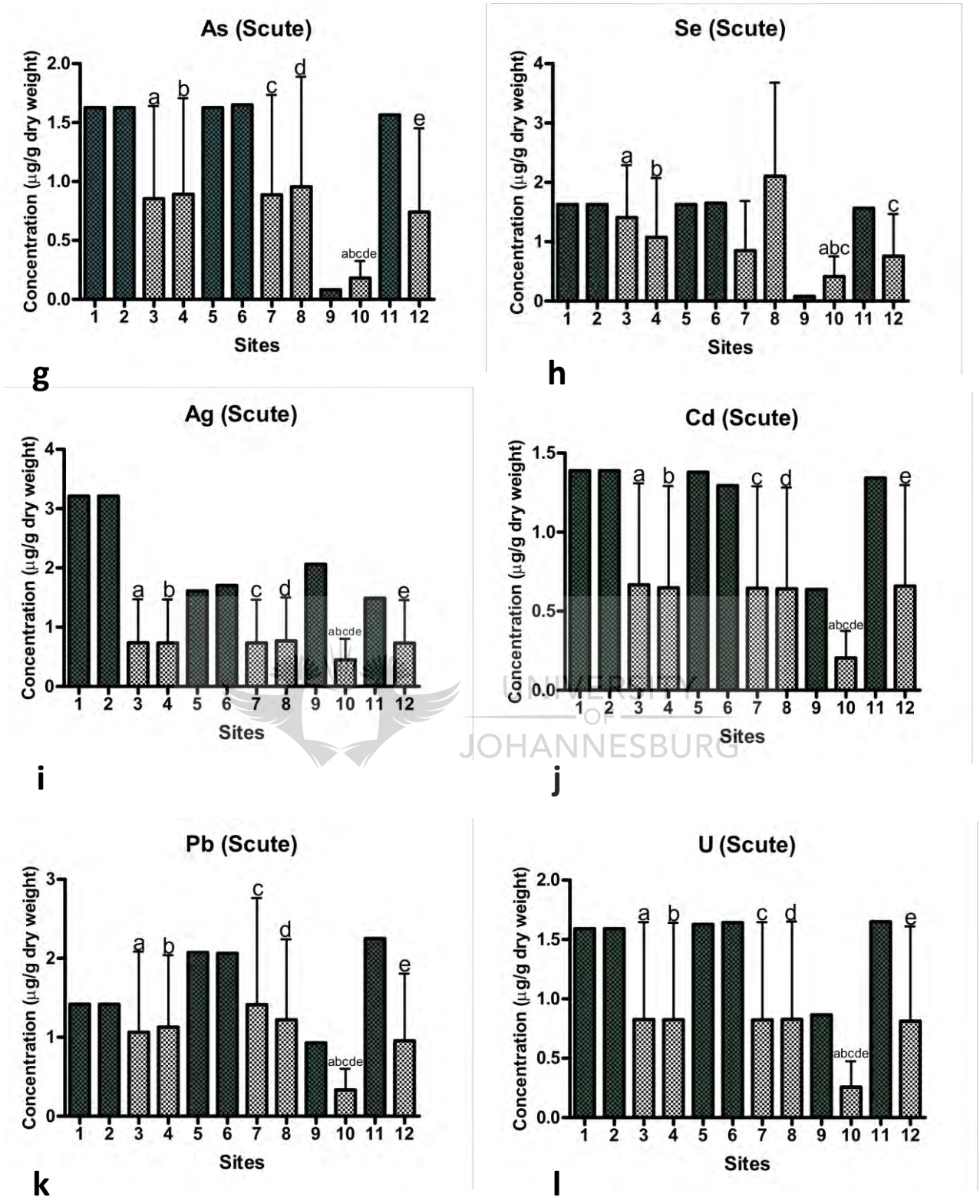


Figure 8.7B. Mean + standard error concentrations (µg/g dry weight) in tortoise scute samples collected from 12/12 (100%) sites used within this study. **(g)** As, **(h)** Se, **(i)** Ag, **(j)** Cd, **(k)** Pb and **(l)** U. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

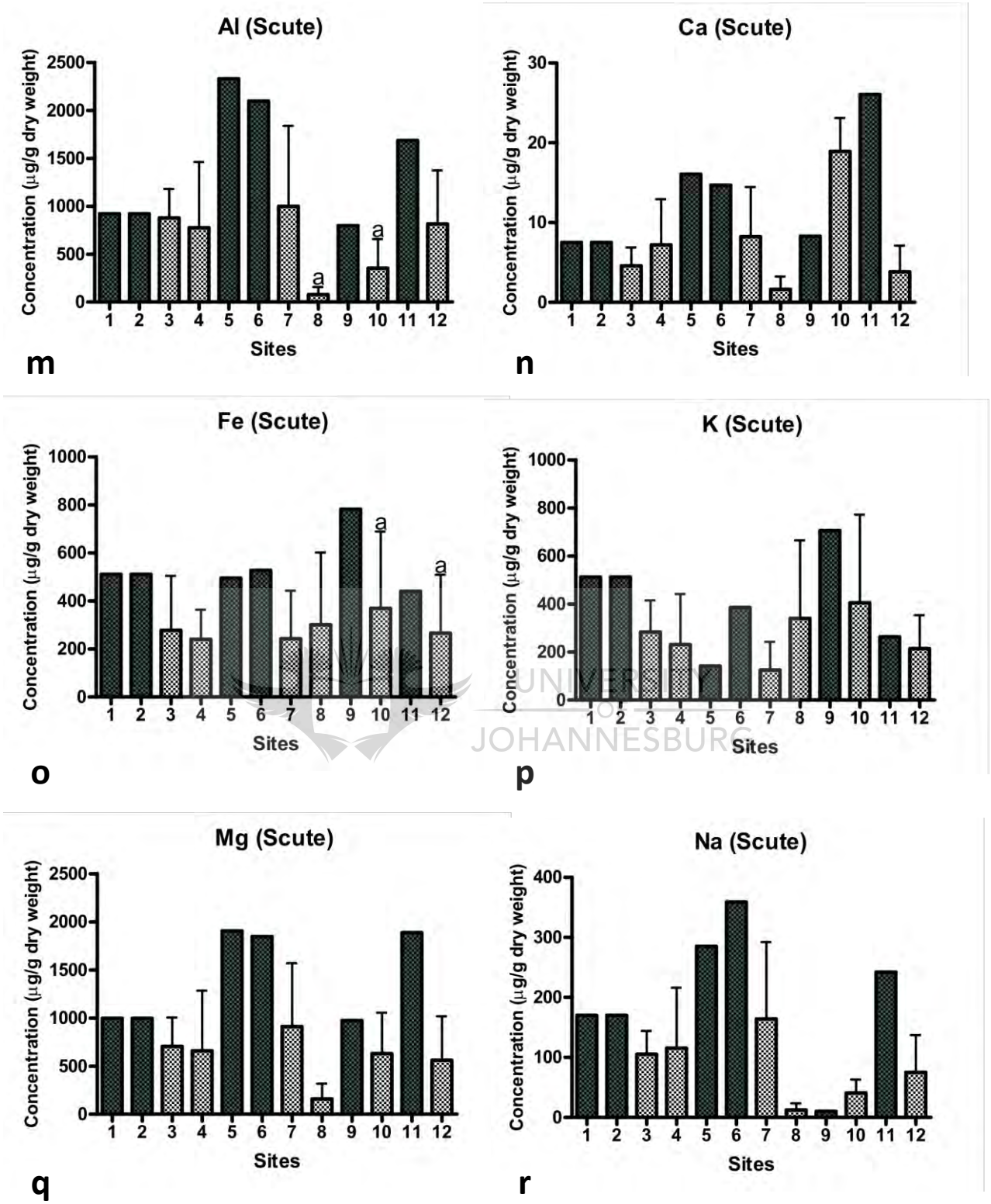


Figure 8.7C. Mean + standard error concentrations (µg/g dry weight) in tortoise scute samples collected from 12/12 (100%) sites used within this study. **(m)** Al, **(n)** Ca, **(o)** Fe, **(p)** K, **(q)** Mg and **(r)** Na. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

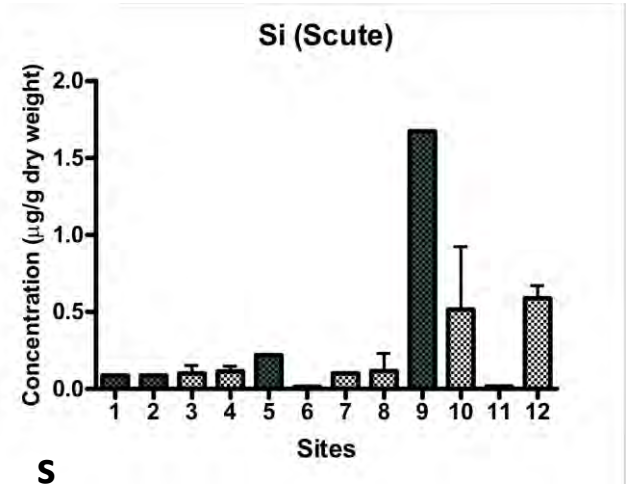


Figure 8.7D. Mean + standard error concentrations (µg/g dry weight) in tortoise scute samples collected from 12/12 (100%) sites used within this study. **(s)** Si. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 8=Paarl (WC), 9=Elandsberg (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites. Colour bars represent sites for which a single sample was collected.

Metal concentrations in nails were also significantly different between sites (Fig. 8.8 A, B, C: a – s). Paternoster (WC) and Namaqualand (NC) for six metals: Mn, Cu, Zn, Pb, U and Fe; Mkuze/Pongola (KZN) and Namaqualand (NC) for four metals: Mn, Cu, Zn and U; Gouritzmond (WC) and Namaqualand (NC) for two metals: Zn and U. The following sites were all significantly lower in Zn concentration compared to Namaqualand (NC): Tswalu (NC), Britstown (NC), De Hoop (WC), Arniston (WC), Gouritzmond (WC) and Paternoster (WC), whilst Namaqualand was significantly lower in Zinc concentration for De Mond (WC). Arniston (WC) and Gouritzmond (WC) were significantly different in concentration for five metals: Co, Se, Cd, U and Na. Arniston (WC) was also significantly different to Paternoster (WC) for six metals: Cu, U, Fe, K, Mg and Na. Paternoster (WC) and Gouritzmond (WC) were significantly different for four metals: Ag, U, Fe and Na. Gouritzmond (WC) was significantly different to Tswalu (NC), Britstown (NC), De Hoop (WC) and Mkuze (KZN) for U, and significantly different to De Mond (WC) for Fe, as well as significantly different to Tswalu (NC) for Na. Tswalu (NC) was different from Paternoster (WC) for three metals: Pb, Mg and Na. Britstown (NC) was significantly different to Arniston (WC), Paternoster (WC) and Mkuze (KZN) for Cd, as well as to Paternoster (WC) for three metals: Ag, U and Fe. Paternoster (WC) was also significantly different to Mkuze (KZN) for three metals: Co, Pb and Al, as well as to De Mond (WC) for three metals: Fe, K and Si. De Mond (WC) nail samples had the highest concentrations for most metals, with the exception of Mn, which was highest for Namaqualand (NC) and Britstown (NC) respectively.

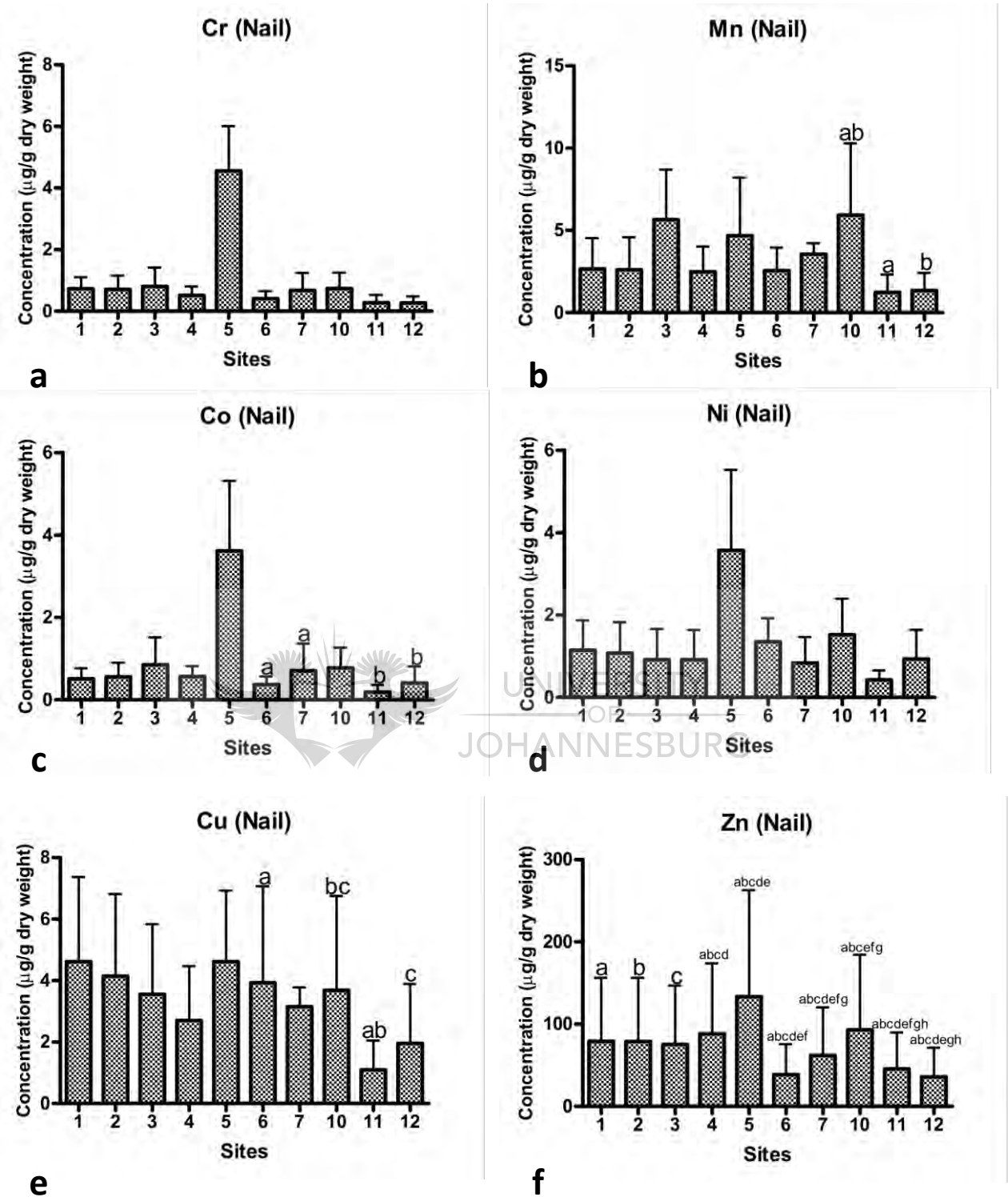


Figure 8.8A. Mean + standard error concentrations (µg/g dry weight) in tortoise nail samples collected from 10/12 (83%) sites used within this study. **(a)** Cr, **(b)** Mn, **(c)** Co, **(d)** Ni, **(e)** Cu and **(f)** Zn. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites.

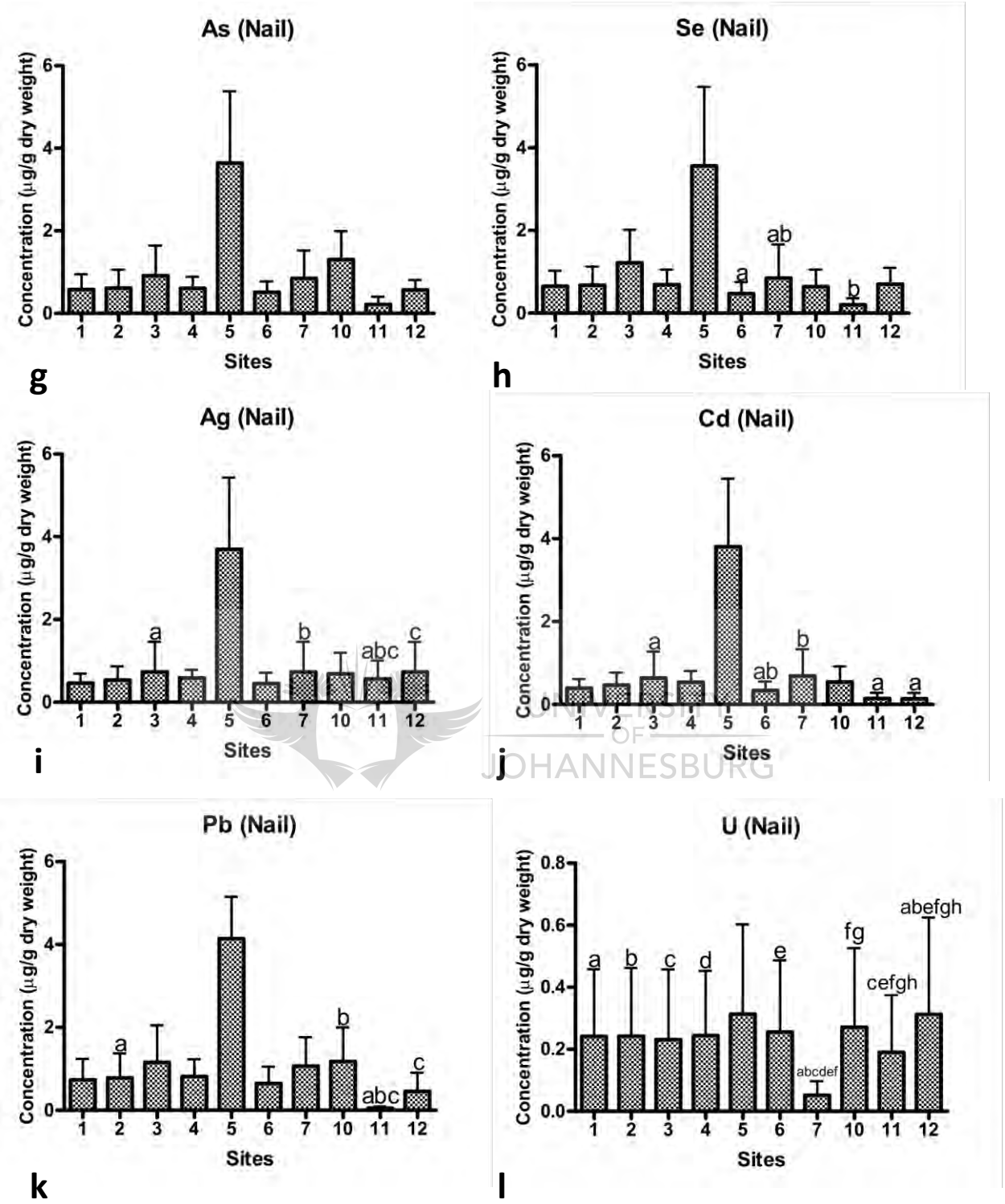


Figure 8.8B. Mean + standard error concentrations (µg/g dry weight) in tortoise nail samples collected from 10/12 (83%) sites used within this study. **(g)** As, **(h)** Se, **(i)** Ag, **(j)** Cd, **(k)** Pb and **(l)** U. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites.

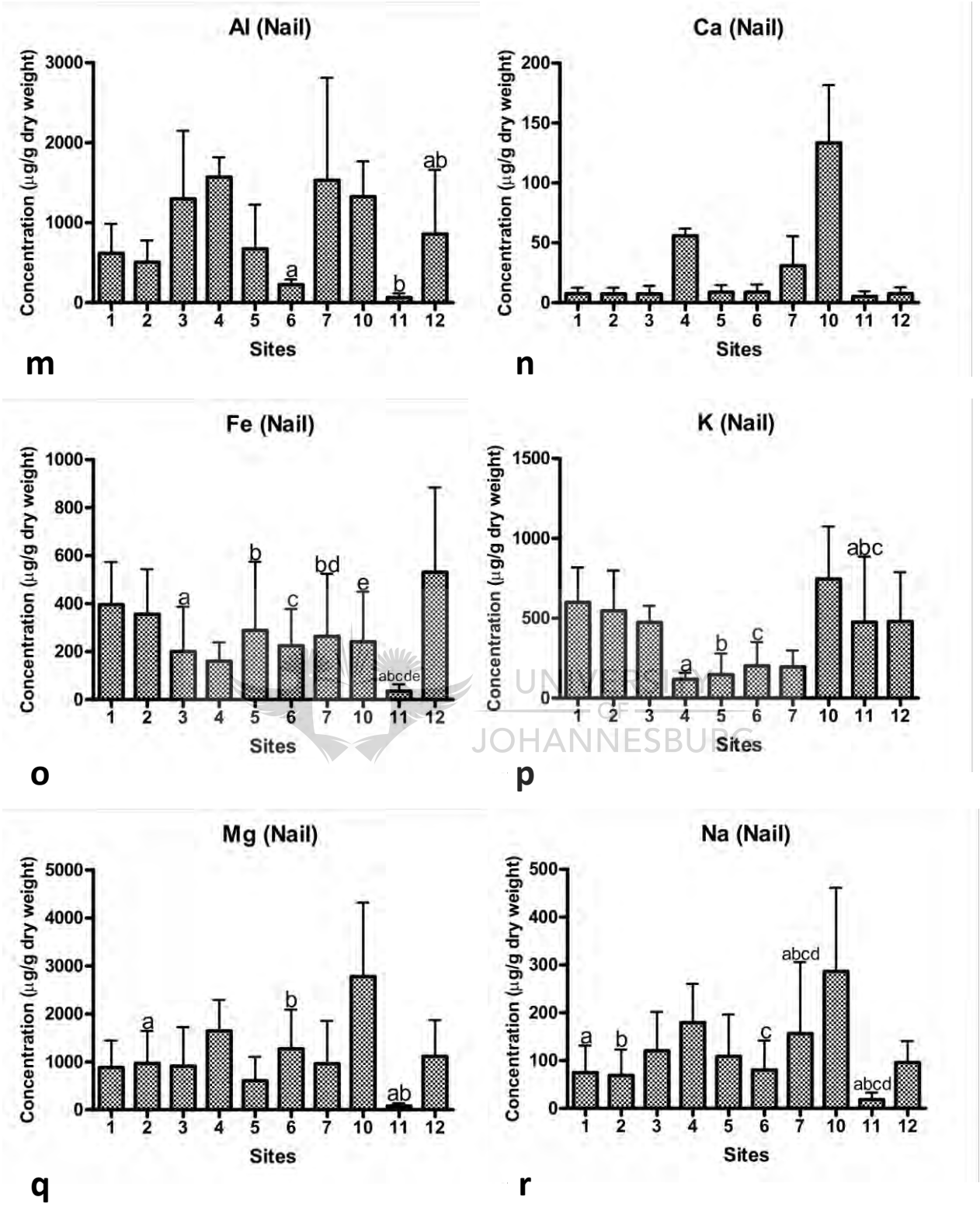


Figure 8.8C. Mean + standard error concentrations (µg/g dry weight) in tortoise nail samples collected from 10/12 (83%) sites used within this study. **(m)** As, **(n)** Se, **(o)** Ag, **(p)** Cd, **(q)** Pb and **(r)** U. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites.

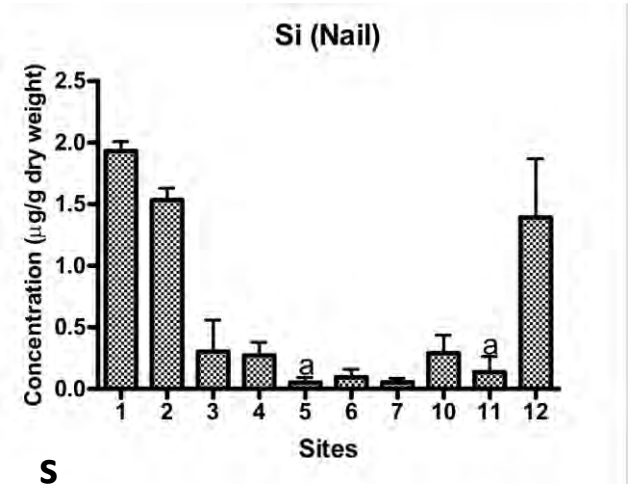


Figure 8.8D. Mean + standard error concentrations (µg/g dry weight) in tortoise nail samples collected from 10/12 (83%) sites used within this study. **(s)** Si. Sites: 1 and 2=Tswalu (NC), 3=Britstown (NC), 4=De Hoop (WC), 5=De Mond (WC), 6=Arniston (WC), 7=Gouritzmond (WC), 10=Namaqualand (NC), 11=Paternoster (WC) and 12=Mkuze/Pongola (KZN). Common superscript above bars denotes significant difference (P=<0.05) between sites.

In summary, there were significant spatial differences for a number of metals for all three tissue types. Samples from Namaqualand were consistently significantly different to other sites, in particular those sites that are located in the southern WC such as Arniston, De Hoop, De Mond, Gouritzmond, Paarl and Paternoster with 3 – 6 metals (bone), 8 - 10 metals (scute) and 3 - 6 metals (nail). Namaqualand was particularly different to Mkuze/Pongola (KZN) for 11 metals (scute) and 4 metals (nail). From the above data sites that are further from each other were significantly different for more metals than sites in closer proximity to one another. However, unpredictably, Britstown (NC) and Namaqualand (NC) were found to be significantly different for 10 metals when utilising scute, compared to the two when utilising bone. Bone was significantly different for only 4 metals between Namaqualand and WC and KZN sites; similarly, scute was significantly different for 12 metals, and nail for 5 metals. Therefore, scute displayed spatial significance for more metals than the other two tissues.

8.3.3. Tissue differences

All three tissues (bone, scute and nail) from a single species (*C. angulata*) were collected from only three sites, i.e. De Mond (WC) (site 5), Gouritzmond (WC) (site 7) and Namaqualand (NC) (site 10) (Table 8.4). Metal concentrations of tissues were compared against each other using principal component analysis (PCA) to observe the spatial differences between the tissues within each site separately (Fig. 8. 9 A, B, C). All three sites showed a 100% variance between all three of the tissue types. De Mond (WC) bone was high in concetration for the metals: Zn, Ni, As and Se, whilst scute was high in concentration for only the metal Al, and nail was high in concentration for the metals: Co, Cr, Fe, Cd and Ag. Gouritzmond (WC) bone had high metal concentrations of Ni, Se, Ca, K, Mg and As, scute had high metal concentrations for Ag and Cd, and nail had high metal concentrations for only Al. Namaqualand (NC) bone was high in concentration for the metals: Na, Mg, K, Ca, Se, As and U, scute high in concentration for the metals: Fe and Zn, and nail high in concentration for the metals: Cu, Ag and Cd. Evident from the above information and the corresponding triplot (Fig. 8.9 A, B, C) the tissues within each site are not comparable.

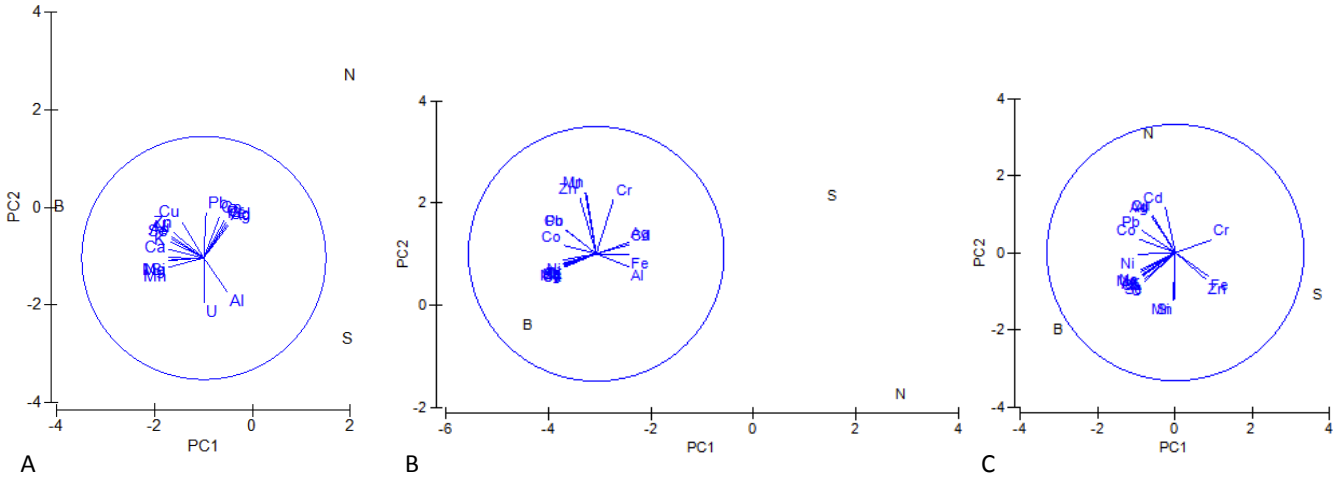
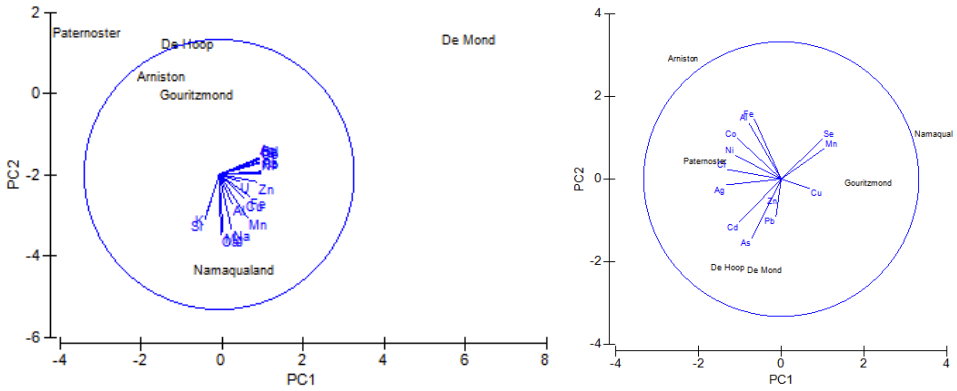


Figure 8.9. Principal component analyses (PCA) plots for metal concentrations in bone, scute and nail (µg/g dry weight) of *Chersina angulata* for **(A)** De Mond (WC), **(B)** Gouritzmond (WC) and Namaqualand (NC). The percentage variation in the data explained by the first three axes was 100%.

8.3.4. Metal concentration comparisons of nail between wild tortoise species and the correlation of these to soil from the same collection sites

To determine whether spatial differentiation between tortoise populations could be determined based on metal concentrations in tissues, nail metal concentrations were compared between sites for the same species using a principal component analysis (PCA). All data were normalised prior to analysis. *Chersina angulata* (n = 149) and *S. pardalis* (n = 79) were the most abundant collected and utilisable species for the above analyses, especially since both species have a wide geographical range (Table 8.4).

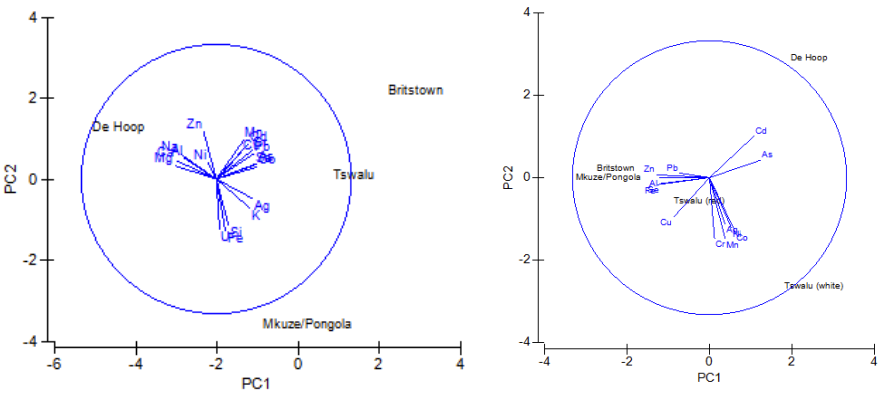
The metal concentrations in the nails of *C. angulata* explained 80.3% of the variability in the data. Namaqualand was very different from the rest of the sites (Fig. 8.10 A) with high concentrations in Mn, Ca, Mg and Na being responsible for the separation of this site from the others. The metal concentration pattern in the nails from De Mond was also different to the other WC sites due to the higher Ag, As, Cr, Co, Zn, Pb, Ni, Se, Cd concentrations. Paternoster tortoises were also different from the other sites due to the lowest concentrations of all metals. De Hoop and De Mond (Figure 8.10 B) were seen to group together for the soil metal concentrations. The other sites grouped separately. Compared to the groupings found for *C. angulata* nail, there would appear to be no relationship between soil and nail samples for this species.



A B

Figure 8.10. Principal component analyses (PCA) plots for metal concentrations in nail **(A)** of *Chersina angulata* (µg/g dry weight) and **(B)** soils (µg/g dry weight) from the respective sites. The percentage variation in the data explained by the first two axes was 80% and 76% respectively.

Similarly to *C. angulata*, the nail metal concentration PCAs for *S. pardalis*, the biplot represents a high percentage of the variation explained in the data, i.e. 86% for four different sites: Britstown, De Hoop, Mkuze/Pongola and Tswalu (Fig. 8.11 A). Nail tissue from Britstown and to a lesser degree, Tswalu, were characterised by high Mn, Pb, Cr, Co, As, Se and Cd concentrations. Mkuze/Pongola was high in: Ag, K, Si, Fe and U, whilst De Hoop was highest in: Zn, Ni, Na, Al, Mg and Ca. Similarly, there was no relationship in the metal accumulation patterns for nail of *S. pardalis* and the soil from the collection sites (Fig. 8.11 B). The two different soil types from Tswalu did not group together and were distinctly different due to higher Cr, Mn, Co and Ag in the white/calcrete soil of Tswalu.



A B

Figure 8.11. Principal component analyses (PCA) plots for metal concentrations in nail **(A)** of *S. pardalis* (µg/g dry weight) and **(B)** soils (µg/g dry weight) from the respective sites. The percentage variation in the data explained by the first two axes was 86% and 75% respectively.

When the two species nail concentration data were combined the PCA biplots together only explained 70% of the variation. The *S. pardalis* metal patterns were more similar than those for *C. angulata*, and Namaqualand, De Hoop and Paternoster were seen to group separately due to very specific metals: K, Se and Ag, and Si respectively (Fig. 8.12).

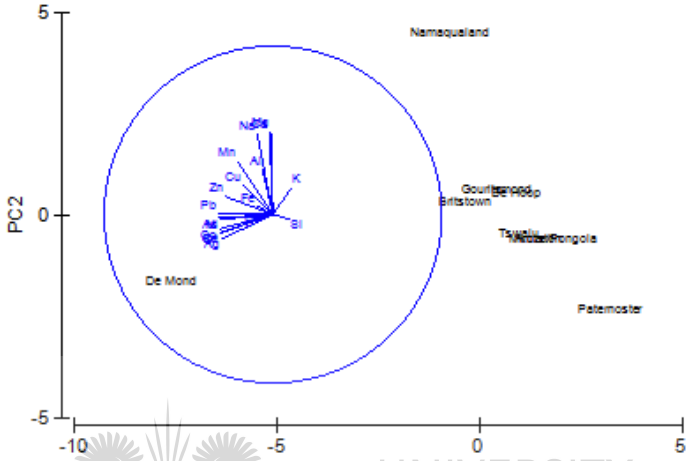


Figure 8.12. Principal component analysis (PCA) plot for different metal concentrations (µg/g dry weight) found in nail samples of both *Chersina angulata* and *Stigmochelys pardalis* combined from a total of nine collection sites.

8.3.5. Discussion

There were significant spatial differences for a number of metals for all three tissue types. Namaqualand was observed as the most consistently different site as shown by all three tissue types. Such differences are likely attributable to the different soil or geological make-up of the regions in which sites are based (see Figs. 3.1 and 3.2, Chapter 3, section 3.1.1.). Namaqualand is the only collection site situated in an area comprised mostly of weakly developed soil on mostly gneiss rock with lime (DEA, 2012). Britstown and Tswalu, in comparison, are close, but appear to fall into an area that is dominated by shale and sandstone rock and lithosols, as well as shifting sands and quartzite rock respectively (DEA, 2012). Other collection sites fall into areas dominated mostly by coastal weakly developed soils and shifting sands on shale, sandstone, quartzite and granite rock (DEA, 2012).

Among all three tissues, scute displayed spatial significance for more metals than the other two tissues. The reason for such a finding is unknown, especially since it was expected that the results of scute and nail would be similar, given that both tissues are similar in cellular morphology (McArthur *et al*., 2004). However, this was not the case, possibly a result of the rate of growth or age of the tissues collected. Scute is deposited in rings on a yearly basis, the older scute tissue in the centre and the youngest on the periphery (McArthur *et al*., 2004). It is unknown at what rate tortoise nail grows, however, at the same time it is worn away during the tortoise's daily activities. Thus, nail may only reflect a limited period of metal exposure and sequestration in comparison with the longer period represented by scute. Scute and nail are epidermal tissues, the former deposited in the form of keratinised plates during growth of the tortoise, but unlike bone, scute and nail are inert once deposited and are thus not 'renewable' tissue forms (McArthur *et al*., 2004). Thus, accumulated metals within deposited scute and nail cannot be metabolised, as they might be within the bone, remaining in these primary concentrations indefinitely.

Nonetheless, all three tissue types showed no major similarities for metal concentration results. In terms of the suitability of a particular tissue type for routine non-invasive metal bioaccumulation monitoring, bone is a tissue sample that is not an option for living individuals and scute, even though it may be collected from living individuals, is not practically feasible since it is very difficult to collect in utilisable amounts. Nail is thus still the best option. Important to note is that metal-fingerprinting cannot be used for all three tissues interchangeably, but only one. Nail metal concentrations were totally different to bone or scute concentrations, but did reveal good variation between sites. Compared to both bone and scute, it is more readily collected and it can be easily stored until analysis. Results such as these, suggest nail to be a good tissue for use in metal-fingerprinting.

Metal concentrations within soil and tortoise nail were seen to not correlate for both *C. angulata* and *S. pardalis*, suggesting that it may also not work in future for other species of tortoise. Nail, however, appears to have better resolution when differentiating amongst sites than do soils; a good example being that of De Hoop and De Mond.

De Hoop and De Mond were found to group together for the soil analysis, which would be expected since these two sites are relatively close to each other and have a very similar soil topology. However, for the nail analysis these two sites are very different from each other. Referring back to figures 8.8 A (a, c - f) and 8.8 B (g – l), it can be seen that De Mond has the highest metal concentrations for nail between sites for most of the metals analysed. De

Mond is an estuarine environment, the mouth of the Heuningnes River (pers. obs. and De Mond authorities) and thus likely receives a lot of these metals, which have been accumulated during the river's course and come to settle within the mouth before it enters the ocean. Results of the soil for metal concentrations did not suggest De Mond to have the highest concentrations for most metals, which would have been expected. If however, nail had correlated with soil, it would have been almost impossible to determine from which site a tortoise originated. Nail differentiates between three very close sites: Arniston, De Hoop and De Mond with high resolution. It would thus be better to and it is therefore proposed that instead of comparing nail metal concentrations to a database of soil metal concentrations to rather provide a nail metal concentration database for which to compare only nail. Results from this study thus form the basis of a potential nail metal concentration database, that in future, might aid conservationists to find a solution for the major problem of returning captive tortoise individuals to their origin of capture.

CHAPTER 9

SUMMATIVE DISCUSSION, FUTURE RESEARCH AND CONCLUDING REMARKS

9.1. Summative discussion

In this thesis, studies were undertaken on apicomplexan haematozoan parasites, which included three haemogregarine species and two of haemoproteids. These infected five terrestrial tortoise species (*Chersina angulata*, *Kinixys belliana belliana*, *K. lobatsiana*, *K. natalensis* and *Stigmochelys pardalis*), both wild and captive, from four South African provinces (Gauteng, KwaZulu-Natal, Northern Cape and Western Cape) and four biomes (semi-arid grassland, Kalahari desert, subtropical thorn bushveld, and coastal endemic fynbos). Apicomplexan biodiversity studies were coupled with bio-indicator methods, biological tagging and assessment of accumulated trace and macro-elements, for future assessment of the 'health' and origins of South African tortoise populations. Techniques applied (as in Chapter 3) were light microscopy (using photomicroscopy and accompanying image-analysis software) to enable description and measurement of the morphological characteristics of apicomplexans, transmission electron microscopy (TEM) to compare ultrastructural characteristics and infer possible taxonomic placement, and molecular methods to extract, amplify and purify sections of parasite DNA useful in sequence comparisons and phylogenetic analysis. Molecular methods were applied, especially to better place an apicomplexan species of dubious taxonomic standing, namely *Haemogregarina fitzsimonsi*, secondly to deduce the vectors which might transmit this species, and thirdly to examine vector phylogeny. Techniques also included statistical analyses (for example, one-way ANOVAs, T-Tests, linear regression analyses and principal component analyses). These were used to test the relationship between parasite parasitaemias, prevalence and host body condition, and with inductively coupled plasma (ICP) mass (MS) and optical emission spectrometery (OES) analysis, to determine the significance, if any, of relationships between trace metal concentrations in wild tortoise tissue (bone, scute and nail) and that of the soil from their collection sites (Chapter 3 discussed the above methods in detail).

Chapter 1 introduced the topic broadly and outlined the hypotheses to be tested. Chapter 2 discussed the different biomes within South Africa and how these may have affected the tortoise biodiversity within the Republic over time. South Africa, having the highest biodiversity of tortoises in the world (Branch, 2008), was suggested to harbour an equal diversity of haematozoan parasites. Therefore, one hypothesis to be tested was that the diversity of South African tortoise apicomplexan parasites exceeded those species already recorded by Cook (2008) and Cook *et al*. (2009a). It was noted in Chapters 1 and 2 that reptiles are of an old phyletic age and it is this longevity that has been proposed in the past to have contributed to the great diversity of apicomplexan haematozoans that infect them (see Telford, 2009). In addition, due to this age, the host-parasite relationship between

reptiles and their haematozoans was proposed to be a well-adapted one, causing, in comparison to mammalian relationships of the same kind, fewer pathogenic effects. Certainly, in this thesis, no marked effects on host cells were observed, although only films of tortoise peripheral blood were examined.

Before this work was undertaken around 23 species of haematozoan had been recorded infecting chelonians worldwide (Chapter 2, Table 2.1) and only 11 of these species had been reported infecting terrestrial tortoises. Furthermore, only five of these species had been previously recorded from Southern African tortoises, including the haemogregarines *Haemogregarina fitzsimonsi* and *Haemogregarina parvula*, and the haemoproteids *Haemoproteus testudinalis, Haemoproteus balazuci* and *Haemoproteus* sp. A. These were noted by Cook (2008) and published by Cook *et al*. (2009a; 2010a, respectively). In this thesis it was thus necessary to assess, or at least begin to assess, terrestrial tortoise haematozoan diversity and identity, especially those species already described which might require re-assessment taxonomically using modern techniques.

Chapter 4 detailed those South African tortoise apicomplexans which had been detected and required re-examination, and some possibly new parasite species as well as host and locality records. Previously identified species *H. fitzsimonsi*, *H. parvula, Hp. testudinalis/Hp. balazuci* were recorded infecting five species of South African tortoises, these apicomplexan species having been previously recorded by Cook (2008), Cook *et al*. (2009a), and from the current research published in Cook *et al*. (2010a). As previously, *H. fitzsimonsi* (detailed in Chapter 5) was found to be relatively non-host species specific, and even though the number of known host records did not increase for this parasite during the current study, the locality records increased by nine and by one province (Northern Cape). On the other hand, *H. parvula* (detailed in Chapter 6) appeared to be host species specific, its locality records increasing by just one in the present study. This parasite, during the current research, was always found concurrently with *H. fitzsimonsi*, and the explanation for this remains an enigma and something to be investigated in the future. *Haemoproteus balazuci*, recorded first by Dias (1953) and then by Cook (2008) was synonymised on its morphological characteristics (using light photomicroscopy) with *Hp. testudinalis* (recorded by Laveran in 1905), as published by Cook *et al*. (2010a) and in this thesis (detailed in Chapter 7). This decreased the theoretical apicomplexan biodiversity in South African tortoises by one species (that is, from five to four). However, the host and locality records increased for *Hp. testudinalis* by two host species and two localities, so that they now include *K. b. belliana* and *K. lobatsiana* from Mozambique (see Dias,

1953) and the South African province of Gauteng, in addition Laveran's (1905) original observation from *S. pardalis* from the Cape. The vectors of the above haemoproteids are still unknown.

It was also found that the province with the highest haematozoan diversity, prevalence and parasitaemia was the subtropical KwaZulu-Natal, and this was second in tick prevalence to the Western Cape. Also discovered was that the tortoise species *K. lobatsiana* had the highest prevalence of haematozoans, *K. natalensis* the highest parasitaemia of haematozoans, and *Homopus areolatus* the highest tick prevalence; however these data were based on a small host sample sizes. In hosts with large sample sizes, *C. angulata* and *S. pardalis*, the former species presented with the highest haematozoan and tick prevalences as well as haematozoan parasitaemias compared to the latter species. *Psammobates* species showed no haematozoan infections and had the lowest tick prevalence of all tortoise genera examined. Additionally, adult male tortoises collectively had the highest haematozoan and tick prevalences in comparison to adult females and juveniles, with juveniles having the highest parasitaemias, and adult males and females having very similar parasitaemias.

During the current study one new species, a haemogregarine, *Haemogregarina* sp. A was described (detailed in Chapter 6), and a haemoproteid, previously recorded as *Haemoproteus* sp. A by Cook (2008), was described and published by Cook *et al.* (2010a) as *Hp. natalensis* (detailed in Chapter 7). Both reports were based on morphometrical analysis (by light photomicroscopy). These descriptions increased the biodiversity of named apicomplexan species in South African tortoises by one (restoring the number to four), as hypothesised, but if in future additional specimens of the haemogregarine (*Haemogregarina* sp. A) are discovered then it may be possible to name a fifth. Both *Haemogregarina* sp. A and *Hp. natalensis* appear to be host and locality specific, having been recorded only from KwaZulu-Natal, and parasitizing two *S. pardalis* from Mkuze Nature Reserve and a single captive *K. natalensis* from Ballito respectively.

In total, 8 of the 13 species of tortoise inhabiting South Africa have been examined in this thesis for apicomplexans. It is important that the remaining five tortoise species of South Africa are also examined at some stage soon, however, this may prove to be a monumental task, requiring considerable effort to detect species which are very difficult to locate in the wild.

It was hypothesized that at least two of the above species of apicomplexans, *H. fitzsimonsi* and *H. parvula*, may be taxonomically incorrectly assigned. Siddall (1995) not only assigned them to the

genus *Haemogregarina* (sensu stricto) but also synonymised the two species; this placing also implied that they are transmitted by a leech vector. Since no leeches and only ticks were found infesting tortoises infected with *H. fitzsimonsi* (Chapter 4), it was further hypothesized in the current thesis that ticks may be its vector, the capacity for ticks to host such apicomplexan parasites and aspects of tick evolution being considered initially in Chapter 2. Chapters 5 and 6 aimed to assess, in detail, the taxonomy of *H. fitzsimonsi* and *H. parvula* respectively with the use of morphometrics (using light photomicroscopy and TEM) and by molecular analysis, if feasible. In addition, collected ticks underwent histological assessment (Chapter 5), taking account of research done by Široký *et al*. (2007) on the apicomplexan *Hemolivia mauritanica* which is transmitted to tortoises by the tick *Hyalomma aegyptium*. In the current study, ticks were removed from tortoises infected with *H. fitzsimonsi* from a wide range of sites, and from regions in which only this particular apicomplexan species was found to occur. Any stages of the blood parasite found in the ticks were thus likely to be those of *H. fitzsimonsi.* On the other hand, unlike *H. fitzsimonsi, H. parvula* was restricted in distribution, although found to co-exist with the former species. This presented a potential difficulty in differentiating possible tick stages for *H. parvula*, since an equal probability existed that stages in ticks where *H. parvula* occurred (KwaZulu-Natal) could belong to *H. fitzsimonsi*. Thus, for the current research *H. fitzsimonsi* was the focus in terms of molecular analysis, and studies on *H. parvula* will have to wait until tortoises infected solely with this parasite can be located (but see below).

Impression slides from all tick stages (larvae, nymphs and adults) were prepared, but sporocyst and sporozoite stages occurred only within adult male and female ticks of the species *Amblyomma marmoreum* and *Amblyomma sylvaticum*, when assessed by light microscopy (Chapter 5). Furthermore, sporogonic stages were found within the haemocoel of the infected ticks, a characteristic typical of *Hepatozoon* species, rather than of *Haemogregarina*. This then led to the attempted molecular characterisation of *H. fitzsimonsi* by use of PCR and selected 18S ribosomal DNA primer sets HEMO1/HEMO2, HEPF300/HEPR900 and 4558F/2773R (see Chapter 5). It was found that *H. fitzsimonsi* did indeed appear to belong genetically within the genus *Hepatozoon*, and this was corroborated by the apparent lack of dividing stages within the peripheral blood of tortoises in the current study, stages that are common with species of *Haemogregarina* and *Hemolivia*. Thus, the hypothesis that *H. fitzsimonsi* was taxonomically incorrectly placed in the genus *Haemogregarina* has been accepted and it is recommended that in future the name *Haemogregarina fitzsimonsi* is replaced by *Hepatozoon fitzsimonsi.*

Phylogenetically, 18S ribosomal DNA *H. fitzsimonsi* sequences, when compared with similar sequences from other *Hepatozoon* species, grouped within one of three major clades, specifically within the clade which clustered other species of reptilian *Hepatozoon* (Chapter 5). However, the apicomplexan perhaps did not group as closely as expected to the other reptile *Hepatozoon* species within this clade. It was suggested that this might result from the phyletic age of chelonian hosts. Also suggested in Chapter 5, was the possibility of phyletically older species of *Hepatozoon* being more species specific than phyletically younger species. This suggestion will, however, require further research in future and is, at best, speculative at present.

Also in Chapter 5, parasite DNA was also successfully extracted, but unfortunately not successfully sequenced, from the two tick species named above. However, based on sporogonic findings by light microscopy and the apparent extraction/amplification of *Hepatozoon* 18S rDNA as viewed on the gels, these two tick species (*A. marmoreum* and *A. sylvaticum*) have been assigned as vectors of *H. fitzsimonsi* as was hypothesized. Also mentioned in Chapter 5 was the possible perpetuation of infection especially through adult male ticks, since males may survive into the next year, unlike adult females which die directly after egg-laying. Discoveries documented in Chapter 5 were additionally useful in hypothesizing a life cycle for *H. fitzsimonsi*. It was proposed that tortoises accidently ingest *H. fitzsimonsi* infected ticks (with sporocysts/sporozoites) at communally utilized tortoise resting sites becoming themselves infected with the haematozoan. The haematozoan would then be transferred back to a tick vector during a blood meal, when parasite gamonts would be taken up along with the tortoise peripheral blood.

Along with examination of tick parasite stages and molecular analysis of these and tortoise peripheral blood stages, ultrastructural examination was carried out on the gamont stages of *H. fitzsimonsi* (Chapter 5) as well as *H. parvula* (Chapter 6). Comparisons of these stages revealed a further conundrum. At the ultrastructural level, *H. parvula* did not resemble *H. fitzsimonsi*, which supported the likelihood that they are two different species. However, *H. parvula* did not resemble other species of *Haemogregarina* or *Hepatozoon* either, but species of *Hemolivia* in its ultrastructure. Unfortunately, no sequences have been uploaded onto GenBank for *Hemolivia* as yet. Therefore, even if *H. parvula* DNA had been extracted and amplified using HEMO1/HEMO2 primers and sequences obtained, comparisons of such data with uploaded sequence data of species of *Hemolivia* would not have been possible, although comparison with *Hepatozoon* sequences could be undertaken. Nonetheless, the hypothesis that *H. parvula* is taxonomically incorrectly assigned to the

genus *Haemogregarina* is accepted in a preliminary sense, based on its distribution, structure and ultrastructure, with molecular analysis pending.

Based on previous observations (Cook *et al*., 2009a), it was hypothesized that a correlation might exist between the prevalence and parasitaemia levels of *H. fitzsimonsi* and tortoise body condition, the relationship possibly being of use in future conservation assessments of population 'health'. As noted earlier, tortoise life spans are generally long, depending on the species, and haemogregarine infections may be chronic and not fatal, and therefore tortoises and their infections may be long lived enough to provide a database tracking infection versus body condition over time.

Only two tortoise species were used for this section of the work (Chapter 8), *C. angulata* and *S. pardalis*, since they were commonly occurring and readily collectible during the current study. In addition, average body condition, calculated using the method of Loehr *et al*. (2006), was the same (1.2) for both species of tortoise. This meant that the two species of tortoise could be readily compared when assessing the relationship between body condition, prevalence and parasitaemia. Both species of tortoise were also parasitized by ticks, *A. marmoreum* on *S. pardalis* and *A. sylvaticum* on *C. angulata*. In addition, with ticks likely being responsible for the perpetuation of infection (Chapter 5), it was theorised that anthropogenically affected areas could increase tick density or, alternatively, completely eradicate tick populations. This might result in either concentrating parasitaemias or reducing them to either an unrecognizable level in tortoise peripheral blood, or eradicating them completely.

However, no significant relationship was evident between the body condition of tortoises, prevalence and the parasitaemia of the infection, for *C. angulata* or *S. pardalis* individually, or for both tortoise species together. Although prevalence and parasitaemia of infection were found to be on average higher in areas that were anthropogenically affected than in un-impacted (reserve) areas, these differences were once again insignificant statistically. The hypotheses that prevalence and parasitaemia of *H. fitzsimonsi* would be good bio-indicators of tortoise body condition and potentially population 'health' were therefore rejected.

Conversely, during this thesis, it was proposed that the relationships between haematozoan and ectoparasite load, along with tortoise body condition could possibly predict environmental status. Thus tortoises inhabiting a natural (un-impacted) environment were perhaps more likely to have a high prevalence of infection with a low parasitaemia, high prevalence owing to a relatively high

prevalence of the vectors (ticks) and a low parasitaemia resulting from an un-suppressed immunological host status. However, this proposal will require further investigation in future, based on more extensive data collection and analysis than was feasible in the current study.

In addition, in Chapter 8, the potential use of South African tortoise haematozoans as biological tags was briefly assessed. Host range and species were taken into consideration when assessing apicomplexan species as well as the average prevalence and parasitaemia of these parasites. Data showed that even though *H. fitzsimonsi* was relatively prevalent and parasitaemias generally high compared to other haematozoan species, the apicomplexan had a wide geographical and host range, and therefore could not be used as a tag unless future molecular assessments of *H. fitzsimonsi* from different tortoise populations reveal different strains of this parasite. In contrast to *H. fitzsimonsi*, the haemogregarines *H. parvula* and *Haemogregarina* sp. A, as well as the haemoproteids *Hp. testudinalis* and *Hp. natalensis* all appear to be locality and largely species specific. These species could theoretically then prove useful in tagging. However, *Haemogregarina* sp. A and *Hp. natalensis* present with such low prevalences, the former along with a very low parasitaemia, that their use as biological tags is not promising at present.

It was hypothesized that through metal-fingerprinting of wild tortoises, a method for the future determination of captive tortoise origin might be determined. Using statistical analyses, such as those detailed in Chapter 3, the relationships between metal concentrations within wild tortoise tissues (bone, scute and nail) and soil from the collection sites were compared. The results of these comparisons were discussed in Chapter 8 and it was found that all three tissue types could be used to differentiate between different sites, using a single tissue at a time. Namaqualand was found to be consistently significantly different from other sites examined for all three tissue types' metal-fingerprints. The metal-fingerprint of scute was found to significantly differentiate between sites for most of the metals analysed, compared to bone and nail, which differentiated between sites, but for fewer of the metals analysed. However, the different tissue types could not be compared with each other since each had a different metal-fingerprint for the same site, for instance, the metal-fingerprint of bone and nail, both collected from Namaqualand, were different. It was thus concluded that only a single tissue could be used for future tortoise site comparisons and since this tissue should be collected non-invasively, nail was selected as the tissue of choice.

Nail was found to provide a high degree of differentiation between sites, even more so than that of the soil samples against which it was compared. Unfortunately, nail and soil type did not correlate

with each other in terms of metal-fingerprinting and it was thus proposed that a nail trace-metal database be set up for different wild tortoise populations and from this it is anticipated that captive individuals' nail samples can be evaluated. Even though a few problems arose with using nail, the method was considered a useful and repeatable approach, and thus it was possible to accept the above hypothesis, and with further investigation it may be used in future for the conservation of tortoises.

9.2. Future research

Further research examining peripheral blood films from the remaining five tortoise species found in South Africa will need to be undertaken to assess the true apicomplexan biodiversity amongst these tortoise species.

Since *H. fitzsimonsi* was found during this thesis to belong morphologically, developmentally and genetically within the genus *Hepatozoon*, this species will require official renaming as *Hepatozoon fitzsimonsi*.

Additional specimens of the tortoise *K. b. belliana* will need to be examined for peripheral blood infections of *H. parvula* and tick specimens collected. The close resemblance of *H. parvula* gamonts to those of *Hemolivia* species at an ultrastructural level suggests strongly that it too may be a species of *Hemolivia*. Since *Hm. mauritanica* is transmitted through the ingestion of tortoise blood by a tick vector, it is probable that *H. parvula* may itself be transmitted in such a way. Dias (1953) collected ticks of the species *Amblyomma nuttalli* from *H. parvula* and *H. fitzsimonsi* infected tortoises. This tick species is apparently endemic to subtropical regions (Chapter 2, section 2.4.2), correlating with the geographical range of *H. parvula* infections. It would be useful in future to find this tick species and master the molecular methods necessary for extracting, amplifying and purifying parasite DNA for *H. parvula*, from sporocyst-infected ticks, if these exist. Ticks will thus need to be examined for sporogonic stages and these compared to similar stages of *H. fitzsimonsi*. This would be particularly important, since the finding of single peripheral blood infections of *H. parvula* may be extremely difficult. However, if tick stages are not a possibility, a very high peripheral blood parasitaemia of *H. parvula* may prove useful in molecular studies, similar to that for *H. fitzsimonsi* as detailed in Chapter 5, particularly if comparative *Hemolivia* sequences can be obtained eventually from elsewhere. In addition, increased sampling effort may aid in discovering further blood stages of *H. parvula*, allowing the taxonomic placement of this parasite to be determined.

The morphological and morphometric similarities of *Hp. testudinalis* to *Haemoproteus roumei* will need to be reassessed by modern photomicroscopy and possibly by molecular techniques, including sampling of *K. b. belliana* from Mozambique for *Hp. testudinalis*. Sequences of the above *Haemoproteus* species will need to be compared to published sequences of *Haemoproteus* on GenBank as well as to one another to determine sequence identity and thus, whether all three (*Hp. roumei*, Dias' (1953) and the current study's *Hp. testudinalis*) can be combined in a single species, or if they represent more species than are apparent at a light microscopy level. Also of importance is the mode of transmission of these parasites. The vectors for the above haemoproteids are unknown and therefore require future investigation. It has been proven that dipteran biting flies of the family Tabanidae transmit *Haemoproteus* of terrapins (see Telford, 2009) and thus if such vectors can be collected from tortoises, they will be well worth investigation.

More samples will need to be obtained for both *Haemogregarina* sp. A and *Hp. natalensis* for molecular analyses. It is hoped that in future prevalence and parasitaemia of *H*. sp. A will be high, so that extraction and amplification of parasite DNA may be successful. Unfortunately, the wild origin of *Hp. natalensis* is unknown and further samples may be very difficult to obtain.

In future, it may be better to perform quantitative PCR on tortoise blood in addition to, or as an alternative to screening blood films. Quantitative real-time PCR is a method that uses measurement of emitted fluorescence during the run to determine the amount of PCR product (parasite DNA). It is a method that can be sensitive, precise and apparently decreases the possibility of contamination by eliminating post-PCR steps (see Klein, 2002). This method may provide a more accurate process by which to assess the relationship between body condition index and parasitaemia of tortoises. However, the expense of such a method may prohibit use by Nature Conservation.

The wide host and geographical range of *H. fitzsimonsi* suggests that it may not be a good species for use in biological tagging. However, molecular analysis of the parasite in different tortoise populations using more sensitive methods (such as those using highly variable regions of parasite DNA) may differentiate various strains of the parasite. This in itself may prove to be quite useful. Tortoises are also known to harbour intestinal parasites such as oxyurids (Nematoda) (Morand *et al*., 1996; McArthur *et al*., 2004) which have been observed during this research and past studies (Cook, 2008) in a large number of wild and captive tortoises. Genetic differentiation of tortoise population

oxyurids and their use as bioloigical tags may be another initiative worth investigating in future for use in the conservation of these animals.

Metal-fingerprinting will need to be assessed further as a conservation tool by determining, with the input of conservation authorities, its practical worth in terms of time required for the process and its overall cost. Also, a number of captive tortoises will need to undergo the same procedure and be compared against the results for wild tortoises obtained during this thesis before any firm conclusions can be drawn.

9.3. Conclusion

Overall, the current research has highlighted the necessity of re-assessing the apicomplexan species of tortoises with the aid of modern techniques (Chapters 5, 6 and 7). It has also argued for the importance of describing species, especially species of dubious taxonomic standing, using not only morphological characteristics, but life cycle and molecular information as well (Chapter 5). Acquisition of morphological data is a necessary primary step in identifying how further histological and molecular work may be employed to assess accurately species taxonomy and therefore biodiversity, and its importance should thus not be overlooked. The use of parasites, particularly apicomplexan parasites, as bio-indicators and biological tags may still prove in future to be feasible. Metal fingerprinting of wild tortoise tissues has shown to be a promising technique and may with further research be offered as a tortoise conservation tool for use by Nature Conservation authorities.

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APPENDICES

APPENDIX 1

TABLES

Table 1a. Wild tortoises collected and examined for haematozoans. Recorded below is the province, sampling site, tortoise species and sex (M=male, F=female and J=juvenile), haematozoan recorded and parasitaemia (%) for each species respectively, as well as tick species and life stage collected from infested tortoises (L=larvae, N=nymphs, A=adult males and females). Subtotals are provided for individual sites and totals per province.

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
KZN	**Mkuze**					
		Kinixys belliana belliana	M	*Haemogregarina fitzsimonsi, Haemogregarina parvula*	5.4, 10.6	
		K. b. belliana	J			
		K. b. belliana	F	*H. fitzsimonsi, H. parvula*	10, 11	
		Stigmochelys pardalis	F			*Amblyomma marmoreum* (L, N, A)
		S. pardalis	M	*H. fitzsimonsi*	2.2	
		S. pardalis	M	*H. fitzsimonsi, Haemogregarina* sp. A.	0.02, 0.005	
		S. pardalis	M			
		S. pardalis	F (J)			
		S. pardalis	F (J)			
		S. pardalis	M	*H. fitzsimonsi*	14.2	
		S. pardalis	F	*H.* sp. A.	0.002	
		S. pardalis	F	*H. fitzsimonsi*	46	
		S. pardalis	F (J)			
		S. pardalis	M	*H. fitzsimonsi*	5	
		S. pardalis	M			
		S. pardalis	M	*H. fitzsimonsi*	2.4	*A. marmoreum* (N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
KZN	**SUBTOTAL**	**16 individuals representing 2/8 (25%) of endemic tortoise species**	**M 8/16 (50%); F 7/16 (44%); J 1/16 (6%)**	**9/16 (56.3%) infected individuals**	**~10.6% *H. fitzsimonsi*; ~10.8% *H. parvula*; ~0.004% *H.* sp. A**	**2/16 (13%) individuals with *A. marmoreum***
	Pongola					
		Kinixys natalensis	F			*A. marmoreum* (N)
		S. pardalis	F			*A. marmoreum* (L)
		S. pardalis	F			*A. marmoreum* (L, A)
		S. pardalis	M			*A. marmoreum* (N, A)
	SUBTOTAL	**2/8 (25%) tortoise species**	**M 1/4 (25%); F 3/4 (75%)**	**0/4 (0%) infected**	**~0%**	**4/4 (100%) individuals with *A. marmoreum***
TOTAL FOR KZN		**20 individuals representing 3/8 (38%) of endemic tortoise species**	**M 9/20 (45%); F 10/20 (50%); J 1/20 (5%)**	**9/20 (45%) infected individuals**	**~10.6% *H. fitzsimonsi*; ~10.8% *H. parvula*; ~0.004% *H.* sp. A**	**6/20 (30%) individuals with *A. marmoreum***

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
NC						
	Britstown					
		S. pardalis	M			*A. marmoreum* (A)
	SUBTOTAL	**One of 8 (13%) of endemic tortoise species**	**M 1/1 (100%)**	**0/1 (0%)**	**~0%**	**1/1 (100%) *A. marmoreum***
	De Beers					
		C. angulata	M	*H. fitzsimonsi*	0.4	*Amblyomma. sylvaticum* (L, N, A)
		C. angulata	M	*H. fitzsimonsi*	0.3	*A. sylvaticum* (L, N, A)
		C. angulata	J	*H. fitzsimonsi*	15.6	*A. sylvaticum* (L, N, A)
		C. angulata	F	*H. fitzsimonsi*	20	*A. sylvaticum* (L, N, A)
		C. angulata	M	*H. fitzsimonsi*	0.02	*A. sylvaticum* (L, N, A)
	SUBTOTAL	**5 individuals representing 1/8 (13%) of endemic tortoise species**	**M 3/5 (60%); F 1/5 (20%); J 1/5 (20%)**	**5/5 (100%) infected individuals**	**~7.3% *H. fitzsimonsi***	**5/5 (100%) individuals with *A. sylvaticum***
	Namaqualand					
		C. angulata	F			
		C. angulata	F			*A. sylvaticum* (L, N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
NC	**Namaqualand**	*C. angulata*	M			
		C. angulata	F			
		C. angulata	M			
		C. angulata	F			
		C. angulata	M	*H. fitzsimonsi*	0.3	
		C. angulata	M			
		C. angulata	M			
		C. angulata	F			
		C. angulata	M			
		C. angulata	M			
		C. angulata	F			
		C. angulata	M			*A. sylvaticum* (L, N, A)
		Psammobates tentorius trimeni	F			
		P. t. trimeni	M			
	SUBTOTAL	**16 individuals representing 2/8 (25%) of endemic tortoise species**	**M 9/16 (56%); F 7/16 (44%)**	**1/16 (6%) infected individual**	**~0.3% *H. fitzsimonsi***	**2/16 (13%) individuals with *A. sylvaticum***
	Tswalu					
		Psammobates oculifer	M			
		P. oculifer	M			

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
NC	**Tswalu**	*P. oculifer*	M			*Ornithodorus compactus* (A)
		P. oculifer	F			
		P. oculifer	F			
		P. oculifer	F			
		P. oculifer	F			
		P. oculifer	M(J)			
		P. oculifer	M			
		P. oculifer	M			
		S. pardalis	F			
		S. pardalis	F			*A. marmoreum* (N, A)
		S. pardalis	F			
		S. pardalis	M			
		S. pardalis	M			
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	F			*A. marmoreum* (N, A)
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	J			
		S. pardalis	M			*A. marmoreum*, *O. compactus* (A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
NC	**Tswalu**	*S. pardalis*	M			*A. marmoreum* (N, A)
		S. pardalis	M			
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	F			*A. marmoreum* (A)
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	M			*A. marmoreum* (A)
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	M			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	M			

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
NC	**Tswalu**	*S. pardalis*	F			
		S. pardalis	F			
		S. pardalis	M			
		S. pardalis	J			
		S. pardalis	F?			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	J			
		S. pardalis	J			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F(J)			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	F(J)			

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
NC	**Tswalu**	*S. pardalis*	F(J)			
		S. pardalis	M			
		S. pardalis	F			
		S. pardalis	M			
		S. pardalis	F(J)			
		S. pardalis	F			
		S. pardalis	F			
		S. pardalis	M			*A. marmoreum* (N, A)
		S. pardalis	M			*A. marmoreum* (A)
	SUBTOTAL	**70 individuals representing 2/8 (25%) of endemic tortoise species**	**M 25/70 (36%); F 41/70 (59%); J 4/70 (6%)**	**0/70 (0%) infected**	**~0%**	**2/70 (3%) individuals with *O. compactus*; 14/70 (20%) with *A. marmoreum***
TOTAL FOR NC		**92 individuals representing 4/8 (50%) of endemic tortoise species**	**M 38/92 (41%); F 49/92 (53%); J 5/92 (5%)**	**6/92 (7%) infected**	**~3.8% *H. fitzsimonsi***	**2/92 (2%) individuals with *O. compactus*; 15/92 (16%) with *A. marmoreum*; 7/92 (8%) with *A. sylvaticum***
WC						
	Arniston					
		C. angulata	F			*A. sylvaticum* (L, N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC	**Arniston**	*C. angulata*	F(J)			*A. sylvaticum* (L, N, A)
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F			
		C. angulata	M			
		C. angulata	F	*H. fitzsimonsi*	2	*A. sylvaticum* (L, N, A)
		C. angulata	F	*H. fitzsimonsi*	3.3	
		C. angulata	M	*H. fitzsimonsi*	60	*A. sylvaticum* (L, N, A)
		C. angulata	M			
		C. angulata	M(J)			
		C. angulata	M	*H. fitzsimonsi*	0.8	*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	M	*H. fitzsimonsi*	0.4	
		C. angulata	F	*H. fitzsimonsi*	0.8	
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	M			

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC		*C. angulata*	F			
		C. angulata	F			
	SUBTOTAL	**20 individuals representing 1/8 (13%) of endemic tortoise species**	**M 10/20 (50%); F 10/20 (50%)**	**6/20 (30%) infected individuals**	**~11.2% *H. fitzsimonsi***	**10/20 (50%) individuals with *A. sylvaticum***
	De Hoop					
		C. angulata	F			
		C. angulata	M			*A. sylvaticum* (N)
		C. angulata	M			
		C. angulata	M			
		S. pardalis	F	*H. fitzsimonsi*	5	
		S. pardalis	F			
	SUBTOTAL	**6 individuals representing 2/8 (25%) of endemic tortoise species**	**M 3/6 (50%); F 3/6 (50%)**	**1/6 (17%) infected individual**	**~5% *H. fitzsimonsi***	**1/6 (17%) individuals with *A. sylvaticum***
	De Mond					
		C. angulata	M	*H. fitzsimonsi*	0.8	*A. sylvaticum* (N, A)
		C. angulata	M			*A. sylvaticum* (N, A)
		C. angulata	M	*H. fitzsimonsi*	0.4	*A. sylvaticum* (N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC		*C. angulata*	M(J)	*H. fitzsimonsi*	0.8	*A. sylvaticum* (N, A)
	SUBTOTAL	**4 individuals representing 1/8 (13%) of endemic tortoise species**	**M 4/4 (100%)**	**3/4 (75%) infected individuals**	**~0.6% *H. fitzsimonsi***	**4/4 (100%) individuals with *A. sylvaticum***
	Elandsberg					
		Psammobates geometricus				
	SUBTOTAL	**Not examined**	-	-	-	-
	Gouritzmond					
		C. angulata	M			
		C. angulata	M			
		C. angulata	F			
	SUBTOTAL	**3 individuals representing 1/8 (13%) of endemic tortoise species**	**M 2/3 (66%); F 1/3 (33%)**	**0/3 (0%)**	**0%**	**0/3 (0%)**
	Paarl					
		Homopus areolatus	M			*A. marmoreum* (N, A)
		H. areolatus	F			*A. marmoreum* (N, A)
		H. areolatus	F			

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC	**Paarl**	*H. areolatus*	F			
		H. areolatus	F			*A. marmoreum* (L, N, A)
		H. areolatus	M			*A. marmoreum* (L, N, A)
		H. areolatus	F			
		H. areolatus	M			*A. marmoreum* (L, N, A)
		H. areolatus	F			*A. marmoreum* (L, N, A)
		H. areolatus	M			*A. marmoreum* (L, N, A)
		H. areolatus	F			
		H. areolatus	F			*A. marmoreum* (L, N, A)
		H. areolatus	F			*A. marmoreum* (L, N, A)
		H. areolatus	F			*A. marmoreum* (L, N, A)
		H. areolatus	F			
		H. areolatus	F			
		H. areolatus	M			*A. marmoreum* (L, N, A)
		H. areolatus	F			*A. marmoreum* (L, N, A)
		H. areolatus	F			*A. marmoreum* (L, N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC	**Paarl**	*H. areolatus*	F			*A. marmoreum* (L, N, A)
	SUBTOTAL	**20 individuals representing 1/8 (13%) of endemic tortoise species**	**M 5/20 (25%); F 15/20 (75%)**	**0/20 (0%) infected**	**0%**	**14/20 (70%) individuals with *A. marmoreum***
	Paternoster					
		C. angulata	M			
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F	*H. fitzsimonsi*	0.3	*A. sylvaticum* (L, N, A)
		C. angulata	M	*H. fitzsimonsi*	3	*A. sylvaticum* (L, N, A)
		C. angulata	M	*H. fitzsimonsi*	0.3	*A. sylvaticum* (L, N, A)
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	F	*H. fitzsimonsi*	0.6	*A. sylvaticum* (L, N, A)
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	F	*H. fitzsimonsi*	40	*A. sylvaticum* (L, N, A)
		C. angulata	M	*H. fitzsimonsi*	1.7	*A. sylvaticum* (L, N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC	**Paternoster**	*C. angulata*	F			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F(J)			*A. sylvaticum* (L, N, A)
		C. angulata	M			*A. sylvaticum* (L, N, A)
		C. angulata	M			
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	M(J)			*A. sylvaticum* (L, N, A)
	SUBTOTAL	**19 individuals representing 1/8 (13%) of endemic tortoise species**	**M 10/19 (53%); F 9/19 (47%)**	**6/19 (32%) infected individuals**	**~7.7% *H. fitzsimonsi***	**17/19 (89%) individuals with *A. sylvaticum***
	West Coast					
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
		C. angulata	F	*H. fitzsimonsi*	2.5	*A. sylvaticum* (L, N, A)

Table 1a. continued

Province	Site	Tortoise species	Sex	Peripheral blood infection	Parasitaemia (%)	Infestation
WC	**West Coast**	*C. angulata*	M			*A. sylvaticum* (L, N, A)
		C. angulata	F			*A. sylvaticum* (L, N, A)
	SUBTOTAL	**6 individuals representing 1/8 (13%) of endemic tortoise species**	**M 1/6 (17%); F 5/6 (83%)**	**1/6 (17%) infected individual**	**~2.5% *H. fitzsimonsi***	**6/6 (100%) individuals with *A. sylvaticum***
TOTAL FOR WC		**78 individuals of 3/8 (38%) of endemic tortoise species**	**M 35/78 (45%); F 43/78 (55%)**	**17/78 (22%) infected individuals**	**~5.4% *H. fitzsimonsi***	**14/78 (18%) individuals with *A. marmoreum*; 38/78 (49%) with *A. sylvaticum***

Table 1b. Captive tortoises collected and examined for the presence of haematozoans. Recorded below is the province, sampling site, tortoise species and sex (M=male, F=female and J=juvenile), haematozoan recorded and parasitaemia (%), tick species are not included since all sites had already removed any ticks when tortoises were first received into the institutions. Subtotals are provided for each site and totals per province.

CAPTIVE					
Province	**Site**	**Tortoise species**	**Sex**	**Infection**	**Parasitaemia (%)**
GP					
	JHB Zoo				
		Kinixys lobatsiana	M	*Haemoproteus testudinalis*	1.3
		K. lobatsiana	F	*Hp. testudinalis*	2
		K. lobatsiana	M		
		K. lobatsiana	M		
		K. lobatsiana	F		
		K. lobatsiana	M		
		Stigmochelys pardalis	F		
		S. pardalis	F	*Haemogregarina fitzsimonsi*	0.4
		S. pardalis	M	*H. fitzsimonsi*	0.7
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	J		
	SUBTOTAL	**16 individuals representing 2/8 (25%) of endemic tortoise species**	**M 9/16 (56%); F 6/16 (38%); J 1/16 (6%)**	**4/16 (25%) infected individuals**	**~1.7% *Hp. testudinalis*; ~0.6% *H. fitzsimonsi***
	Private				
		S. pardalis	M		
	SUBTOTAL	**One of 8 (13%) of endemic tortoise species**	**M 1/1 (100%)**	**0/1 (0%) infected**	**~0%**
TOTAL FOR GP		**17 individuals representing 2/8 (25%) of endemic tortoise species**	**M 10/17 (59%); F 6/17 (35%); J 1/17 (6%)**	**4/17 (23.5%) infected individuals**	**~1.7% *Hp. testudinalis*; ~0.6% *H. fitzsimonsi***
KZN	**Ballito** [follow up study to Cook (2008)]	*Kinixys natalensis*	M	*Haemoproteus* sp. A	15
TOTAL FOR KZN		**1/8 (13%) of endemic tortoise species**	**M 1/1 (100%)**	**1/1 (100%) infected individual**	**~15% *Hp.* sp. A**
WC					
	Butterfly Park				
		Chersina angulata	M		
		C. angulata	M		
		C. angulata	M		
		C. angulata	M		
		C. angulata	M		

Table 1b. continued

Province	Site	Tortoise species	Sex	Infection	Parasitaemia (%)
WC	**Butterfly Park**	*C. angulata*	M		
		C. angulata	F		
		C. angulata	M		
		C. angulata	M		
		C. angulata	F		
		C. angulata	F		
		C. angulata	F		
		C. angulata	F		
		C. angulata	M		
		C. angulata	M		
		C. angulata	F		
		C. angulata	F		
		C. angulata	M		
		C. angulata	F		
		C. angulata	F		
		C. angulata	M		
		C. angulata	F		
		K. lobatsiana	M		
		Homopus areolatus	F		
		H. areolatus	M		
		H. areolatus	M		
		H. areolatus	M		
		H. areolatus	M		
		H. areolatus	M		
		Psammobates tentorius trimeni	M		
		Testudo graeca	F		
		T. graeca	F		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	M		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	F	*H. fitzsimonsi*	0.1

Table 1b. continued

Province	Site	Tortoise species	Sex	Infection	Parasitaemia (%)
WC	**Butterfly Park**	*S. pardalis*	F		
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	F	*H. fitzsimonsi*	0.07
		S. pardalis	F		
		S. pardalis	F		
		S. pardalis	F	*H. fitzsimonsi*	0.01
		S. pardalis	M		
		S. pardalis	F		
		S. pardalis	M		
		S. pardalis	M		
		S. pardalis	M		
TOTAL FOR WC		**63 individuals representing 5/8 (63%) of endemic tortoise species plus one alien species *(T. graeca)***	**M 31/63(50%); F 32/63(51%)**	**3/63 (4.8%) infected individuals**	**~0.06% *H. fitzsimonsi***

APPENDIX 2

Permits

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

ORIGINAL

PERMIT

Permit Fee: R 50.00
Receipt No: Direct deposit

Permit No: 942/2009
Contact: Miss S.M. Hughes

This permit is issued in pursuance of the provisions of the Nature Conservation Ordinance No 15 of 1974 Chapter 7 , and the Regulations framed thereunder.

The permit is issued to:

ID Number: 8309020193089

Ms C. Cook
Department opf Zoology
University of Johannesburg
P. O. Box 524
Auckland Park
2006

Residential Address

Department opf Zoology
University of Johannesburg
P. O. Box 524
Auckland Park
2006

KwaZulu-Natal

To Collect and Export the following species of Amphibians Invertebrates and Reptiles

ALL SPECIES

Of Ticks on tortoises to be taken as required on the following Species of Tortoises: Stigmochelys pardalis babcocki - Babcock's Leopard Tortoise; Kinixys belliana belliana - Bell Hinged Tortoise and Kinixys natalensis - Natal hinged tortoise may be collected in Ndumo Game Reserve

TORTOISE

0.5ml Blood may be collected from the following Tortoises : Stigmochelys pardalis babcocki - Babcock's Leopard Tortoise; Kinixys belliana belliana - Bell Hinged Tortoise and Kinixys natalensis - Natal hinged tortoise may be collected in Ndumo Game Reserve

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 25 February 2009

for CHIEF EXECUTIVE

Permit Holder

KZN WILDLIFE PERMITS OFFICE
P.O. Box 13053, Cascades, 3202, Pietermaritzburg, KwaZulu-Natal. Tel:+27 33 8451320/1324
Fax: +27 33 845 1747. E-mail: permits@kznwildlife.com. Website: www.kznwildlife.com

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

TERMS AND CONDITIONS UNDER WHICH THIS PERMIT IS ISSUED

1. It is valid only:
   (i) from : 25 February 2009
   to : 31 December 2009
   (ii) in the original
   (iii) if all **3** pages are signed by the permit holder named above
   (iv) to the permit holder named above

2. Permit to be returned to E KZN Wildlife, P O Box 13053, Cascades, 3202, upon expiry for renewal or cancellation.

3. Permit shall be carried by holder, or the specified nominees, at all times during use.

4. Outside of E KZN Wildlife areas, use of this permit is subject to landowner's or controlling authority's written permission.

5. Prior to collecting in areas under the control of the E KZN Wildlife the holders shall contact the Officer-in-Charge of the area at least 48 (Forty-eight) hours before commencing, and shall comply with any conditions which the Officer may impose at his discretion.

6. At least one representative specimen (preferably at least one male and one female) of each species collected from each locality must be lodged with a recognised South African museum/herbarium. Holotype specimens, and half the number of paratype specimens, of any new species MUST BE DEPOSITED with a recognised South African museum/herbarium, and may only leave South Africa on a loan basis. These specimens are to be deposited in the SA museums within a year of publishing the description of the new species. The holder shall provide the Chief Executive Officer, KZNNCS with the name of the museum at which the specimens have been lodged, and the accession number of each specimen. This condition relates to unavoidable by-catch of non-target organisms as well.

7. A copy or copies of any publication arising from the authority herein contained will be made available to E KZN Wildlife.

8. Should renewal of this permit be desired, a minimum of one month's notice is required.

9. (i) Reserving accommodation within E KZNWildlife areas is entirely the responsibility of the permit holder. Booking is obtainable at the Central Booking Office, Telephone 033 8451000.(ii) Any assistance required from Board staff will be subject to other demands on the

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 25 February 2009

for CHIEF EXECUTIVE

Permit Holder

KZN WILDLIFE PERMITS OFFICE
P.O. Box 13053 Cascades, 3202 Pietermaritzburg, KwaZulu-Natal. Tel.+27 33 8451320/1324
Fax: +27 33 845 1747 E-mail: permits@kznwildlife.com Website: www.kznwildlife.com

EZEMVELO KZN WILDLIFE
Conservation, Partnerships & Ecotourism

Officer's time and must be arranged in advance with him/her.

10. This permit is valid only if an export/import permit has been issued by the export/import province/country if the legislation applicable in such province/country prescribes such permit.

11. Valid for one consignment only.

12. Holders shall provide the Chief Executive, with a named list of every specimen collected (including the class, order, family, gender and age), the geographical co-ordinates (to seconds accuracy) of each collection locality and dates of collection, as laid out in the following table. A Global Positioning System with the WGS84 Datum should be used wherever possible to determine the geographical co-ordinates of the collection sites; please state the method used.

13. SPECIMEN - COLLECTION DATE - SPECIES - LOCALITY - LATITUDE - LONGITUDE
(museum Accession) (ddmmyy) (Seconds Accuracy) (Seconds Accuracy).
Holders are requested to provide additional information, such as the habitat in which each specimen was collected and abundance or relative abundance data (providing standardised sampling methods are used) with the list.

14. The transportation of any live specimen by air shall be done in accordance with the International Air Transport Association live animals regulations and any other Act relevant to the transport, keeping, handling, transport, care and/or welfare of the said species.

15. No collecting is permitted within the road reserve which is a strip 30 (thirty) metres either side of a public road, no matter how small or remote the road may be.

16. No collecting is permitted in the wilderness areas within the Protected Area. For confirmation of boundaries of the wilderness area contact the Officer in Charge.

Please read the Terms and Conditions under which this Permit is issued

ISSUED at PIETERMARITZBURG, KwaZulu-Natal, on 25 February 2009

for CHIEF EXECUTIVE

Permit Holder

942/2009

IUCN
The World Conservation Union
MEMBER: IUCN

KZN WILDLIFE PERMITS OFFICE
P.O. Box 13053, Cascades, 3202 Pietermaritzburg, KwaZulu-Natal. Tel +27 33 8451320/1324
Fax +27 33 845 1747. E-mail: permits@kznwildlife.com. Website: www.kznwildlife.com

CapeNature

HEAD OFFICE

postal	Private Bag X29 Rondebosch 7701
physical	CapeNature House Belmont Office Park Belmont Rd Rondebosch 7701
website	www.capenature.co.za
enquiries	Danelle Kleinhans
telephone	+27 21 659 3420 **fax** +27 21 659 3415
email	dkleinhans@capenature.co.za
reference	1/2/1/6/5/C
date	18/01/2010

Ms C Cook
University of Johannesburg
PO Box 1383
SAXONWORLD
2193

Dear Ms Cook

APPLICATION TO COLLECT FAUNA SPECIMENS FOR SCIENTIFIC RESEARCH

I refer to your application to collect fauna specimens in the Western Cape Province.

Attached is permit Nr. **AAA004-000383-0035** dated **17 December 2009** to collect specimens in the Western Cape Province. Please take special note of the standard conditions attached to the permit. I specifically draw your attention to permit condition (i). **It is imperative that you make contact with the Reserve Manager BEFORE you intend collecting on any nature reserve, conservation area, wilderness area and / or state forest**. No deviation is allowed from the fore-mentioned conditions without the prior written approval of the Chief Executive Officer: Western Cape Nature Conservation Board.

Please also take note of the *pro forma* (copy attached), which must please be used when submitting your collection / distribution records to CapeNature as per the conditions to your permit. Please feel free to add columns for extra data to the *pro forma* but no columns should be deleted. This pro forma is also available electronically from CapeNature.

Should you have any queries please do not hesitate to contact this office.

Yours faithfully,

CHIEF EXECUTIVE OFFICER

CapeNature
FAUNA • FLORA • HUNTING • CITES

Western Cape Province
Head Office

Telephone No: 021 6593400
EMail: dhignett@capenature.co.za

CapeNature

Facsimile No: 021 6593415
Internet: www.capenature.co.za

CapeNature House
Belmont Office Park
14 Belmont Road
Rondebosch
7700

Private Bag x29
Rondebosch
7701

PERMIT TO HUNT WITH PROHIBITED HUNTING METHOD OF WILD ANIMALS

(Issued in terms of the provisions of the Nature Conservation Ordinance 1974, (Ord 19 of 1974)Section29&33)

Not Transferable

Holder

Full Name	Ms C Cook	**Identity No.**	8309020193089
Trade Name	University of Johannesburg	**Registration No.**	AAA004-00882
Postal Address	PO Box 1383 SAXONWORLD 2193	**Physical Address**	14 Cecil Avenue Melrose JOHANNESBURG 2196

In terms of and to the provisions of the abovementioned Ordinance and the Regulations framed thereunder, the holder of this permit is hereby authorised to Hunt (capture/disturb/stampede/kill) the protected wild animal(s) specified below on the property mentioned on this permit. See conditions on last page:

Details

Permit/Licence No	**0035-AAA004-00383**
Expiry Date	31/01/2012
Date Issued	17/12/2009
Amount Paid	R 0.00
Reference	NO CHARGE
File Code	1/2/1/6/5/C

Stamp:

CapeNature
FAUNA • FLORA • HUNTING • CITES

Description	Property
Organization	University of Johannesburg
Person	Cook, C Ms
ID	8309020193089
Properties	In the Western Cape Province only.
District	Not applicable
Province/State	Western Cape
Country	South Africa

Species(Scientific Name)	Qty	ID	Note
(Note)	0		FOR EXAMINATION ONLY, NO COLLECTION.
Karoo Padloper(Homopus boulengeri)	0		None
Tortoise, Angulate(Chersina angulata)	0		None
Tortoise, Common Pad(Homopus areolatus)	0		None
Tortoise, Geometric(Psammobates geometricus)	0		None
Tortoise, GreaterPad(Homopus femoralis)	0		None
Tortoise, Tent(Psammobates tentorius tentorius)	0		None
Tortoise, Western Te(Psammobates tentoruis trimeni)	0		None
Tortoise,Bushmanland(Psammobates tentorius verroxii)	0		None
Tortoise,Southern Sp(Homopus signatus cafer)	0		None

Issued by:
Danelle Kleinhans

Approved on Behalf CEO
Western Cape Nature Conservation Board

18/01/2010
Effective Date

Signature of Holder
I acknowledge, accept and understand fully the permit conditions as described

Standard Conditions

1. When the holder of this permit *kills/captures/collects any wild animal in terms thereof, he shall, before leaving the above-mentioned property, or if he does not leave it, after each day's *hunt/capture/collection, record the particulars regarding the date, species and number of each sex of each species, or if it is impossible to distinguish the sex, the total number of each species of such wild animals which he had *killed/captured/collected.

2. The holder of this permit shall return it to the Chief Executive Officer: Western Cape Nature Conservation Board, Private Bag X29, RONDEBOSCH, 7701, within 14 days of the date of expiry thereof.

3. THIS PERMIT IS SUBJECT TO THE SPECIAL CONDITIONS AS SET OUT IN THE ADDENDUM HERETO.

Special Conditions

CONDITIONS:
1. All locality data GPS'ed and dated must be submitted to CapeNature's Scientific Services on completion of the project;

METHOD: On foot and by hand. No traps will be used.

NAMES OF PERSONS ENGAGED IN THE RESEARCH PROJECT:
Ms Courtney Cook - University of Johannesburg (ID 83090201930089)
Prof Nico J Smit - (Supervisor) University of Johannesburg (ID 7206075012084)

CONDITIONS APPLICABLE TO RESEARCHERS UNDERTAKING RESEARCH OR OTHER COLLECTING WORKS ON PROVINCIAL CONSERVATION AREAS AND / OR PRIVATELY OWNED LAND IN THE PROVINCE OF WESTERN CAPE:

1. THE MANAGER OF THE RELEVANT CONSERVATION AREA(S) (IF ANY) MUST BE INFORMED TIMEOUSLY BEFORE ANY CONSERVATION AREA IS ENTERED FOR COLLECTING OR RESEARCH PURPOSES AND THE MANAGER'S WRITTEN PERMISSION TO ENTER SUCH RESERVE MUST BE ACQUIRED BEFOREHAND. THIS PERMIT DOES NOT GRANT THE PERMIT HOLDER AUTOMATIC ACCESS TO ANY NATURE RESERVE, CONSERVATION AREA, WILDERNESS AREA AND / OR STATE FOREST. ANY OTHER / FURTHER CONDITIONS OR RESTRICTIONS THAT THE MANAGER MAY STIPULATE AT HIS / HER DISCRETION MUST ALSO BE ADHERED TO. THIS PERMIT MUST BE AVAILABLE TO BE SHOWN ON DEMAND.
2. The owner of any other land concerned (be it privately or publicly owned land) must give WRITTEN consent allowing the permit holder to enter said property to collect flora / fauna. This written permission must reflect the full name and address of the property owner (or of the person authorised to grant such permission), the full name and address of the person to whom the permission is granted and the number and species of the flora / fauna, the date or dates on which such flora / fauna may be picked / collected and the land in respect of which permission is granted. Copies of this written permission must be made available to The Western Cape Nature Conservation Board upon request.
3. Type-specimens of any newly described / discovered species or other taxon collected must be lodged with a recognised South African scientific institution / museum / herbarium (preferably within the Province of Western Cape) where such material will be available to other researchers. For every flora specimen collected on a Western Cape Nature Conservation Board nature reserve, one additional (extra) herbarium specimen must be forwarded to the Western Cape Nature Conservation Board Herbarium at Jonkershoek (c/o MJ Simpson, Private Bag X5014, Stellenbosch 7599).
4. A list of all collected specimens / material including the; species name, the number collected, the collection date and the precise locality of the collection must be submitted within 14 days from the date of expiry of your permit to The Chief Executive Officer: CapeNature, Private Bag X29, Rondebosch, 7701
5. The maximum number of specimens per species specified in the permit (if at all) may not be exceeded without the prior permission of The Chief Executive Officer: Western Cape Nature Conservation Board.
6. For projects of more than one year's duration a progress report must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board before 31 December of each year.
7. One copy of all completed reports, publications, or articles (including books, videos, CDs, DVDs etc.) resulting from the project/collection must be submitted to The Chief Executive Officer: Western Cape Nature Conservation Board free of charge.
8. Should a report, publication, article or thesis arise from this project/collection, an acknowledgement to Western Cape Nature Conservation Board must be included.
9. The Forest Act 1984 (Act 122 of 1984) and regulations, the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) and all regulations in terms of the Ordinance must be adhered to.
10. Should it be envisaged to export any material / specimens across the boundaries of the Western Cape Province, an export permit will be required in respect of certain species and a further application form will have to be completed. The permit holder must confirm with the Western Cape Nature Conservation Board whether an export permit is required BEFORE exporting any material / specimens from the Western Cape Province.
11. No species that appear on the Red Data List or species listed as endangered in terms of the Nature Conservation Ordinance, 1974 (Ordinance 19 of 1974) may be collected, except for those mentioned on the permit.
12. 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.
13. IF APPLICABLE, ETHICS CLEARANCE MUST BE ACQUIRED FROM YOUR RESEARCH INSTITUTE PRIOR TO COLLECTION.

CHIEF EXECUTIVE OFFICER
WCNCB

CapeNature
FAUNA • FLORA • HUNTING • CITES

AFFIDAVIT: MASTER'S AND DOCTORAL STUDENTS
TO WHOM IT MAY CONCERN

This serves to confirm that I, Courtney Antonia Cook
Full Name(s) and Surname

ID Number 8309020193089

Student number 920203595 enrolled for the

Qualification Ph. D. (Zoology)

Faculty of Science

Herewith declare that my academic work is in line with the Plagiarism Policy of the University of Johannesburg which I am familiar.

I further declare that the work presented in the Ph. D. (Zoology) (minor dissertation/dissertation/thesis) is authentic and original unless clearly indicated otherwise and in such instances full reference to the source is acknowledged and I do not pretend to receive any credit for such acknowledged quotations, and that there is no copyright infringement in my work. I declare that no unethical research practices were used or material gained through dishonesty. I understand that plagiarism is a serious offence and that should I contravene the Plagiarism Policy notwithstanding signing this affidavit, I may be found guilty of a serious criminal offence (perjury) that would amongst other consequences compel the UJ to inform all other tertiary institutions of the offence and to issue a corresponding certificate of reprehensible academic conduct to whomever request such a certificate from the institution.

Signed at Johannesburg University on this _____ of September 2012

Signature_____ Print name_____

STAMP COMMISSIONER OF OATHS
Affidavit certified by a Commissioner of Oaths
This affidavit conforms with the requirements of the JUSTICES OF THE PEACE AND COMMISSIONERS OF OATHS ACT 16 OF 1963 and the applicable Regulations published in the GG GNR 1258 of 21 July 1972; GN 903 of 10 July 1998; GN 109 of 2 February 2001 as amended.