



**Prevalence of *Babesia* species  
and associated ticks (Acari: Ixodidae)  
in captive cheetah (*Acinonyx jubatus*) populations  
in South Africa**

**By**

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This work is dedicated to  
all those who have laboured before me and to those  
who endured my many moods while I composed.

Without their wisdom, perseverance, patient, and understanding,  
this study would not have been possible.

I present this work to our peers and students, who continually  
challenged me to learn, to rethink, and to explain

My efforts were inspired by  
my love of the God; my father, mother and sister and my profession.

## Declaration

Apart from the assistance received that has been reported in the acknowledgements and in the appropriate places in the text,  
this thesis represents the original work of the author.

No part of this thesis has been presented for  
any other degree at any other university.

Candidate ...Habib Golezardy.....

Date.....February 2012.....

## Summary

# **Prevalence of *Babesia* species and associated ticks (Acari: Ixodidae) in captive cheetah (*Acinonyx jubatus*) populations in South Africa**

By

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Due to prevailing environmental and climatic conditions South Africa hosts one cheetah subspecies (*Acinonyx jubatus jubatus*) and a wide range of tick-borne protozoa such as *Babesia*. Blood samples collected from 143 cheetahs at four study sites, namely the Ann van Dyk Cheetah Breeding Center-De Wildt (Brits and Shingwedzi), the Cheetah Outreach and the Hoedspruit Endangered Species Centre, were examined for *Babesia* infection. The V4 hypervariable region of 18S rRNA gene was amplified and subjected to the Reverse Line Blot (RLB) hybridisation assay. Hybridisation of the parasite DNA with *Babesia* genus and species-specific probes was evident. The results showed a predominance of *Babesia lengau* (n=63, 44.1%), followed by *Babesia felis* (n=3, 2.1%) and *Babesia canis rossi* (n=7, 4.8%).

Unfed ixodid ticks (n=10,432), collected from the vegetation by drag-sampling, represented five species: *Amblyomma hebraeum*, *Amblyomma marmoreum*, *Haemaphysalis elliptica*, *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus simus* and *Rhipicephalus zambeziensis*. The monthly occurrence of ixodid ticks at the De Wildt Cheetah Breeding Centre (Brits) showed a higher activity in the warm months of the year. Recovery of ticks decreased during the warm

hours of the day, suggesting that free-living ticks are humid dependent. The presence of birds, rodents, free-ranging antelopes such as nyalas (*Tragelaphus angasii*), kudu (*Tragelaphus strepsiceros*), bushbucks (*Tragelaphus scriptus*) and impalas (*Aepyceros melampus*), as well as Burchell's zebras (*Equus burchellii*) and leopard tortoises (*Geochelone pardalis*) can contribute to the availability of various tick species at the breeding centres.

Mice as the host for immature instars of ixodid tick species and unfed ixodid ticks were studied for presence of *Babesia* species. *Babesia lengau* was detected in 22 (39.2%) mice as well as in *Haemaphysalis elliptica* larvae, nymphs and adults. The presence of *B. lengau* in mice suggests a long-term association since the host preference of *B. lengau* for mice remains unclear. However, the presence of this parasite in unfed immature and adult *H. elliptica* is indicative of a transstadial transmission suggesting that this tick species may be a potential vector for *B. lengau*.

The correlation between *Babesia* infection and various parameters such as gender, age, tick burdens and location, in two different breeding farms belonging to the De Wildt Cheetah Breeding Centre was analysed using the Fisher's exact test analysis. The prevalence of *Babesia* species in cheetahs was associated with tick burden suggesting a strong positive correlation between the prevalence of infection and presence of suspected vector ticks. Regardless of tick burden, age could be related to prevalence of infection, meaning that the fact that older cheetahs had a higher prevalence of infection with *Babesia* species. These findings were of considerable interest especially since at the time of study the cheetahs in both populations did not show clinical signs of infection with *Babesia* species.

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## Chapter 1:

### General introduction

Parasitism is regarded as the most successful and dominant life-style of living organisms. Parasites, which include representatives from many phyla, are among the factors that can influence the natural equilibrium of host populations ([Zelmer, 1998](#)). Various recent studies have highlighted the role of parasitism in the dynamics of animal populations and the structure of animal communities ([Irvine, 2006](#)).

Parasites were traditionally identified and distinguished on the basis of their morphological features, the host(s) that they may infect, transmission patterns, pathological effects on the host(s) and/or geographical distribution. In recent developments, molecular characterization has important implications for accurate identification of parasites, irrespective of developmental stages and sex ([Poulin & Morand, 2000](#); [Cruickshank, 2002](#); [Prichard & Tait, 2001](#); [Gasser, 2006](#)). Molecular techniques as diagnostic tools are increasingly used to study the ecology of an assortment of organisms ([Conraths & Schares, 2006](#)) as well as in diagnosis of arthropod-transmitted diseases ([Shaw, Birtles & Day, 2001](#); [de la Fuente, Estrada-Peña, Venzal, Kocan & Sonenshine, 2008](#)).

These molecular diagnostic techniques represent important tools for the study of the systematics, population genetics, biogeography and ecology of parasites. The techniques used with eukaryotic

cells are generally relevant and applicable to the study of parasites and their hosts (Prichard, 1997). The Polymerase Chain Reaction (PCR), one of the first nucleic acid amplification systems developed, is used extensively for identification of parasites (Mullis, Faloona, Scharf, Saiki, Horn & Erlich, 1986). PCR is applied broadly due to its sensitivity which permits the enzymatic amplification of gene fragments of small quantities of nucleic acids (Paxson, 2008). Great advances have been made in the field of molecular characterization of tick-transmittable pathogens and also in the understanding of some of the key immune responses that they elicit, although the objective of inducing protection is proving elusive (Tress, 1999). For this reason, particular molecular tools such as PCR which facilitate amplification of the target genomic DNA of the parasite by using specific markers, have brought about a broad revolution in detection and identification of the pathogenic tick-borne organisms (Sparagano, Allsopp, Mank, Rijpkema, Figueroa & Jongejan, 1999).

Ticks (Acari: Ixodidae) exhibit a range of associations with their vertebrate hosts through blood-feeding. As non-permanent, haematophagous ectoparasites, ticks are widely distributed from Arctic to temperate, subtropical and tropical regions of the world (Hoogstraal, 1956; Sonenshine, 1991). The variety in specificity of tick-host associations was based on comprehensive worldwide information on the natural tick-host feeding associations displayed by adult ticks and immature stages (Hoogstraal & Aeschlimann, 1982).

The evolution of ticks has equipped them with the capacity of transmitting a wider spectrum of pathogenic micro-organisms (such as obligate intracellular organisms) than other arthropod vectors (Dennis & Piesman, 2005). These include tick-borne bacteria, protozoa, rickettsia and viruses of both medical and veterinary importance. Since blood parasites rely on their tick vectors for transmission, occurrence of tick-borne diseases in a region undergo seasonal fluctuations (Bashir, Chaudhry, Ahmed & Saeed, 2009). In South Africa, theileriosis and babesiosis are important tick-borne protozoan diseases, with babesiosis being an important disease in felids (Ayoob, Prittier & Hackner, 2010). Pathogens in hosts can be identified by conventional methods such as indirect fluorescence antibody test (IFAT), isolation in cell culture or histological staining techniques (Blewett & Branagan, 1973).

In order to prevent economic losses and reduce mortalities, detection of pathogen DNA, especially of haemoparasites of different animals in ticks and identification of potential tick vectors, has been the subject of study in many countries (Lewis, Penzhorn, Lopez-Rebollar & De Waal, 1996; Camacho, Pallas, Gestal, Guitián, Olmeda, Telford III & Spielman, 2003; Rar, Fomenko, Dobrotvorsky, Livanova, Rudakova, Fedoros, Astanin & Morozaova, 2005; Spitalska, Namavari, Hosseini, Shad-del, Amrabadi & Sparagano, 2005; Aktas, Altay & Dumanli, 2006; Liu, Zhou, Zhou, Liu, Du, Chen, Yao & Zhao, 2007).

Despite low levels of infection, the presence of *Babesia* species in various tick species (vectors) has been studied in many countries including Russia (Rar *et al.*, 2005), South Africa (De Waal & Potgieter, 1987) and Turkey (Altay, Aktas & Dumanli, 2008). The key elements involved in vector-borne infectious diseases are the infectious micro-organisms, the vector and the reservoir from which the vector became infected (Klempner, Unnasch & Hu, 2007). Possible control strategies should therefore be based on understanding the complex dynamics of vector–host interactions and the ways in which the environments of both the vector and host intersect to produce a disease. Very little literature is available about the means of transmission of piroplasms in cheetahs as well as their potential vectors.

The overall objective of this survey was to understand the epidemiology of *Babesia* infection in captive cheetah populations in South Africa and to detect the *Babesia* species infecting cheetahs using molecular techniques.

Due to the high level of competition with large predators such as lions, cheetahs were not doing well in protected wildlife reserves. Since their habitat has a wide range, a large population of cheetahs remains outside protected reserves ([http://www.cheetah.org/?nd=cheetah\\_facts](http://www.cheetah.org/?nd=cheetah_facts)). In addition to the conservation areas, cheetahs are kept at few breeding centers (Fig. 1), namely:

1. *The Ann Van Dyk Cheetah Breeding Centre - De Wildt/Brits*

This is a private research and breeding centre best known for its highly successful captive-breeding program that contributed to the cheetah being removed from the endangered list of the *South African Red Data Book-Terrestrial Mammals* in 1986. The centre comprises an area of about 65 ha in extent situated in the foothills of the Magaliesberg in North West

Province, 20 km west of Pretoria ( $25^{\circ} 40' S$ ,  $27^{\circ} 55' E$  – 1211 m). The cheetahs are kept individually in enclosures with natural vegetation. The habitat surveyed is defined as terrestrial in a woodland biome, hence terrestrial and arboreal mammal species can be expected, but no rupicolous or wetland-reliant species. The area has vegetation coverage of marikana thornveld type (Mucina & Rutherford, 2006).

## 2. *The Ann Van Dyk Cheetah Breeding Centre - De Wildt/Shingwedzi*

This centre lies 56 km the north west of Bela-Bela in Limpopo Province ( $24^{\circ} 40' S$ ,  $28^{\circ} 2' E$  – 1500 m). The vegetation of the area, a semi-arid zone with a typical inland subtropical climate, consists of central sandy bushveld (Mucina & Rutherford, 2006). A variety of antelopes as well as carnivores and birds are kept at the centre.

## 3. *The Hoedspruit Endangered Species Centre*

This centre was initially established as a breeding program for cheetahs. It holds facilities including a farm of 100 ha in extent which is located approximately 32 km south east of Hoedspruit, Limpopo Province ( $30^{\circ} 89' S$ ,  $24^{\circ} 28' E$  – 515 m). The Centre mainly focuses on the conservation of rare, vulnerable or endangered animals. The vegetation is typical of the lowveld, mixed woodland (Mucina & Rutherford, 2006).

## 4. *The Cheetah Outreach*

This was established as an education and community-based program to raise awareness of the dilemma of the cheetah and their challenges for survival. The Cheetah Outreach is situated on the Spier Wine Estate, Western Cape Province ( $33^{\circ} 49' S$ ;  $18^{\circ} 28' E$  – 745 m), with the vegetation, typical of grassy fynbos (Mucina & Rutherford, 2006). Cheetah Outreach was established in January 1997 on a hectare of land donated by Spier. An education facility aims to increase global awareness of the cheetah and to raise funds for the centre.

### *Breeding management and husbandry at the centers*

The cheetahs are kept individually in wire-fenced camps. The trees and natural vegetation provide a suitable environment for ticks to breed and quest. The cheetahs are usually fed in a cemented cage twice a day with either raw meat (chicken and horse meat) or supplemented

commercial food, depending on availability, metabolic diseases and nutrient requirements. The populations change continually due to mortalities or sales of cheetahs. Medical and dental examination under general anaesthesia is performed annually on the cheetahs. When a female is in oestrus, a male is allowed into the enclosure temporarily for mating to take place. The cubs are kept with their mothers until the age of three months after which they are sold to the zoos or translocated to the other farms. Disregarding the newborns, the age of cheetahs ranges from two to 12 years. Due to the high tick infestation, the cheetahs are placed in a cage and are sprayed with an acaricide at least once a month.

This thesis initially covers blood sampling of cheetahs at various above cheetah breeding centers, followed by detection of tick-borne haemoparasites using PCR and Reverse Line Blot (RLB) hybridization assay. Additionally, we also aimed to detect the *Babesia* species in ixodid tick species, collected from the vegetation at the study sites in an attempt to identify the potential tick vector. Tick burdens on the vegetation, cheetahs and rodents were also studied at each breeding center. The monthly variation in prevalence of ticks on the vegetation at the study sites was determined in a one-year study. Hourly dragging of vegetation was performed to assess daily behaviour of ticks on the vegetation and the potential time of the day for possible infestation. The integrity of the tick vector and the phylogenetical relationship with other tick species was subject to study using mitochondrial 12S and 16S ribosomal RNA genes. Considering variables such as age, sex, locality and tick burdens, the risk factors for babesial infection at the Ann van Dyk Cheetah Breeding Center-De Wildt (Brits and Shingwedzi) were analysed.



Fig 1: Map of South Africa indicating the study sites

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## Chapter 2:

### Literature review

#### 1. Cheetah

##### *1.1. The cheetah in history*

Throughout history, there has constantly been conflict between humans and large carnivores, which hunt livestock and therefore represent a challenge to humans. As a result, the numbers of large carnivores have gradually diminished. There are 37 species in the cat family (Felidae), and all except the domestic cat are considered as threatened or endangered (Hutton & Dickinson, 2000). The cheetah (*Acinonyx jubatus*) was named and described by the Swedish biologist Carolus Linnaeus in the 1750s (Hunter & Hamman, 2003). The name "cheetah" is derived from the Hindi word "Chita" (Labuschagne, 1979). The cheetah is the world's fastest mammal (achieving speeds of up to 112 km per hour) and probably the most specialized of the felids. In contrast to the genus *Panthera*, with a close relationship between lion (*Panthera leo*), leopard (*Panthera pardus*) and jaguar (*Panthera onca*), the genus *Acinonyx* comprises only the cheetah (Hunter & Hamman, 2003).

## 1.2. Classification of cheetahs

Cheetahs are the most specialised cat in terms of morphology and behaviour. The cheetah's taxonomy is presented in [Table 1](#).

**Table 1:** Classification of the cheetah ([Hunter & Hamman, 2003](#))

<b>Kingdom</b>	Animalia
<b>Phylum</b>	Chordata
<b>Class</b>	Mammalia
<b>Order</b>	Carnivora
<b>Family</b>	Felidae
<b>Genus</b>	<i>Acinonyx</i>
<b>Species</b>	<i>jubatus</i>
<b>Subspecies</b>	<i>jubatus</i>

Subspecies represent significant differences between isolated populations of the same species. Cheetahs were historically classified into 17 subspecies due to close genetic ties, but only six subspecies are currently recognised ([Table 1](#)). The Asiatic cheetah, *Acinonyx jubatus venaticus* is confined to that continent. The five African subspecies are distinguished by subtle differences in their coats: *Acinonyx jubatushecki* (West Africa), *A. jubatus jubatus* (Southern Africa), *A. jubatus raineyi* (eastern East Africa), *A. jubatus ngorongorensis* (East Africa) and *A. jubatus soemmeringii* (Central Africa) ([Alderton, 1998](#); [Hunter & Hamman, 2003](#)). Most of the subspecies designations are probably the result of over enthusiasm on the part of taxonomist. The physical differences between subspecies have been based on hair length and colour variation, spot size and separation, and overall body size.

For most African countries, information on the occurrence of cheetahs is scanty. In the southern African subregion, cheetahs occur widely in the countries neighbouring South Africa. In South Africa, they are observed sporadically in the northern provinces ([Fig. 1](#)), but they have also been

observed in the KwaZulu-Natal Province (Bourlière, 1963; Skinner & Smithers, 1990). In general, cheetahs prefer savanna woodland, whereas in the southern part of Africa, they are found in the South West Arid and the Southern savanna zones (Smithers, 1983).

### *1.3. Distribution of cheetahs*

The cheetah, as generally solitary and predominantly diurnal species (Labuschagne, 1979), was originally described from a specimen in southern Africa (Friedmann & Daly, 2004). They naturally occur in low densities. The distribution of cheetahs has been modified over historical times by modern man's colonisation of the Asian and African continent (Hunter & Hamman, 2003). Sporadic sightings of cheetahs occurred in India until the 1960s, but they are now extinct in that country. Today the Asiatic cheetah, *A. jubatus venaticus*, occurs only in Iran, where it is critically endangered (Hunter & Hamman, 2003).

Cheetahs occur in 29 Africa countries, with the largest populations in East and southern Africa, where they bear a great value for their high visibility. Although suitable cheetah habitat in South Africa, comprises 125 150 km<sup>2</sup> (Boitani, Corsi, De Biase, D'Inzillo Carranza, Ravagli, Reggiani, Sinibaldi & Trapanese, 1999), they are observed sporadically only in an area of 55 654 km<sup>2</sup> under formal conservation (Fig. 1; Friedmann & Daly, 2004). Formal conservation areas comprise 44.5% of the area that is suitable for cheetahs in South Africa (Marnewick, Beckhelling, Cilliers, Lane, Mills, Herring, Caldwell, Hall & Meintjes, 2007).



Fig. 1: Distribution of cheetahs in South Africa (Friedman & Daly, 2004)

#### 1.4. Threats and status

Although cheetah populations have low genetic variation, they have been able to compete and survive in the wild (O'Brien, Wildt, Goldman, Merrill & Bush, 1983). In their recent history, cheetahs experienced a severe population bottleneck followed by inbreeding. Cheetahs are threatened on many fronts (other carnivores, humans and their own genes). As the planet's dominant predatory species, humans destroy cheetah habitats and prey and persecute cheetahs to protect livestock and agriculture. Agriculture destroys wildlife habitats and expels prey species from the region. Cheetah cubs are easily killed by other predators (Hunter & Hamman, 2003). Adaptation to running at high speed has made cheetahs poor combatants. As a result, cheetahs are globally listed as vulnerable on the International Union for Conservation of Nature (IUCN) Red List (<http://www.iucnredlist.org>) due to persecution and illegal trade (Labuschagne, 1979; Friedmann & Daly, 2004). Successful captive breeding of cheetahs has been a difficult task,

since they have a poor reproduction history and their infant mortality rate is fairly high (O'Brien, Roelke, Marker, Newman, Winkler, Meltzer, Colly, Evermann, Bush & Wildt, 1985). The National Zoological Gardens of South Africa began an all-inclusive study on propagation of cheetahs in captivity in 1971 (Brand, 1980).

### *1.5. Diseases in cheetahs*

Since cheetahs lack genetic diversity, they are especially vulnerable to diseases (Munson, Terio, Worley, Jago, Bagot-Smith & Marker, 2005). Exposure of wild cheetahs to pathogens does not produce the same disease condition as of that in captive cheetahs (Munson, 1993; Munson, Marker, Dubovi, Spencer & Evermann, 2005). Cheetahs suffer from a prolonged elevation of corticosteroids in response to continuous environmental changes in captivity (Wells, Terio, Ziccardi & Munson, 2004). The genetic basis as well as modulation of the immune response to chronic environmental stress may have a great impact on the health status of cheetahs in captivity, compared to free-ranging cheetahs (Terio, Marker & Munson, 2004). This may subsequently influence successful breeding management. An overview of the diseases which affect cheetahs in a population can serve as a basis for a more suitable health-care program. Some infectious agents infecting multiple carnivore species can cause notably more severe and vigorous forms of diseases in cheetahs (Munson, Meltzer & Kriek, 1998).

*Helicobacter*-induced gastritis, feline herpes virus dermatitis, feline corona virus/feline infectious peritonitis, feline leukaemia virus (FeLV), feline immunodeficiency virus (FLV), sclerosing disease, veno-occlusive disease of the liver, glomerulosclerosis, leukoencephalomalacia, demyelinating disease and oxalate nephrosis are of unusual diseases occur in high prevalence in captive cheetahs (Munson, 1993).

Little information has been published on clinical babesial infections in cats. Feline babesiosis presents clinically as gastrointestinal, haematological, mental, renal and respiratory disorders. Concurrent diseases may attribute to the body temperature elevation (Futter & Belonje, 1980; Ayoob *et al.*, 2010).

## 2. *Babesia*

### 2.1. *Babesia* species

According to their phylogenetic classification, *Babesia* species are placed taxonomically in the phylum Apicomplexa (also called Sporozoa), class Aconoidasida (Piroplasma), and order Piroplasmida (Levine, Corliss, Cox, Deroux, Grain, Honigberg, Leedale, Loeblich III, Lom, Lynn, Merinfeld, Page, Poljansky, Sprague, Vavra, & Wallace, 1980). Piroplasms are characterised by intraerythrocytic forms which can be pear-shaped (Levine, 1971). Babesiidae and Theileriidae are two families within the order Piroplasmida. The primary distinction between them is usually defined as the absence of a preerythrocytic cycle in *Babesia* and the absence of transovarial transmission in *Theileria* (Riek, 1968; Kakoma & Mehlhorn, 1993). Piroplasms (so called due to their pear-shaped intraerythrocytic stages), comprising the genera *Theileria* and *Babesia*, are protozoa with high infectivity rates (Levine, 1971). Along with *Plasmodium* and *Theileria*, which also belong to the phylum Apicomplexa (Table 2), *Babesia* parasites develop inside erythrocytes.

Table 2: Classification of *Babesia*:

<b>Phylum</b>	Apicomplexa
<b>Class</b>	Piroplasma
<b>Order</b>	Piroplasmida
<b>Family</b>	Babesiidae
<b>Genus</b>	<i>Babesia</i>

As far as is known, all *Babesia* species are infective to various ixodid tick species and are regarded as tick-transmitted parasites which have the ability to infect a wide range of vertebrate hosts such as wild and domestic mammals, birds and reptiles (Levine, 1971; Bush, Fernandes, Esch & Seed, 2001). They cause a severe disease followed by various lethal haematological, neurological and respiratory complications in wild and domestic animals (Kuttler, 1988).

More than 100 *Babesia* species have been described (Levine, 1971; Telford, Gorenflot, Brasseur & Spielman, 1993), from many different mammalian hosts and several avian species. The order

Rodentia has the greatest variety (Kakoma & Mehlhorn, 1993; Levine, 1971). Almost any mammal that serves as a host for a *Babesia*-infected tick is regarded as a potential reservoir (Telford *et al.*, 1993).

## 2.2. Background of *Babesia* species in felids

The literature on feline babesiosis is rather meagre (Futter & Belonje, 1980; Schoeman, Lobetti, Jacobson & Penzhorn, 2001; Schoeman & Leisewitz, 2006). The first report of babesiosis in wild and domestic felids contained no description of morphology or pathology (Lingard & Jennings, 1904). Since then, eight felid piroplasms have been named in various published papers. *Babesia felis* was initially described from an African wild cat, *Felis sylvestris* (syn: *Felis ocreata*) (Davis, 1929) after which it has consistently been detected in domestic cats in South Africa (Robinson, 1963; Bosman, Venter & Penzhorn, 2007). It was initially called *Nuttallia felis* (Davis, 1929). *Babesiella felis* was detected in a captive puma, *Felis concolor* (Carpano, 1934) in Egypt. *Nuttallia felis* var. *domestica* (Jackson & Dunning, 1937) was described in a domestic cat in South Africa. *Babesia cati* was observed in blood smear of a wild cat, *Felis catus*, in India (Mudaliar, Achary & Alwar, 1950). *Babesia herpailuri* was observed in a jaguarondi, *Herpailurus yaguarundi*, in South America (Dennig, 1967). *Babesia pantherae*, a large species which was initially named *B. pantheri* (Dennig, 1967; Dennig & Hebel, 1969), was isolated from a leopard in Kenya (Dennig & Brocklesby, 1972). A small piroplasm was detected in blood smears of lions in the Kruger National Park by López-Rebollar, Penzhorn, de Waal and Lewis (1999). The indirect immunofluorescent antibody test (IFAT) was negative to *B. felis* antigen, which indicated a possible distinct species. *Babesia leo* was described as a distinct species based on a phylogenetic analysis of the 18S rRNA gene (Penzhorn, Kjemtrup, López-Rebollar & Conrad, 2001). In a recent survey, *Babesia leo* was detected in three Namibian cheetahs (Bosman *et al.*, 2007). *Babesia canis* sub. *presentii* was identified in two domestic cats in Israel (Baneth, Kenny, Tasker, Anug, Ahksp, Levy & Shaw, 2004) based on the study of the 18S rRNA gene sequence and phylogenetic analysis. The cats were suffering from viral co-infections and showing severe clinical manifestations of the disease. Dennig and Brocklesby (1972) proposed that *B. felis*, *Babesiella felis*, and *Nuttallia felis* var. *domestica* should all be considered as a single species, *B. felis*. Levine (1973) regarded *Babesiella felis*, *Nuttallia felis* var. *domestica* and *Babesia cati* as synonyms of *B. felis*. Although babesiosis of domestic cats has been reported in

various countries, such as France (Leger, Ferte, Berthelot, Nourry & Brocvielle, 1992; Bourdeau 1996), Germany (Moik & Gothe, 1997), Thailand (Jittapalapong & Jansawan, 1993) and Zimbabwe (Stewart, Hackett & Collett, 1980), it does not appear to be a regularly occurring clinical disease in any country other than South Africa.

In a recent study, a novel *Babesia* species, namely *B. lengau* (from the Setswana name for the cheetah) was detected in South African cheetahs (Bosman, Oosthuizen, Peirce, Venter & Penzhorn, 2010). The organism is a typical small *Babesia* species with trophozoites usually observed in a central to subcentral position within the host's red blood cells. Together with other *Babesia* species, *B. lengau* formed a monophyletic group with *B. conradae* (Kjemtrup, Wainwright, Miller, Penzhorn, & Carreno. 2006), a small canine *Babesia* identified in California.

### 2.3. Life cycle of *Babesia* species

There is a tremendous diversity of life cycles of protozoa in their vectors and hosts. Protozoa undergo a series of modifications in their morphology as well as internal structure, metabolism and antigenicity in order to adapt themselves to the new microenvironment (guts and salivary glands which is completely different from the vertebrate blood in terms of temperature, pH-range, gas concentration, etc.) in the host body for various period of time (Fig. 2; Friedhoff, 1987). Despite the range of surveys on a variety of *Babesia* species (Kakoma & Mehlhorn, 1993; Telford *et al.*, 1993; Homer, Aguilar-Delfin, Telford, Krause & Persing, 2000) their small size and indistinguishable morphological details (by light microscopy), have limited the knowledge on the life cycles of these parasites.

The life cycle of all *Babesia* species includes three types of reproduction, namely schizogony (or merogony which is an asexual reproduction in the vertebrate host), gamogony (formation and fusion of gametes inside the tick gut) and sporogony (asexual reproduction in the tick salivary glands) (Mehlhorn & Schein, 1984; Kakoma & Mehlhorn, 1993). Much of what is known about the life cycle of *Babesia* species in the tick is based on studies on *B. microti* (Telford *et al.*, 1993). Piroplasms are detectable in the tick up to 10 hrs after the tick begins to feed on an infected vertebrate after which they gradually transform to gametocytes.

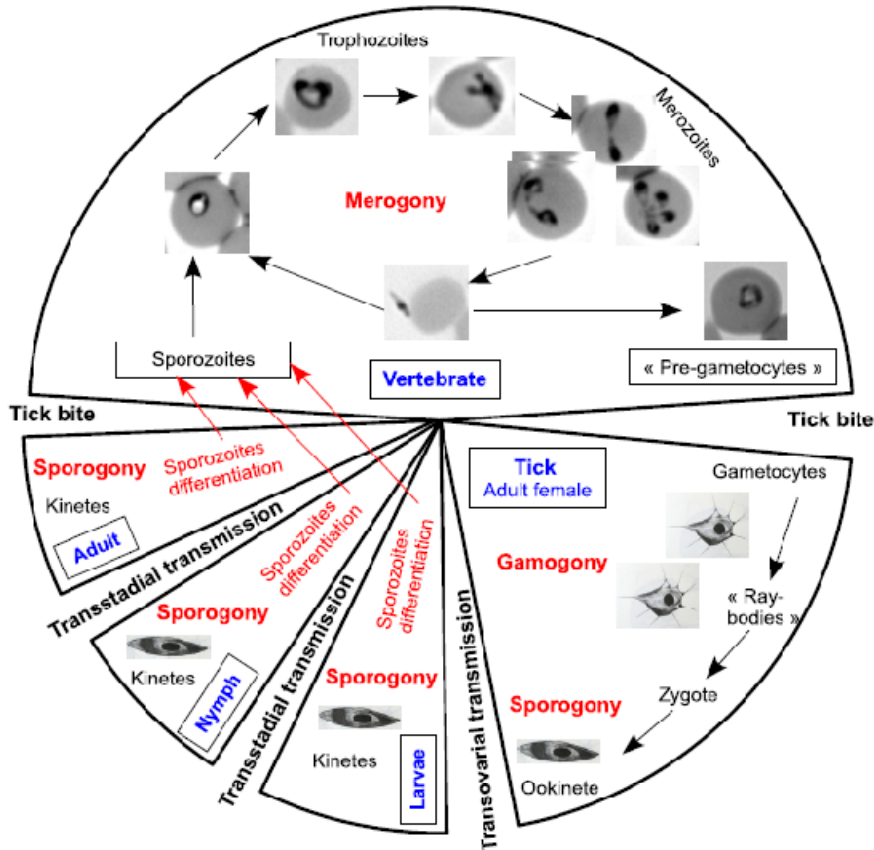


Fig. 2: Typical life cycle of *Babesia* species (Gardiner, Fayer & Dubey, 1998)

After asexual reproduction, the piriform merozoites enter other erythrocytes and become pleomorphic trophozoites (Schoeman & Leisewitz, 2006). The merozoites of *Babesia* species develop within the erythrocytes of vertebrate hosts while they are completing the asexual stage (binary fission) of their reproduction cycle (Friedhoff, 1987; Friedhoff, 1990; Homer *et al.*, 2000). Characteristics such as location, size and shape of intraerythrocytic developmental stages can be used for diagnostic purposes (Mehlhorn & Schein, 1984). Penetration of merozoites into erythrocytes includes five active phases, initiated by physical contact of the merozoite and the erythrocyte (Friedhoff & Scholtyseck, 1977; Ward & Jack, 1981).

Merozoites of some *Babesia* species such as *B. caballi* and *B. canis* are typically pear-shaped, whereas others such as *B. microti* tend to be generally polymorphic (Mehlhorn & Schein, 1984). After entering erythrocytes, merozoites soon undergo division, leading to the characteristic

appearance of paired parasites. In some instances, however, four merozoites are formed simultaneously, resulting in the formation of a tetrad or so-called ‘Maltese cross’ arrangement (Mehlhorn & Schein, 1984). At this stage, the parasites do not develop further until they are ingested by engorging tick vectors. In the vector, these parasites form ray-bodies, two of which then fuse and form the zygote which develops further and leads to formation of a kinete which will penetrate the salivary glands where it gives rise to a large number of sporozoites (Mehlhorn & Schein, 1984).

#### 2.4. Babesiosis in felids

Babesiosis (also referred to as piroplasmiasis), is one of the most common infections of domestic animals worldwide and is regarded as an emerging zoonosis in humans. In dogs and cats, babesiosis was originally viewed as a tropical and subtropical disease, but in recent times it has been diagnosed with increasing frequency in temperate regions of the world (Irwin, 2003). Babesiosis occurs naturally in domestic cats at any age, but cats younger than 3 years are more susceptible. There does not appear to be any sex and/or breed predilection (Schoeman *et al.*, 2001). Babesiosis in domestic cats has primarily been reported in South Africa where infection is mainly due to *B. felis* (Jacobson, Schoeman & Lobetti, 2000). *Babesia felis* is a very small intra-erythrocytic but highly pathogenic, haemoprotozoan parasite (Levine, 1971). In a survey conducted by Futter and Belonje (1980), *B. felis* in erythrocytes varied from less than 1 µm up to 2.25 µm in diameter, with the majority being 1.25 µm. Jackson and Dunning (1937) and McNeil (1937) first reported babesiosis in domestic cats in South Africa.

#### 2.5. Epidemiology of feline babesiosis in South Africa

Babesiosis in cats has not received much attention worldwide due to the sporadic occurrence of clinical cases. Feline babesiosis in domestic cats has mainly been reported from South Africa where it is a significant clinical entity (Ayoob *et al.*, 2010). A survey conducted by Jacobson *et al.* (2000) revealed that more than 3000 cases of feline babesiosis occur each year in various provinces in South Africa (Figs 3 & 4). Although the distribution is largely coastal (Schoeman, 2001; Penzhorn, Schoeman & Jacobson, 2006), the disease has been reported from relatively far inland in endemic areas. Due to a paucity of published data, the geographical distribution of cheetah-associated babesiosis in South Africa has not been well established.



Fig 3: Map of South Africa

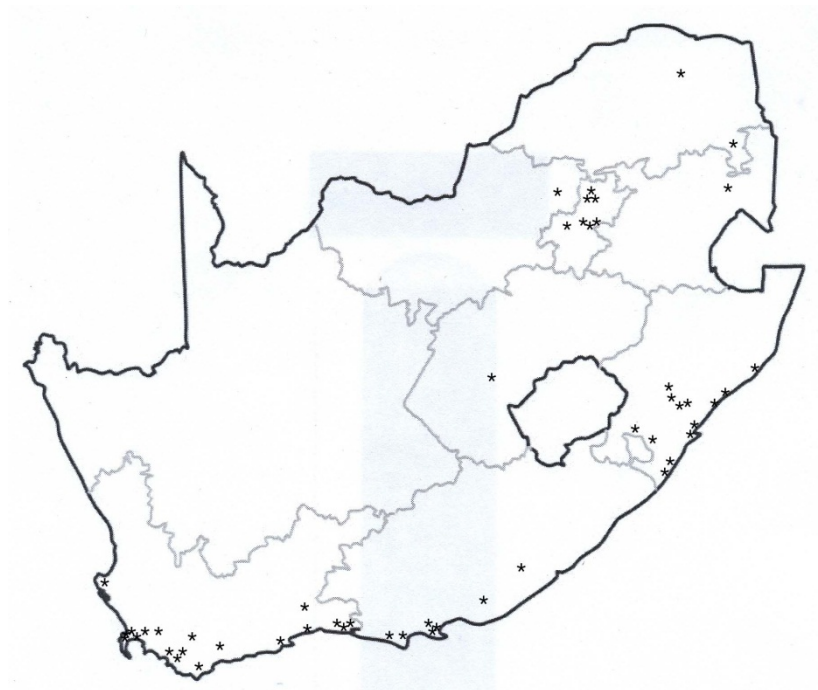
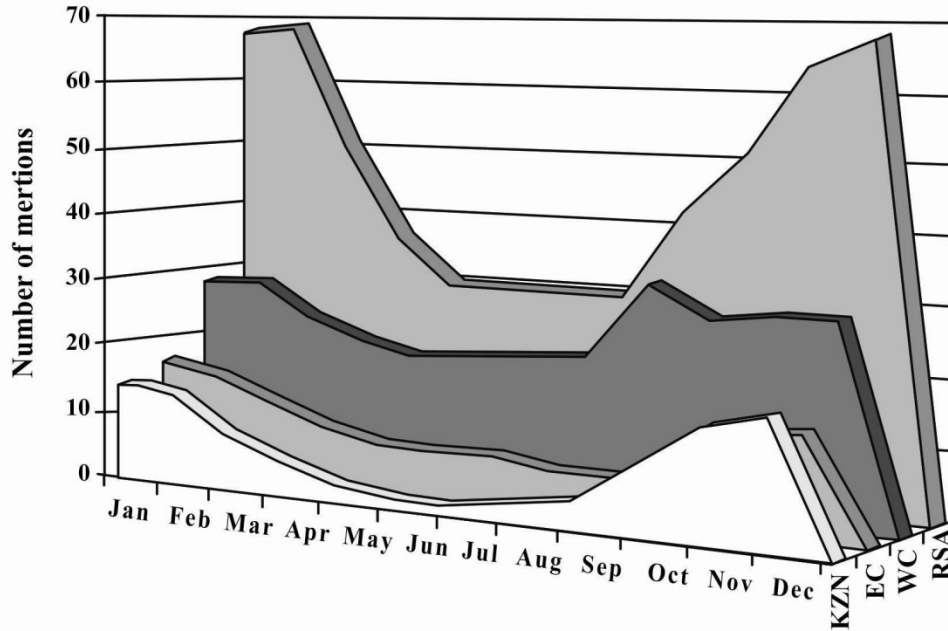


Fig 4: Distribution of feline babesiosis in domestic cats in South Africa, showing the endemic provinces

In an epidemiological study based on questionnaires (Jacobson *et al.*, 2000) three provinces, Western Cape, Eastern Cape and KwaZulu-Natal, had the highest number of positive responses and the highest number of cases throughout the year. Practitioners reported cases with typical clinical manifestation of babesiosis such as depression, anorexia, weight loss, anaemia, weakness, vomiting, pica and icterus (Taboada & Lobetti, 2005).

Previously, there were a number of reports on the incidence of the disease in the Western and South-Western Cape Province (McNeil, 1937; Brownlie, 1954; Futter & Belonje, 1980), in Port Elizabeth in the Eastern Cape Province (Robinson, 1963), and in KwaZulu-Natal (Potgieter, 1981). The occurrence of babesiosis in cats was also reported sporadically in the Free State Province (Jacobson *et al.*, 2000) and even in the northern part of the country such as Mpumalanga (Penzhorn, Stylianides, Coetzee, Viljoen & Lewis, 1999; Jacobson *et al.*, 2000), Gauteng (Jacobson *et al.*, 2000) and the North West Province (Jacobson *et al.*, 2000), but they were regarded as non-endemic areas. It was assumed that the diseased cats had previously visited endemic areas, but some practitioners stated that their cases never left the Gauteng region (Jacobson *et al.*, 2000), indicating that the distribution of the tick vectors may expanded into previously non-endemic areas.

A country-wide survey of the monthly incidence of feline babesiosis, showed that the disease is frequently diagnosed in the warmer months of the year (October - March) (Fig. 5), regardless of breed, age and sex (Jacobson *et al.*, 2000). There was a pronounced variation in the seasonal incidence of the disease amongst the three endemic provinces, with the seasonal pattern being more pronounced in KwaZulu-Natal. This pattern should correspond with annual pattern of the activity of tick vector/s in the regions. It could be argued, however, that factors such as rainfall, misdiagnosis, tick control and lack of response to treatment could have had a negative impact on the results of the survey.



**Fig. 5:** Seasonal incidence of feline babesiosis in the whole South Africa, and in endemic provinces (Jacobson *et al.*, 2000). EC = Eastern Cape Province, KZN = KwaZulu-Natal Province, RSA = Republic of South Africa, WC = Western Cape Province

## 2.6. Diagnostic tests

The definitive diagnosis of babesiosis depends on demonstration of the organisms in the infected erythrocytes, amplification of babesial DNA extracted from infected blood or tissue, or positive serology results (Taboada, 1995; Taboada & Lobetti, 2005). Laboratory diagnostic tests for various *Babesia* species include:

### 2.6.1. Microscopic identification

Demonstration of the parasites in the red blood cells on Diff-Quick stained, thin capillary blood smears can lead to a rapid diagnosis. In addition to parasitaemia, a blood smear indicates underlying regenerative, haemolytic anaemia, marked anisocytosis, polychromasia, reticulocytosis and normoblastaemia (Voigt, 2000).

### 2.6.2. Serological test

Since it is very difficult to detect *Babesia* parasites on blood smears in chronic carriers, immunodiagnosics (indirect immunofluorescent antibody test) is used to identify infected hosts. It is a suitable method of detecting the parasites indirectly in either patent or occult infections (Taboada & Lobetti, 2005). Recent or active infection can be detected by demonstrating rising antibody titres over a period of two or three weeks. The enzyme-linked immunosorbent assay (ELISA) technique has been developed to meet various requirements in the field of protozoal diseases (Taboada & Merchant, 1991). A clinical diagnosis should not be based solely on seropositivity, since animals in or from endemic areas can be seropositive without showing clinical signs.

### 2.6.3. Nucleic acid detection

Genetic methods are the most sensitive and specific means of detecting infection. Screening for *Babesia* can be performed by Polymerase Chain Reaction (PCR) tests via extraction of DNA from blood samples (Ano, Makimura & Harasawa, 2001; Matjila, Leisewitz, Jongejan & Penzhorn, 2008). For instance, application of a seminested PCR can result in the detection and differentiation of *Babesia canis* and *Babesia gibsoni* DNA in canine blood (Birkenheuer, Levy & Breitschwerdt, 2003).

There are some problems with interpretation of the results, such as sample cross contamination and difficulty in detection of sub-clinical infection (Willoughby, 2003). The high sensitivity and specificity of a newly developed PCR probe assay accompanied by Reverse Line Blot (RLB) hybridization assay should allow detection of low parasitaemias in sub-clinically infected cases and may be the most useful test in screening dogs newly introduced into a *Babesia*-free region.

Reverse Line Blot hybridisation assay, where multiple samples can be analysed against multiple probes to enable simultaneous detection and differentiation of *Theileria* and *Babesia* species, was introduced as a standard molecular tool for diagnostic and epidemiological studies in a number of laboratories all over the world (Gubbels *et al.*, 1999).

### 2.7. Chemotherapy and control of babesiosis in felids

Treatment of feline babesiosis has not yet been evaluated critically. Drug treatment, irrespective of the parasitaemia, is often initiated when the haematological values are life-threatening. Chemotherapeutic drugs such as primaquine phosphate, diminazene, doxycycline, imidocarb and oxytetracycline are listed as having antibabesial properties. Primaquine, a member of the 8-aminoquinoline group of anti-malarial compounds, is the drug of choice for the treatment of *B. felis* infection in domestic cats (Potgieter, 1981; Jacobson *et al.*, 2000). The effective dose is close to the lethal dose. A decline in parasitaemia and concomitant rise in the PCV are regarded as positive response to the treatment. In addition to chemotherapy, supportive treatment such as fluid therapy, administration of corticosteroids, antibiotics and on occasional basis even blood transfusion is required (Jacobson *et al.*, 2000; Ayoob *et al.*, 2010).

To date, all described *Babesia* species are transmitted from the vector to their vertebrate hosts via bites, therefore, removal of all possibility of exposure to tick vector is the best way to prevent the disease (Irwin, 2003). Regular tick control through application of topical anti-tick compounds should therefore be practiced in endemic areas where there is continual tick challenge.

## 3. Vector

### 3.1. Vector-borne diseases

Ectoparasites are important causes of disease in animals, either through direct pathological effects, or as vectors of viral, bacterial, rickettsial or protozoal diseases. The emergence of arthropod-transmitted microparasitic diseases has always been a challenge in veterinary medicine. The number of arthropod species capable of transmitting pathogens in animal populations is fairly high (Rawson, 1934). The impact of arthropod infestation is not always immediate, but may increase with time since infestation or the onset of the host-arthropod interaction. Climate change and easy access to niche environments is expanding the geographic range of arthropod and arthropod-transmitted diseases (Shaw, Birkes & Day, 2001).

Tick-borne haemoparasitic diseases, often the most neglected of vector-borne disease (VBD) systems (Gayle & Ringdahl, 2001; Randolph, 2009), remain some of the most important diseases

in animals. Ticks have been implicated as a source of disease for more than 100 years. The first confirmation of a tick-borne disease was demonstration that the cattle tick (*Rhipicephalus (Boophilus) microplus*) can transmit the protozoan *Babesia bigemina*, the causative pathogen of Texas cattle fever (Smith & Kilbourne, 1893). Tick-borne diseases such as theileriosis and babesiosis create a variety of problems in veterinary medicine from the epidemiological, health and breeding management points of view. The epidemiology of tick-borne diseases often involves a range of hosts (Cumming, 1998), all of which harbouring parasitaemias high enough to infect ticks.

### 3.2. Tick (Acari: Ixodidae)

Ticks, as obligate and non-permanent feeders, have a characteristic large body size (2-30 mm) and specialised mouthparts for attachment and blood feeding (Sonenshine, 1991). They are mainly classified in the suborder Ixodida under the order Parasitiformes (Norton, Kethley, Johnston & O'Connor, 1993) and can potentially survive off their host for extended periods of time. Ticks are obligate blood-feeding ectoparasites of terrestrial vertebrates at some stage of their life cycle (Walter & Proctor, 1998) and vary widely in terms of morphology, hosts, habits and habitats on which the phylogeny for tick families, subfamilies and genera is based.

Most species of ticks have a propensity to live in open environments, even though feeding on vertebrate hosts is one of the survival factors. Ixodid ticks are designed to take up the large portion of the meal on the last day of attachment, where their size will increase to almost 100 times the original size. Certain characteristic of ticks (adaptability, firm attachment, high agent dispersal, high reproductive potential, slow feeding, starvation resistance, wide host range and versatile saliva) make the act of pathogen transmission possible (<http://www.bada-uk.org/homesection/about/ticks/diseasetransmission.php>), after which an active biological process takes place (Wilson, 2002). The tick must first find a suitable host. Many species are of considerable importance and interest as vectors of a wide variety of debilitating pathogens to both domestic and wild animals (Hoogstraal, 1985; Sonenshine, 1991). Ticks commonly occur in any area and since there are few predators to minimise their populations and also the defence mechanism of the host may be conditionally ineffective (e.g. immunosuppressed animals) on them, therefore they can serve as potent vectors for various pathogens (Friedhoff, 1990).

Each instar of tick species has morphologically adapted to various hosts and specific locations on the hosts (Hoogstraal & Kim, 1985). As a natural feature of the tick-host interaction, ticks naturally do not disperse on the host's body but stay in close proximity to each other while feeding. Since the prolonged feeding period may vary from several days (immatures) to weeks (adults), it causes overlapping feeding periods (Hoogstraal & Kim, 1985).

The overdispersed distribution of ticks on the host body will eventually result in large numbers of hosts harbouring feeding ticks. Overdispersion results from indiscriminate distribution of questing ticks in the hosts's natural habitat, host genetic and behavioural and immunological heterogeneities (Randolph, Gern & Nuttall, 1996). Because of the direct and indirect effects on their hosts, ticks are considered to be not only a serious threat to successful stock farming, but also a very real hazard to human economy in many parts of the world, particularly in Africa (Rechav, 1982).

### 3.2.1. Classification of ticks

The taxonomic assemblage referred to as ticks is a relatively small group, comprising 860 species in 22 genera (Oliver, 1989). The superfamily Ixodoidea includes three families, namely Argasidae, Ixodidae and Nuttalliidae (Keirans, 1992; Keirans & Robbins, 1999). Ixodidae (hard ticks) comprising more than 650 species, is the largest of the three families (Hoogstraal, 1956). At least 82 ixodid tick species have been identified in South Africa (Walker, 1991; Walker, Bouattour, Camicas, Estrada-Peña, Horak, Latif, Pegram & Preston, 2003).

The family Nuttalliellidae contains a single genus and species, *Nuttalliella namaqua*, which shares characters of both hard and soft tick families (monotypic) and also has many derived features. *Nuttalliella namaqua* is restricted to South Africa and Tanzania (Bedford, 1934; Keirans, Clifford, Hoogstraal & Easton, 1976).

### 3.2.2. Life cycle of ticks

Four distinct developmental stages occur in the life cycle of an ixodid tick, namely egg, larva, nymph and adult (Sonenshine, 1991). In one-host ticks, the first two instars (larva, nymph) live and moult on the same host; engorged adult females drop off and

lay their eggs on the ground. In two-host ticks, larvae and nymphs share the same host, but the engorged nymphs drop off and moult to the adult stage, which has to find a separate host. In three-host ticks, each stage drops from the host after engorging and the subsequent instar has to find a new host. Ixodid ticks have substantial capacity to ingest and concentrate a large volume of host blood for survival and reproducing (<http://www.bada-uk.org/home/section/about/ticks/lifecycle.php>). The rapid metabolism and body development of ticks can explain the on-host intervals. During off-host periods, ticks may experience environmental distress such as desiccation and high temperatures which affect their survival rates.

### *3.3. Effect of environmental variables on tick populations in a region*

Variables such as host, dispersal ability, environment (climate and vegetation) and human activities which occur over predetermined regions, affect tick species. These factors may affect the localities where ticks are found. The environment changes through either space or time, but in different ways and the position of a given point may be as important as its individual properties in understanding its place in the ecosystem (Legendre, 1993). Variations in the occurrence of organisms can be related to some extent to variations in the properties of the environment, and give valuable insights into the relationships between organism and its environment. Both host specificity and ecological specificity may be significant within the Ixodida.

Potentialities for presence or absence of hosts always vary (George, 1990; Cumming, 1998). Factors such as diverse host preferences of ticks (Hoogstraal & Aeschlimann, 1982), physiological compatibilities of hosts and ticks (Fivaz, Petney & Horak, 1992), survival rate of tick eggs (Dipeolu & Akinboade, 1984), successful attachment of ticks on various hosts (Bonsma, 1981), differences in host movements and habitat use and specific host behaviours such as their tendency to walk through or around clump of undergrowth and bushes (MacLeod, 1975), tremble reflex (Bonsma, 1981) and grooming activities can influence the abundance of ticks in a region (Fivaz & Norval, 1990).

The ability of ticks to disperse throughout a region is related to some extent to preferred hosts (Londt & Whitehead, 1972). If ticks are consistently transported by hosts into areas where their eggs cannot survive, dispersal will instead lead to mortality. Long-range dispersal is always dependent on

the host. Host movements may lead to an increase in tick population in a particular region (Minshull & Norval, 1982).

The dissimilar patterns of tick–habitat associations (Ntiamoa-Baidu, Carr-Saunders, Matthews, Preston & Walker, 2004) and also the patterns of tick–host association in forest-inhabiting ticks provide supporting evidence that such a combination of adaptations to habitats and hosts has occurred. Evidence is mounting that temperature and other climatic variables are driving many ecological processes (Poulin & Mouritsen, 2006). The prospective impacts of global warming on parasitic diseases comprise an expansion of the geographical range of many parasites and the emergence of previously unimportant pathogens (Harvell, Mitchell, Ward, Altizer, Dobson, Ostfeld & Samuel, 2002).

Climate determines both the plant population and herbivore biomass in a habitat (Coe, Cumming & Phillipson, 1976), and also directly affects the tick population. Factors such as rainfall, minimum and maximum daily temperatures, duration of periods of intense heat (Needham & Teel, 1991), and seasonality can play potential roles in confining the tick population to a certain region (Rechav, 1984; Pegram, Perry, Musisi & Mwanaumo, 1986). Vegetation cover and type can influence tick survival by improving environmental boundaries (Tukahirwa, 1976), through their influence on microclimate and interactions with various herbivores in the ecosystem (Coe *et al.*, 1976; Cumming, 1982). Frequent use of the various kinds of acaricides can change the distribution of ticks in a locality (Norval, Perry, Meltzer, Kruska & Booth, 1994). Failure in tick control and the administration of acaricides can lead to rapid proliferation of tick populations (Norval, Short & Chisholm, 1985).

### *3.4. Effect of environmental variables on tick populations on a host*

A defined environment consists of a number of components such as ticks and host/s (de la Fuente, Estrada-Peña, Venzal, Kocan & Sonenshine, 2008). Host-specificity of ixodid ticks has always been questionable, although they have traditionally been regarded as relatively host-specific (Hoogstraal & Aeschlimann, 1982). It is argued that, however, that ticks select any available hosts in a region or select a certain host in a specific given environment. The spectrum of host-specificity is distinguished as each ixodid tick species is subject to individual study.

Tick populations on hosts are limited by wide-ranging mechanisms expressed through natural host-parasite interactions. The presence of certain tick species is closely related to the presence of suitable hosts. An optimised and ideal host-parasite relationship is one where host and parasite coexist without any threat to other living species (Tatchell, 1987; Cumming, 1998). Thus the implications of host-parasite relationships need to be studied and various patterns of these relationships need to be discussed in greater detail. As ticks have always shown remarkable species- and stage-specific predilection for different sites of attachments, ticks feed repeatedly at the same site.

The association between tick species and one or a group of vertebrate species is defined as host specificity which is vital for the completion of ticks' life cycles (Thompson, 2001). Since ticks are comparatively host-specific, their geographic distributions can be determined by that of their host/hosts (Sonenshine, 1991). However, Klompen, Black, Keirans and Oliver (1996) indicated a limited degree of specificity in the tick-host association as a large number of ticks spend a great portion of their life cycle off the host.

Sites of attachments differ with tick species (Ogden, Hailes & Nuttall, 1998), though they are mostly found around the head, neck and the groin. Stimulated grooming activities owing to skin irritation at the attachment sites successfully limit the number of ticks feeding and engorging (Norval, 1979; Hoogstraal & Aeschlimann, 1982; Tatchell, 1987; Cupp, 1991). The outcome of host-acquired immunity against ticks will possibly range from simple rejection of the parasite, increased feeding time, inadequate engorgement, infertility, or decreased viability of eggs, to death of ticks on the host's body (Willadsen, Muller & Baker, 1980; Wikel, 1996).

### *3.5. Ixodid ticks as potential vectors for Babesia species*

Ticks are believed to be designed to carry as well as transmitting disease agents and are the most widespread disease vectors worldwide. Hard ticks transmit the majority of tick-borne diseases. It can be argued that this is due to their prolonged feeding habits, which facilitate both delivery and uptake of blood-borne parasites (Balashov, 1972). Duration of attachment is dependent on key elements such as tick species, host immune response, etc. During this period, infection in the preferred host will take place (Sauer, McSwain, Bowman & Essenberg, 1995).

Independent adaptation to a blood-feeding environment could, however, have determined which ticks the parasite eventually exploited as vectors (Hoogstraal, 1985; Wilson, 2002). Various ixodid tick genera such as *Dermatocentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus* are capable of transmitting blood parasites when feeding on various vertebrate hosts (Piesman, Lewengrub, Rudzinska & Spielman, 1987; Rodríguez Bautista, Ikadai, You, Battsetseg, Igarashi, Nagasawa & Fujisaki, 2001; Kumar, Malhotra, Sangwan, Goel, Kumar & Kumar, 2007; M'ghirbi & Bouattour, 2008; Jongejan, Fourie, Chester, Manavella, Mallouk, Pollmeier & Baggott, 2011).

*Babesia* infection may itself promote transmission of *Babesia* species amongst hosts through enhancing the feeding success and survival of its tick vector (Randolph, 1991). There are two host-mediated mechanisms are suggested for the observed parasite-vector interactions, the first one is the anti-haemostatic effects of *Babesia* species and the second is the interaction of their immunosuppressive effects and the development of immunity to ixodid ticks by their vertebrate hosts (Randolph, 1991). In one study on female *Boophilus annulatus* ticks, infection with *Babesia bigemina* or *B. bovis* had no effect on the time elapsing between engorgement and oviposition by the tick (Ouhelli, Pandey & Aboughal, 1987). On the other hand, a short period of oviposition, laying fewer eggs by infected female ticks and significant reduction of the hatching percentage of *B. bigemina*-infected eggs was evident (Ouhelli *et al.*, 1987).

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## Chapter 3:

# Species diversity and diurnal and seasonal patterns of activity of questing ticks (Acari: Ixodidae) associated with captive cheetah (*Acinonyx jubatus*) populations in South Africa

### Abstract

Despite many studies involving the tick burdens of wild and domestic animals, there is limited information on the ecology of ticks associated with captive populations of cheetahs in South Africa. Free-living ticks were collected by drag-sampling of the vegetation at the Ann van Dyk Cheetah Breeding Center - De Wildt (Brits/Shingwedzi) and the Hoedspruit Endangered Species Centre to assess their seasonal population dynamics. Monthly changes in apparent density of questing immature and adult ticks were shown to be closely related to climatic factors. Ticks were also collected from trapped murid rodents to determine the correlation between the prevalence and abundance of the rodent ticks and the tick burdens of cheetahs. Five ixodid tick species belonging to three genera were collected from the vegetation, mice and cheetahs, of which *Haemaphysalis elliptica* was the most numerous. The abundance of the immature stages of *H. elliptica* on the trapped mice suggests that they by preference feed on these small mammals. Adult ixodid ticks never parasitized the rodents. Very few questing ticks were collected during the warmest hours of the day, while numbers peaked on the vegetation during the warmest months of the year. The results also indicated that, compared to the other species of trapped mice, *Aethomys* species are good hosts of the immature stages of *H. elliptica*.

## Introduction

The study of ticks associated with wild animals is of importance not only as an attempt to expand our limited knowledge on global biodiversity, but also to assess the risk they pose to health as well as their potential impact on wildlife conservation and captive breeding management (McOrist & Smales, 1986; Dantas-Torres, Ferreira, de Melo, Lima, Siqueira, Rameh-de-Albuquerque, de Melo & Ramos, 2009). The presence of ticks on sympatric hosts is consistent with their availability in an area, and the utilization of habitat by hosts is a major determinant of the magnitude of tick burdens on animals. These infestation patterns and their association with host habitat are important elements towards the control of some major tick-borne diseases in Africa (De Garine-Wichatitsky, 2002).

Various factors such as the complexity of a tick's life cycle, the number of eggs it produces, the presence of host species on which it feeds and the survival of its free-living stages can potentiate its ability to disperse. Because of the close relationship between parasites and their natural hosts, opportunities for dispersal would thus also depend on the characteristics of the host species involved (McCoy, Boulinier, Tirard & Michalakis, 2003). It is evident that certain suitable microhabitats such as type of vegetation and even the height of the grass are necessary for the survival of the free-living developmental stages of ticks that eventually support the density of parasitic tick populations (Londt & Whitehead, 1972).

Studying the variation in tick diversity from one wildlife reserve to another can provide several insights into the ecology of these parasites, and much information can be obtained from non-destructive live-sampling in a variety of localities. Studies of ixodid tick burdens have revealed the involvement of either a single or several tick species. In previous surveys a number of domestic and wild felids have been examined for ticks (Horak, Jacot Guillarmod, Moolman & De Vos, 1987a; Horak, Braack, Fourie & Walker, 2000; Horak, Heyne & Donkin, 2010). Recent surveys have reported 14 ixodid tick species on cheetahs in southern Africa, but it would seem that only six species can be regarded as true parasites of these animals (Horak *et al.*, 2000; Horak *et al.*, 2010). Body size may also influence the tick species which occur on animals (Gallivan & Horak,

1997), as some ticks prefer large carnivores, while others prefer small ones (Horak *et al.*, 1987a; Walker, 1991; Horak, Chaparro, Beaucournu & Louw, 1999; Horak *et al.*, 2000; Horak *et al.*, 2010).

Surveys conducted on wild carnivores in southern Africa revealed that wild felids can be hosts of a large number of tick species, of which the most important are the adults of *Haemaphysalis elliptica* (previously misidentified as *H. leachi*) and *Rhipicephalus simus* (Horak *et al.*, 2000; Horak *et al.*, 2010). In warm and moist regions, *H. elliptica* and *R. simus* are widespread, provided that there are sufficient numbers of suitable rodent and carnivore hosts (Howell, Walker & Nevill, 1978; Norval, 1984).

Cheetahs in the Kruger National Park harboured 11 ixodid tick species, with the immature stages of *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* being the most numerous (Horak *et al.*, 2000). The preferred hosts of the adult stages of *A. hebraeum* and *R. appendiculatus* are large domestic and wild ruminants (Horak, MacIvor, Petney & De Vos, 1987b; Walker, Keirans & Horak, 2000), whereas their immature stages are found on large and small mammals, and those of *A. hebraeum* also on birds (Horak *et al.*, 1987b). The presence of more than two adult *A. hebraeum* ticks on a carnivore smaller than a lion is viewed as an unusual incident and may indicate environmental stress in the host animal (Horak *et al.*, 2000).

In assessing the epidemiology of certain tick-borne diseases in South Africa, it has been essential to identify the immature stages of the tick species infesting rodents as well as the level and intensity of infestation (Petney, Horak, Howell & Meyer, 2004). The availability of rodents as hosts for the immature stages of various ixodid ticks has also to be taken into consideration (Anderson, 2002; Matthee, Horak, van der Mescht, Ueckermann & Radloff, 2010). The potential of rodents for rapid reproduction and their ability to maintain a high level of population growth can contribute significantly towards the dynamics of their host status for various tick species (Horak, Spickett & Braack, 2000). The notable differences in tick burdens of rodents are usually the intensity of infestation and the species composition of the ticks infesting them (Rechav, 1982; Horak,

Sheppey, Knight & Beuthin, 1986; Horak, Fourie, Novellie & Williams, 1991; Petney *et al.*, 2004).

## **Materials and methods**

### **1. Survey localities and period**

This study was conducted on animals resident at the Ann Van Dyk Cheetah Breeding Center-De Wildt/Brits (hereafter referred to as De Wildt/Brits), the Ann Van Dyk Cheetah Breeding Center-De Wildt/Shingwedzi (hereafter referred to as De Wildt/Shingwedzi), as well as the Hoedspruit Endangered Species Centre (hereafter referred to as Hoedspruit) (refer to chapter 1, figure 1). Fluctuations in the numbers of free-living ticks at De Wildt/Brits were monitored at monthly intervals for one year from March 2008 to February 2009. This locality was chosen because of its close proximity to Onderstepoort, large cheetah population and heavy tick burdens as detected during the pilot study. The tick burden of each individual cheetah was also assessed at both Cheetah Breeding Centres-De Wildt (Brits and Shingwedzi) while the animals were physically restrained. The tick burdens on the cheetahs and vegetation were studied at De Wildt/Brits from June till August and from October till November in 2008 and at De Wildt/Shingwedzi only in June 2008. The numbers and species of ticks on the cheetahs and vegetation were determined and compared at Hoedspruit in July and November 2008. Rodent trapping as well as drag-sampling for ticks from vegetation were implemented at De Wildt/Brits from July 2010 till May 2011.

### **2. Tick recovery**

- ***Drag-sampling***

The method of tick collection from vegetation was drag-sampling, using a drag-sampling device. This was a great benefit to the survey in terms of collecting all developmental stages of ixodid ticks questing on the vegetation (Sonenshine, Atwood & Lamb, 1966; Petney & Horak, 1987). The device consists of ten flannel strips (10 x 10 x 100 cm) attached adjacent to each other on a 120 cm-long

wooden spar by means of Velcro tape (registered trademark of VELCRO). A piece of steel rod, 9 cm long, is sewn into the end of each strip to keep the strip on the vegetation during dragging. A twine harness is attached to each end of the spar so that the device can be dragged behind the operator (Spickett, Horak, Braack & Van Ark, 1991).

Five cheetah enclosures were randomly chosen monthly and the sampling device was dragged over the vegetation for a distance of 50 m, as described by Zimmerman and Garris (1985). On each occasion the drag-sampling was done on a sunny morning at a site where the questing activity of ticks was expected to be substantial. After each 50 m drag, the flannel strips were inspected for the presence of ticks. The ticks were collected carefully using forceps and placed in labelled glass bottles containing 70% ethanol. They were transported to the ectoparasitology laboratory at the Faculty of Veterinary Science for identification and counting using a stereoscopic microscope. The monthly count represents the mean of five drag counts. Ticks were identified making use of published descriptions and illustrations as well as comparison with voucher specimens.

In a second study, the hourly fluctuation in the numbers of questing ticks recovered from vegetation was monitored at De Wildt - Brits. For this purpose a single drag was performed every hour from 08h00 to 17h00 over the vegetation at different localities. The day-time temperature in the shade at ground level (measured with a maximum/minimum thermometer) as well as the time at which each drag was done, were recorded throughout the day. The ticks collected were identified and counted as described previously.

- *Cheetahs*

The tick burdens of cheetahs were determined at De Wildt/Shingwedzi in June 2008 and subsequently at Brits from October till December 2008. This was done when the cheetahs were restrained for spraying against ectoparasites, and the skin was searched for the presence of ticks. Adult ticks, mostly engorged ones, since they were more easily visible to the naked eye, were collected by application of a

finger and the thumb at the point of attachment, rather than by directly pulling on the tick's body.

Because of the restraint-associated stress and possible self-injury due to physical restraint of the cheetahs in a cage, the researcher did not have adequate time to search the entire body for ticks. Therefore, only a small patch (10 x 10 cm) at various anatomical locations (ventral and dorsal parts of the neck, shoulders as well as the perineal region and tail, where ticks mostly tend to attach) on the skin of each cheetah was searched for ixodid ticks (Bryson, Horak, Höhn & Louw, 2000). The ticks were identified and the numbers collected were recorded and eventually regarded as the tick burdens of each cheetah.

- ***Murid rodents***

As small mammals are the preferred hosts of the immature stages of several tick species they were trapped and examined at De Wildt/Brits at two-month intervals from July 2010 to May 2011 and in two sessions at Hoedspruit (November 2010 and May 2011). Small mammals were trapped in Sherman live traps. A census line of 40 Sherman live-traps was set against the western fence of a series of occupied cheetah enclosures. A control line of 40 Sherman traps equally spaced was set across a 20 meter wide servitude that served as a service road for the two rows of enclosures. The control line was set against the eastern fence of a series of unoccupied enclosures. In all instances traps were spaced 10 meters apart. Traps were baited with a mixture of rolled oats, peanut butter, a dash of sunflower oil and cane syrup. The trap lines were set for three consecutive nights, and checked in the mornings and re-baited in the evenings. At the request of the management of the Centre, the traps were closed during the day to avoid catching yellow-footed squirrels (*Paraxerus cepapi*). This generated 120 trapping nights per line per trapping session.

Animals trapped in the census line were removed for laboratory examination, whereas animals trapped in the control line were released after being marked with a red Aerolac spray paint. Individuals re-trapped during any particular trapping

session were discounted since they had already been recorded as part of the resident rodent population. The rodents were identified taxonomically as proposed by Bronner, Hoffmann, Taylor, Chimimba, Best, Matthee and Robinson (2003) and Skinner and Chimimba (2005) and then placed in labelled bags and transported to the ectoparasitology laboratory at the Faculty of Veterinary Science where they were removed from the bags and euthanised by a rapid non-sterile intraperitoneal injection of 1ml of Eutha-naze (Bayer, Animal Health Division, Germany). Having made sure that the rodents were dead, the carcasses were individually placed in separate labelled clear plastic bags and soaked in suspension of the tick detaching agent Amitix (Schering-Plough, Animal Health Division, USA) at a concentration of 4ml in a litter of water, after which the bags were sealed (Horak *et al.*, 1986).

The following morning each rodent was thoroughly washed and then the skin was scrubbed with brushes with steel bristles and the washings and scrubblings were collected in bottles. At the laboratory one sample at a time was processed. The contents of the bottles were slowly poured into a steel mesh sieve, with 150  $\mu\text{m}$  apertures and washed with a strong jet of water. The contents of the sieve were transferred to a container and from there, bit by bit, into a square perspex tray and examined under a stereoscopic microscope for collection of the ticks that were present (Horak, Boomker, Spickett & De Vos, 1992). The collected ticks were placed in separate labelled glass vials containing 70% ethanol as preservative prior to being examined under a stereoscopic microscope for identification (genus and species), counting, and finally recording. At the conclusion of each trapping session the traps and specimen bags were thoroughly washed.

The vegetation in the cheetah camps along the census line was also drag-sampled for ticks. Ten drag-samples, each 50 meters long, were performed during one of the mornings within each rodent trapping session in order to compare the numbers of ticks and their species recovered from the vegetation and the rodents.

## Results

The species and numbers of all the ticks collected throughout the study from the vegetation at De Wildt (Brits and Shingwedzi) and at Hoedspruit are summarized in [Table 1](#). Ixodid ticks belonging to three genera, and mostly immatures, were collected, namely *Amblyomma hebraeum*, *Amblyomma marmoreum*, *Haemaphysalis elliptica*, *Rhipicephalus simus* and *Rhipicephalus zambeziensis*, with *H. elliptica* being the most abundant species. The aggregate numbers (with standard deviation) of all ixodid ticks collected monthly at De Wildt/Brits are tabulated and also graphically illustrated in [Tables 2 & 3](#), and [Fig. 1](#)). *H. elliptica* was the predominant species collected at De Wildt/Brits and exhibited a seasonal pattern of occurrence ([Fig. 2](#)). The other two species collected, namely *A. hebraeum* and *R. simus* showed no clear pattern of seasonality but were most abundant during the warmest months of the year ([Fig. 3](#)). Aggregated numbers of ticks peaked on the vegetation during the warmest and wettest months of the year ([Fig. 9](#)), whereas a trough was present during the coldest months.

Of the 10, 260 specimens collected from the vegetation during the 12-month survey at De Wildt/Brits ([Table 3](#)), 11.7% were *A. hebraeum*, 85.2% *H. elliptica* and 3.1% *R. simus*. Most species were represented by larvae, as the drag-sampling method usually favours this developmental stage of a tick's life cycle, though several nymphs and adults of *H. elliptica* were also collected on the flannel strips ([Table 1](#)).

The numbers of immature stages of *H. elliptica* collected at different times of the day in June ([Fig. 4 & 5](#); [Table 4](#)) and December 2008 ([Fig. 6 & 7](#); [Table 5](#)) revealed that very few ticks were collected on the flannel strips during the warmest hours of the day, compared to the early hours of the morning and late hours of the afternoon. Comparing the two immature stages, the proportions of larvae collected during the warmest time of the day were considerably lower than those of nymphs. Excluding the other tick species because of their low incidence at the centre, the survey indicated that the coolest hours of the day were the possible time of infestation of rodents since greater numbers of the immature stages of *H. elliptica* are actively questing. In December 2008 at De Wildt/Brits the temperature in the shade at ground level ranged from 19.9°C to 32.7°C at various

times of the day with an average relative humidity of 35% on the day of drag-sampling. The number of ticks collected in relation to change in temperature during the day is illustrated graphically (Fig. 8). As the temperature rose, the number of questing larvae and nymphs rapidly declined. At Hoedspruit, substantially more ticks were collected from the vegetation in summer than in winter (Tables 6 & 7).

Adult ticks belonging to three species were recovered from the cheetahs at the three study sites during the skin search. These were identified as *A. hebraeum*, *H. elliptica* and *R. simus*, with *H. elliptica* being the most numerous. The seasonal comparisons showed that a rise in temperature and commencement of rain can greatly contribute to the tick burdens of cheetahs (Tables 8 & 9 & 10).

The 56 rodents live-trapped at De Wildt/Brits were identified as *Aethomys* species (n=15, 34.1%), *Graphiurus murinus* (n=2, 4.5%), *Mastomys* species (n=26, 59.1%) and *Mus minutoides* (n=1, 2.3%). The rodents trapped at Hoedspruit were identified as *Mastomys* species (n=1, 12.5%), *Mus minutoides* (n=1, 12.5%), *Saccostomys campestris* (n=6, 50%) and *Tatera leucogaster* (n=3, 25%) (Table 11; Fig. 10). A trapping success of 17 individuals (17/240 = 7.1%) in the census line and 27 individuals (27/240 = 11.2%) in the control line was recorded at De Wildt/Brits, and (5/240 = 2.1%) for the census line, and (7/240 = 2.9%) for the control line at Hoedspruit. Immature stages of *H. elliptica* and *R. simus*, with *H. elliptica* being the most numerous, were collected from the rodents at De Wildt/Brits (Table 12; Fig. 11), whereas only *H. elliptica* was recovered from mice at Hoedspruit (Table 13). During the tick drag-sampling performed at the same times as the rodent trapping, 259 ticks of two species, namely *A. hebraeum* (0.8%) and *H. elliptica* (99.2%) were collected from the vegetation at De Wildt/Brits (Fig. 12), and 2 539 ticks of four species, namely *A. hebraeum* (0.4%), *A. marmoreum* (0.04%), *H. elliptica* (60.2%) and *R. (B.) decoloratus* (39.4%) were collected from vegetation at Hoedspruit. The abundance of ticks on vegetation corresponded with the rainfall recorded at the De Wildt-Brits (Fig. 13).

**Table 1:** Diversity and numbers of all the ixodid tick species collected throughout the study by drag-sampling the vegetation at two cheetah breeding centres in South Africa

Tick species	Locations											
	The Ann van Dyk Cheetah Breeding Centre – De Wildt								The Hoedspruit Endangered Species Centre			
	Brits				Shingwedzi							
	LL	NN	♂	♀	LL	NN	♂	♀	LL	NN	♂	♀
<i>Amblyomma hebraeum</i>	1170	96	0	0	0	0	0	0	93	28	5	10
<i>Amblyomma marmoreum</i>	0	0	0	0	37	0	0	0	0	0	0	0
<i>Haemaphysalis elliptica</i>	9743	931	89	25	228	34	13	2	2042	235	15	6
<i>Rhipicephalus (B.) decoloratus</i>	0	0	0	0	0	0	0	0	1002	0	0	0
<i>Rhipicephalus simus</i>	356	7	0	0	0	0	0	0	0	0	0	0
<i>Rhipicephalus zambeziensis</i>	0	0	0	0	88	0	0	0	96	42	0	0

**Table 2:** Seasonality of the mean numbers of all stages of development combined of three ixodid tick species questing from vegetation at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits

Tick species	Mean number of ticks											
	March	April	May	June	July	Aug	Sept	Oct	Nov	Dec	Jan	Feb
<i>Amblyomma hebraeum</i>	34	19	5	3	0	0	2	12	27	51	43	45
<i>Haemaphysalis elliptica</i>	211	144	119	82	31	52	54	61	168	221	284	259
<i>Rhipicephalus simus</i>	0	0	0	0	0	1	5	8	12	11	15	13
Mean total numbers of ticks recovered	245	163	124	85	31	53	61	137	207	283	342	317

**Table 3:** Combined number (and standard deviation) of all stages of development of all ixodid ticks collected per drag-sample in each month from March 2008 to February 2009 at the Ann van Dyk Cheetah Breeding Center – De Wildt/Brits

Months of the year	Number of ticks						± Standard deviation
	Drag 1	Drag 2	Drag 3	Drag 4	Drag 5	Mean	
March	121	394	182	236	292	245	104.6
April	82	234	123	167	209	163	61.9
May	124	205	93	153	45	124	60.4
June	48	111	56	127	83	85	44.7
July	22	39	13	57	24	31	17.2
August	31	88	68	29	49	53	25.1
September	89	36	105	17	58	61	36.3
October	128	111	166	68	212	137	54.7
November	202	162	257	71	343	207	101.9
December	278	191	385	259	302	283	70.4
January	318	253	458	303	378	342	78.7
February	201	442	325	261	356	317	91.9

**Table 4:** The hourly number of immature ixodid ticks collected per drag-sample from vegetation at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits (June 2008)

Tick species (Larvae)	Time									
	08:00	09:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00
<i>Amblyomma hebraeum</i>	8	4	5	2	0	0	0	0	0	1
<i>Haemaphysalis elliptica</i>	81	52	35	19	8	10	15	28	41	54
<i>Rhipicephalus simus</i>	4	5	0	0	0	0	0	1	5	3

Tick species (Nymphs)	Time									
	08:00	09:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00
<i>Amblyomma hebraeum</i>	4	0	0	0	1	0	0	2	0	0
<i>Haemaphysalis elliptica</i>	31	23	15	8	2	3	8	11	21	25
<i>Rhipicephalus simus</i>	0	0	0	0	0	0	0	0	0	0

**Table 5:** The hourly number of immature ixodid ticks collected per drag-sample from vegetation at the Ann van Dyk Cheetah Breeding Center – De Wildt/Brits (December 2008)

Tick species (Larvae)	Time									
	08:00	09:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00
<i>Amblyomma hebraeum</i>	5	2	7	1	0	0	0	0	0	1
<i>Haemaphysalis elliptica</i>	291	183	99	47	24	19	35	81	123	201
<i>Rhipicephalus simus</i>	4	5	0	0	0	0	0	1	1	6

Tick species (Nymphs)	Time									
	08:00	09:00	10:00	11:00	12:00	13:00	14:00	15:00	16:00	17:00
<i>Amblyomma hebraeum</i>	7	0	3	0	0	0	0	5	0	3
<i>Haemaphysalis elliptica</i>	41	29	17	7	6	8	11	19	25	39
<i>Rhipicephalus simus</i>	2	0	3	0	0	0	1	0	0	1

**Table 6:** Ixodid ticks collected from vegetation at the Hoedspruit Endangered Species Centre (July 2008)

Tick species	Number of ticks			
	LL	NN	MM	FF
<i>Amblyomma hebraeum</i>	23	7	2	3
<i>Haemaphysalis elliptica</i>	121	24	5	1
<i>Rhipicephalus zambeziensis</i>	13	5	0	0

LL = larvae; NN = nymphs; MM = males; FF = females

**Table 7:** Ixodid ticks collected from vegetation at the Hoedspruit Endangered Species Centre (November 2008)

Tick species	Number of ticks			
	LL	NN	MM	FF
<i>Amblyomma hebraeum</i>	61	21	3	7
<i>Haemaphysalis elliptica</i>	521	83	10	5
<i>Rhipicephalus zambeziensis</i>	83	37	0	0

LL = larvae; NN = nymphs; MM = males; FF = females

**Table 8:** Ixodid tick species associated with cheetahs at the Ann van Dyk Cheetah Breeding Centre - De Wildt/Brits

Tick species		Number of ticks		Number of infested cheetahs	
		winter	spring	winter	spring
<i>Amblyomma hebraeum</i>	LL	0	0	0	15
	NN	0	0		
	MM	0	22		
	FF	0	8		
<i>Haemaphysalis elliptica</i>	LL	0	0	57	56
	NN	0	0		
	MM	663	882		
	FF	265	356		
<i>Rhipicephalus simus</i>	LL	0	0	0	18
	NN	0	0		
	MM	0	69		
	FF	0	33		
<b>Total number of ticks recovered</b>		<b>928</b>	<b>1370</b>	<b>57</b>	<b>62</b>
<b>Total number of cheetahs infested with ticks at each locality</b>					

LL = larvae; NN = nymphs; MM = males; FF = females; n= number of cheetahs examined

**Table 9:** Ixodid tick species collected from cheetahs at the Ann van Dyk Cheetah Breeding Centre - De Wildt/Shingwedzi

Tick species		Number of ticks	Number of infested cheetahs
<i>Haemaphysalis elliptica</i>	LL	0	17
	NN	0	
	MM	125	
	FF	81	
<b>Total number of ticks recovered</b>		<b>206</b>	<b>17</b>
<b>Total number of infested cheetahs</b>			

**Table 10:** Ixodid tick species collected from cheetahs at the Hoedspruit Endangered Species Centre

Tick species		Number of ticks		Number of infested cheetahs	
		winter	spring	winter	spring
<i>Amblyomma hebraeum</i>	LL	0	0	12	6
	NN	0	0		
	MM	11	31		
	FF	8	13		
<i>Haemaphysalis elliptica</i>	LL	0	0	19	23
	NN	0	0		
	MM	32	425		
	FF	59	287		
<i>Rhipicephalus simus</i>	LL	0	0	0	0
	NN	0	0		
	MM	0	0		
	FF	0	0		
<b>Total number of ticks recovered</b>		<b>110</b>	<b>756</b>	<b>26</b>	<b>29</b>
<b>Total number of cheetahs infested</b>					

**Table 11:** Rodents trapped at the De Wildt/Brits and Hoedspruit Cheetah Breeding Centres (2010 – 2011)

Mouse species	Locations			
	De Wildt/Brits		Hoedspruit	
	Census	Control	Censes	Control
<i>Aethomys</i> sp.	8	7	0	0
<i>Graphiurus murinus</i>	1	1	0	0
<i>Mastomys</i> sp.	8	18	1	0
<i>Mus minutoides</i>	0	1	0	1
<i>Saccostomus campestris</i>	0	0	4	2
<i>Tatera leucogaster</i>	0	0	0	3
<i>Crocidura hirta</i> *	0	0	0	1
<b>Total catch</b>	<b>17</b>	<b>27</b>	<b>5</b>	<b>7</b>

sp.= species ; \* not a rodent

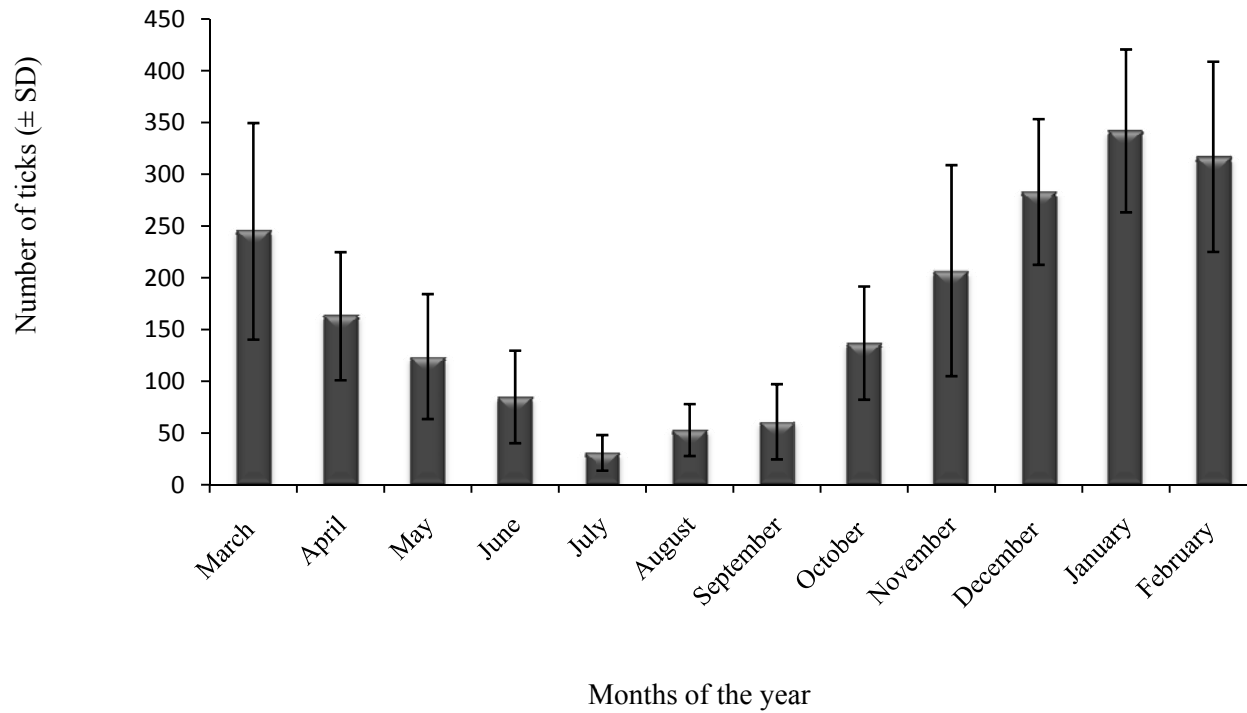
**Table 12:** Number of mice infested with ticks at the Ann van Dyk De Wildt/Brits Cheetah Breeding Centre

Mouse species	Tick species							
	<i>H. elliptica</i>		<i>R. (B.) decoloratus</i>		<i>R. simus</i>		<i>R. zambeziensis</i>	
	LL	NN	LL	NN	LL	NN	LL	NN
<i>Aethomys</i> sp. (n=5)	227	27	0	0	22	17	0	0
<i>Graphiurus murinus</i> (n=1)	19	0	0	0	1	0	0	0
<i>Mastomys</i> sp. (n=8)	28	7	2	0	0	1	2	0

*B* = *Boophilus*; *H* = *Haemaphysalis*; n = number of infested mice; *R* = *Rhipicephalus*

**Table 13:** Number of mice infested with ticks at the Hoedspruit Cheetah Breeding Centre

Mouse species	Tick species	
	<i>Haemaphysalis elliptica</i>	
	LL	NN
<i>Mastomys</i> sp. (n=1)	8	0
<i>Saccostomus campestris</i> (n=4)	86	5



**Fig. 1:** Mean monthly abundance (with standard deviation) of all stages of development of all ixodid ticks collected by drag-sampling vegetation from March 2008 to February 2009 at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits

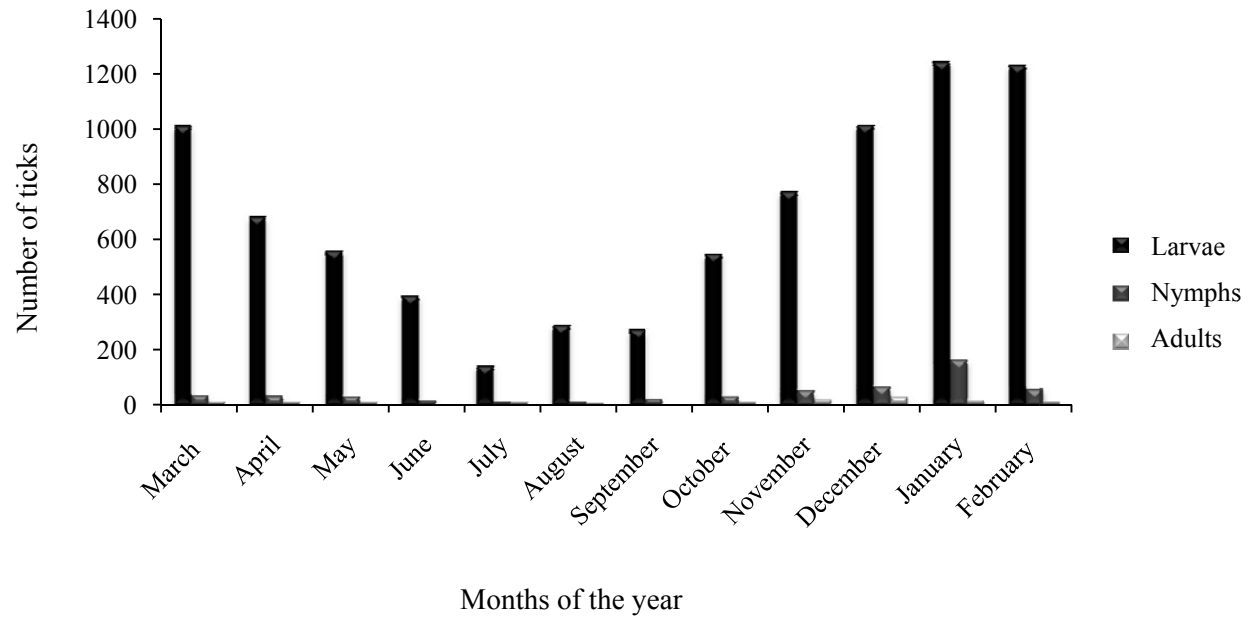


Fig. 2: Seasonal abundance of all stages of development of *Haemaphysalis elliptica* collected by drag-sampling the vegetation at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits from March 2008 to February 2009

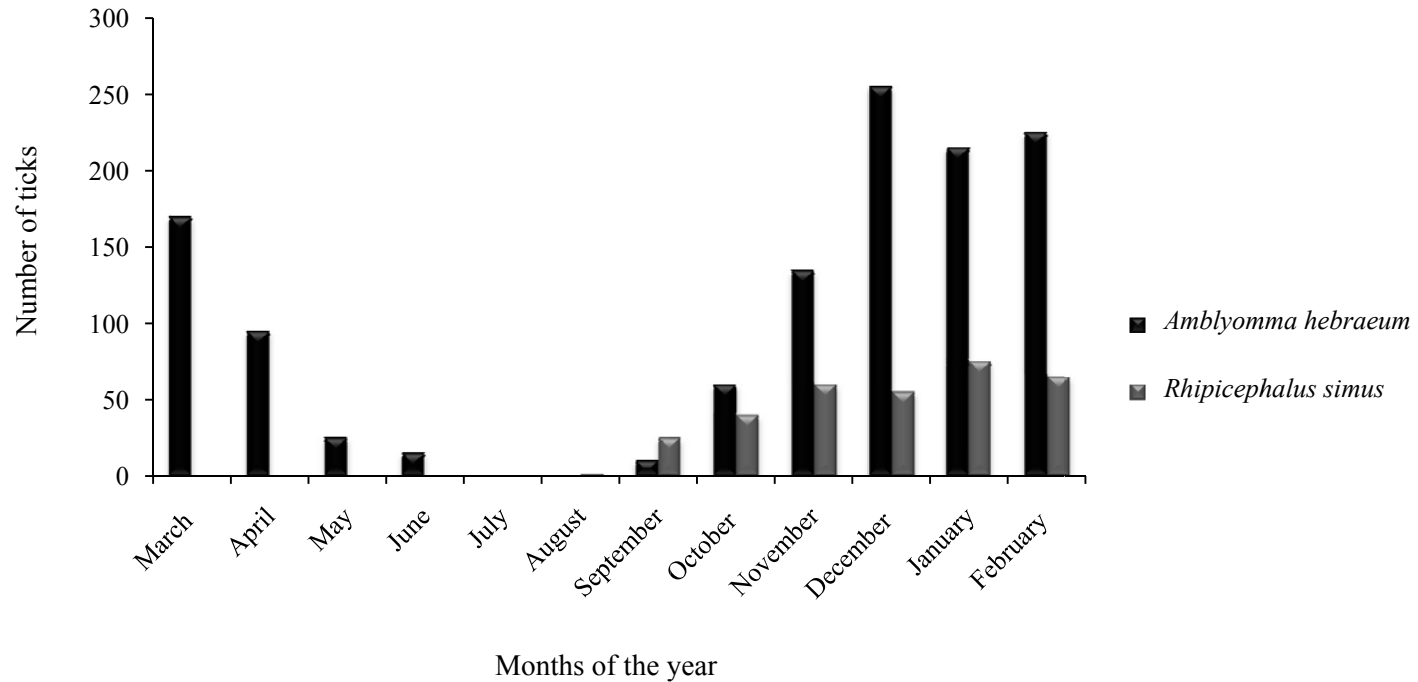


Fig. 3: Seasonal abundance of *Amblyomma hebraeum* and *Rhipicephalus simus* collected by drag-sampling vegetation at the Ann van Dyk De Wildt/Brits Cheetah Breeding Centre from March 2008 to February 2009

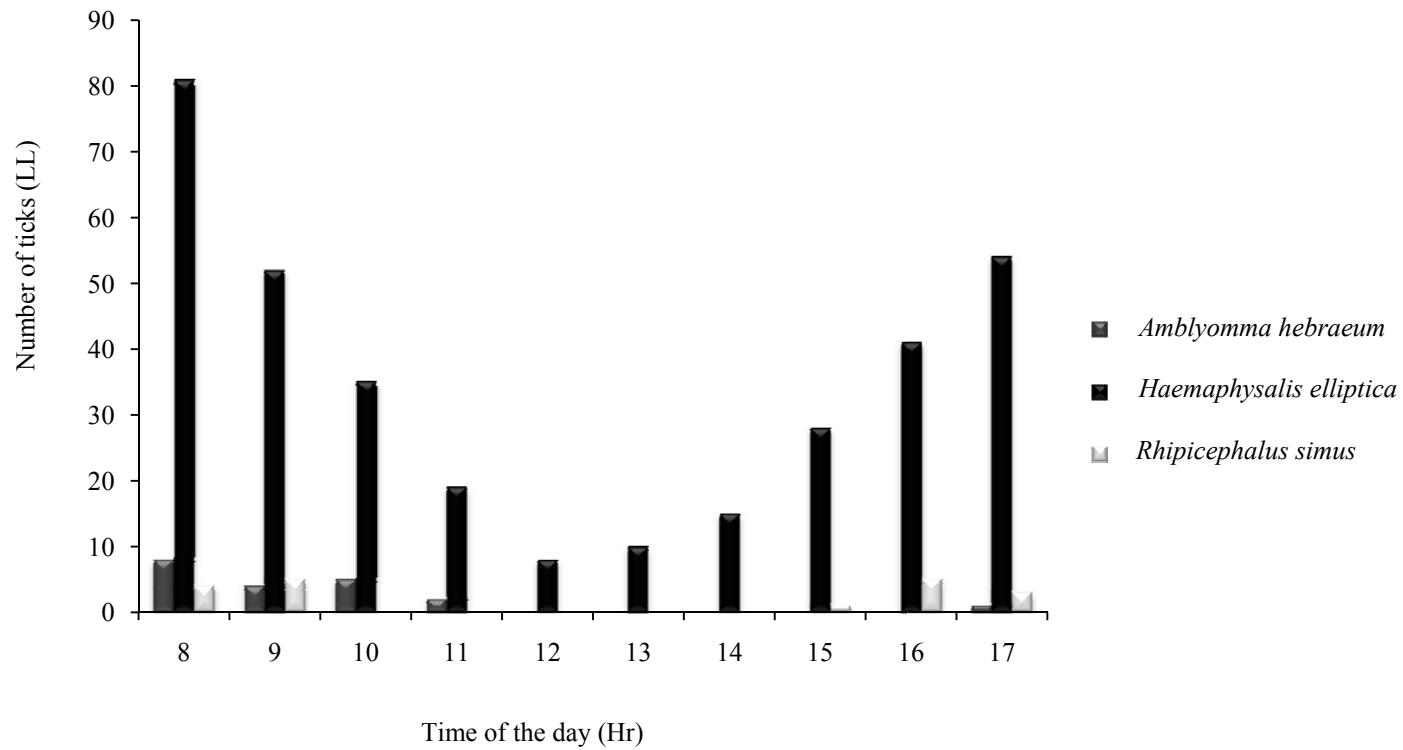


Fig. 4: Numbers of *Amblyomma hebraeum*, *Haemaphysalis elliptica* and *Rhipicephalus simus* larvae recovered hourly from vegetation at the De Wildt/Brits Cheetah Breeding Centre (June 2008)

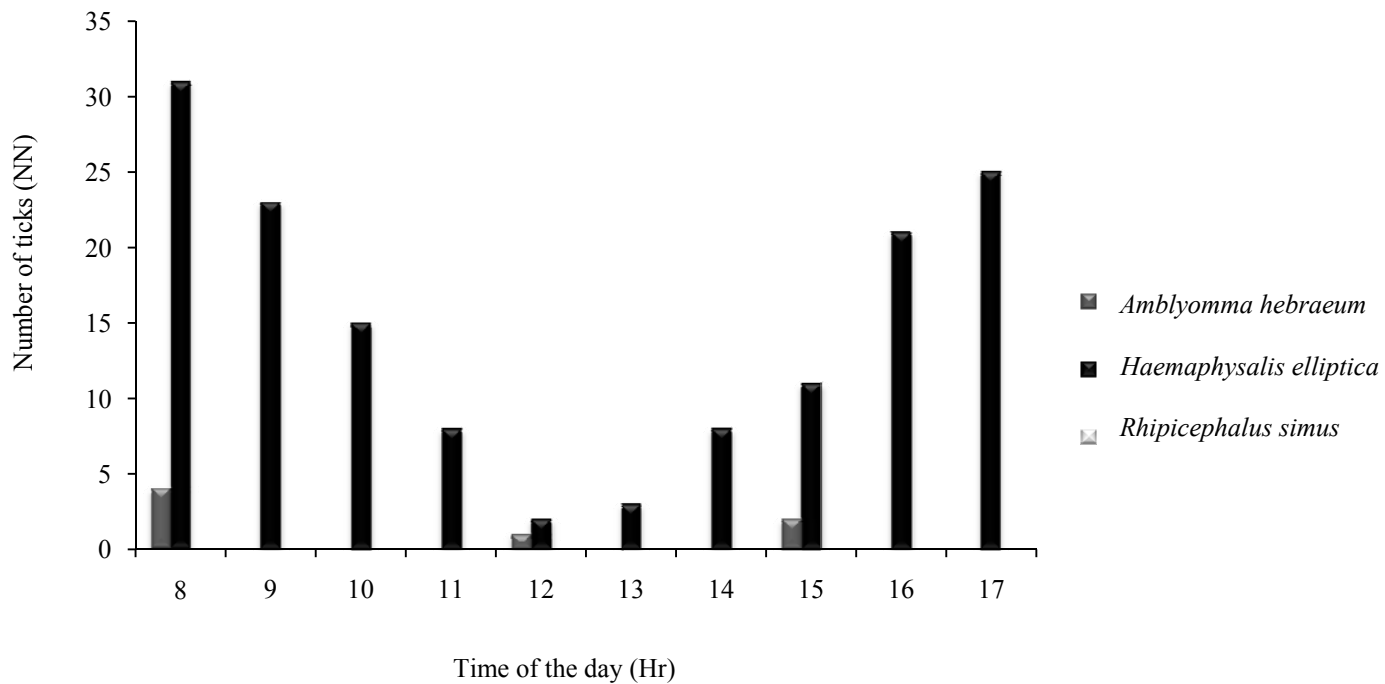
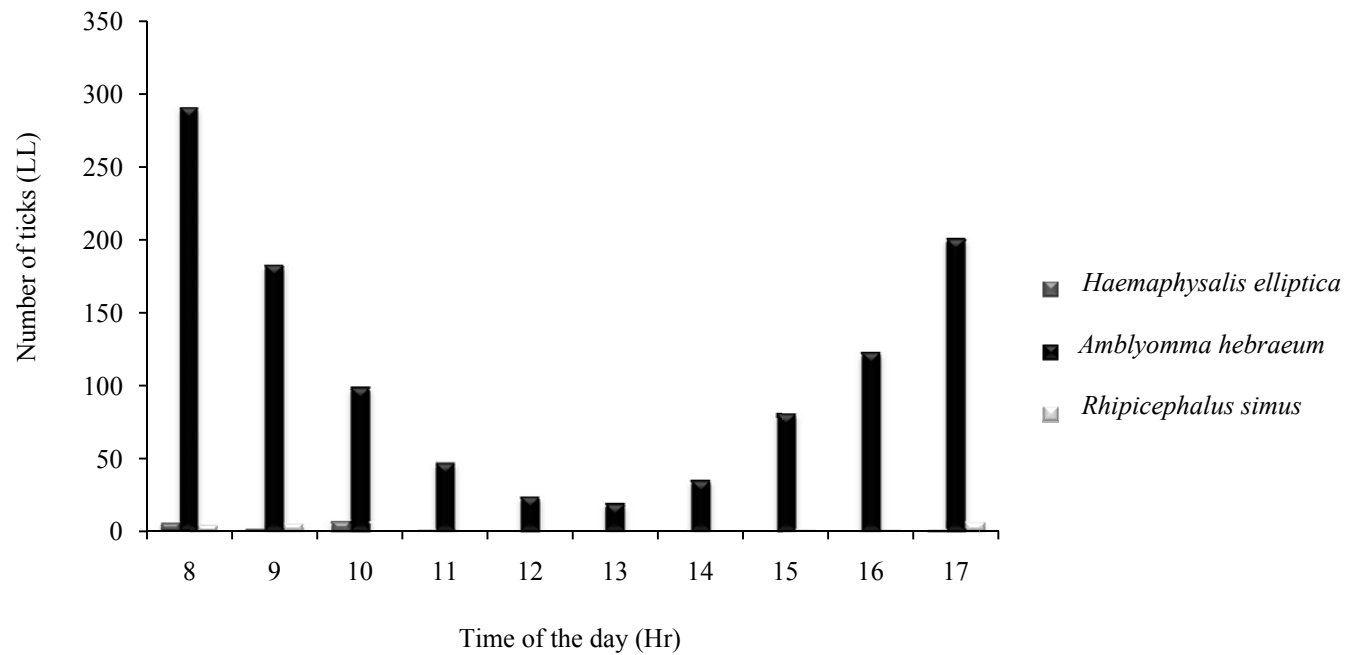


Fig. 5: Numbers of *Amblyomma hebraeum*, *Haemaphysalis elliptica* and *Rhipicephalus simus* nymphs recovered hourly from vegetation at the Ann van Dyk Cheetah Breeding Centre-De Wildt/Brits (June 2008)



**Fig. 6:** Numbers of *Amblyomma hebraeum*, *Haemaphysalis elliptica* and *Rhipicephalus simus* larvae recovered hourly from vegetation at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits (December 2008)

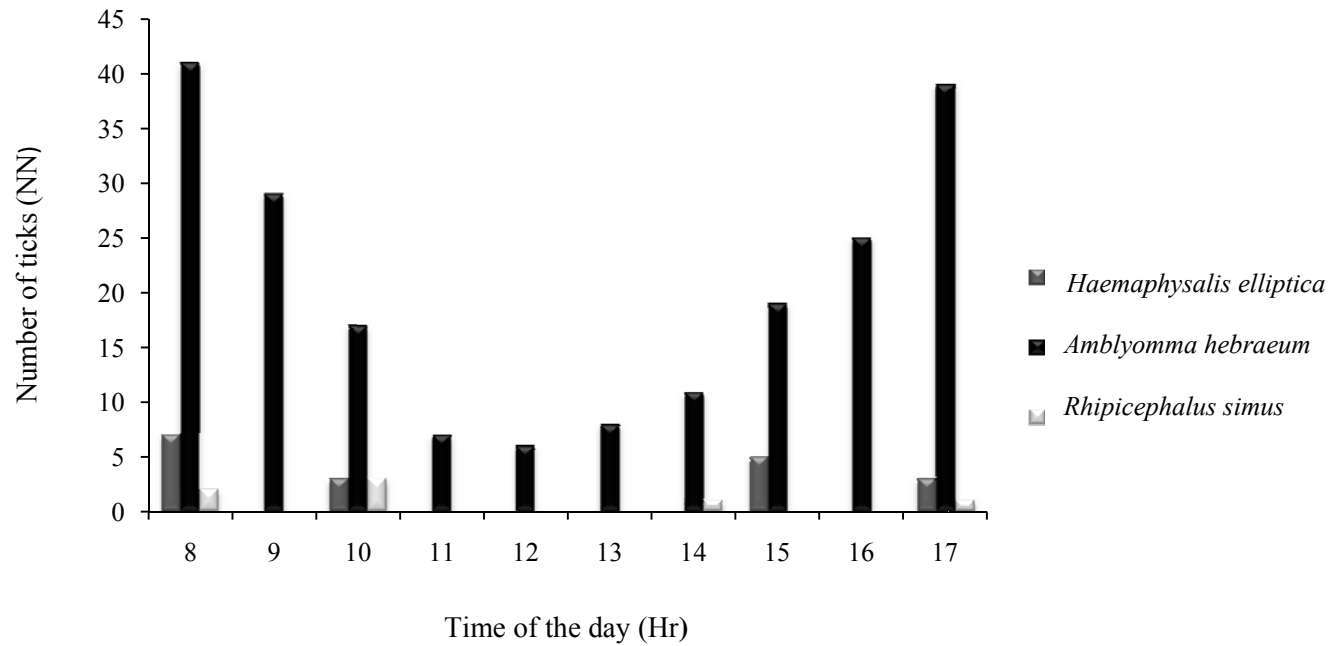


Fig. 7: Numbers of *Amblyomma hebraeum*, *Haemaphysalis elliptica* and *Rhipicephalus simus* nymphs recovered hourly from vegetation at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits (December 2008)

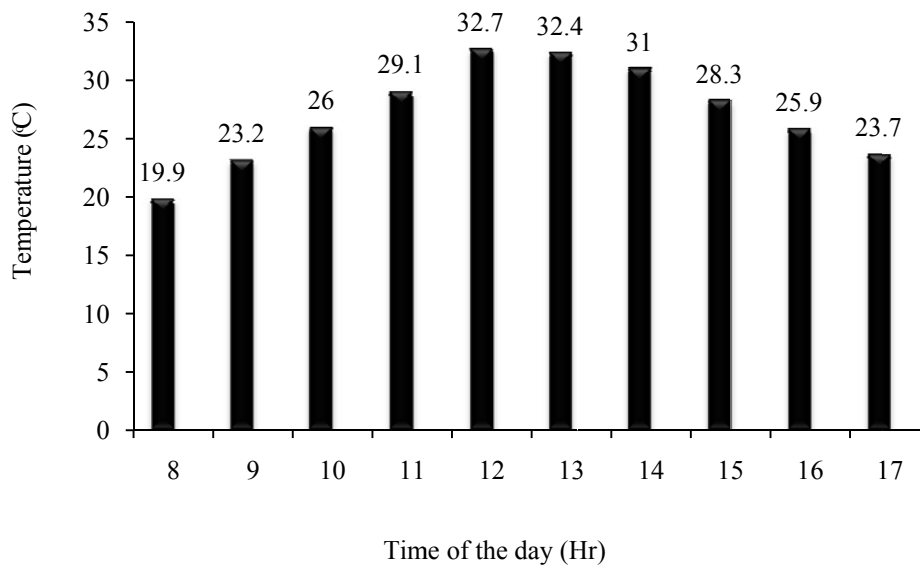


Fig. 8: Atmospheric temperature changes at various times of the day at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits (December 2008)

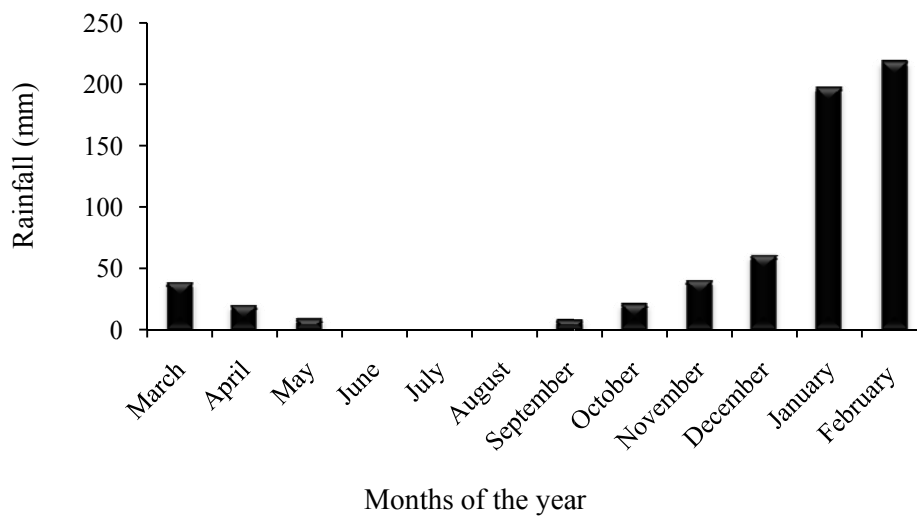
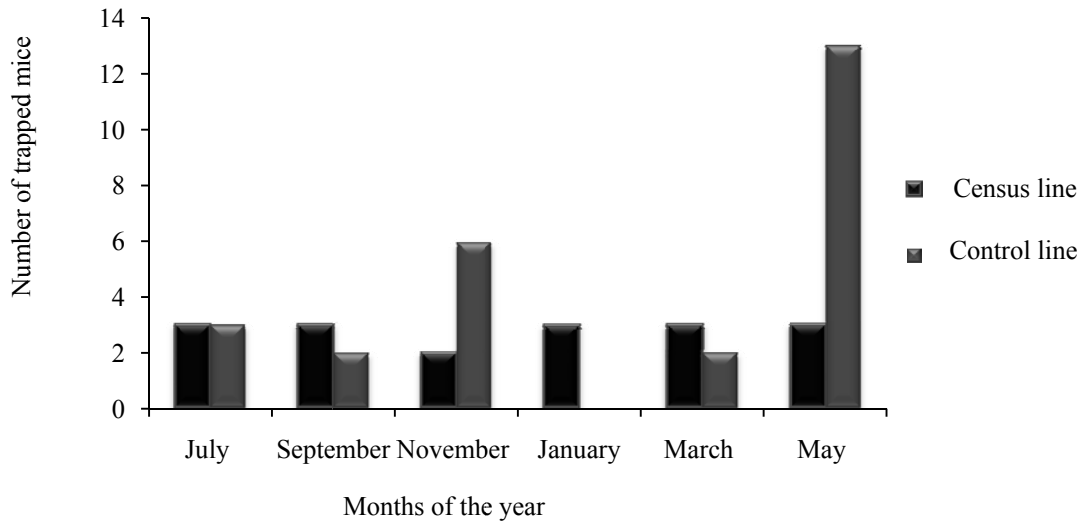
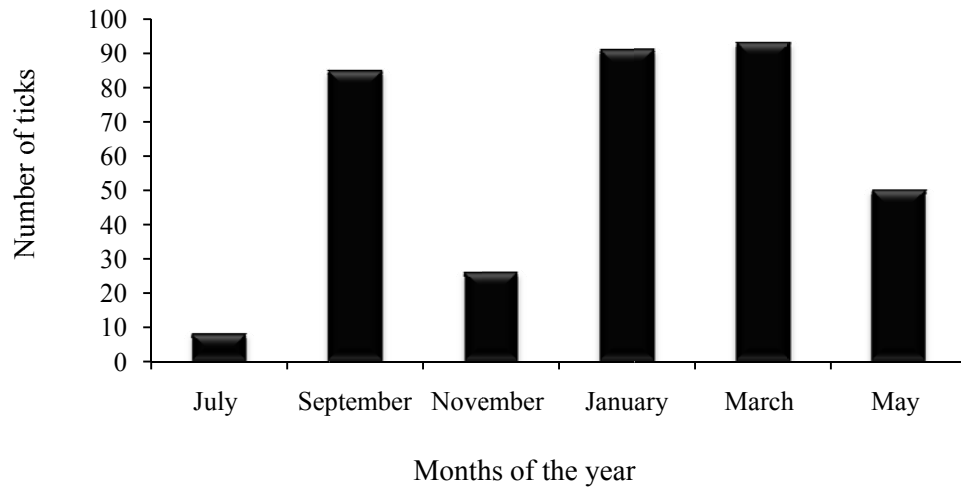


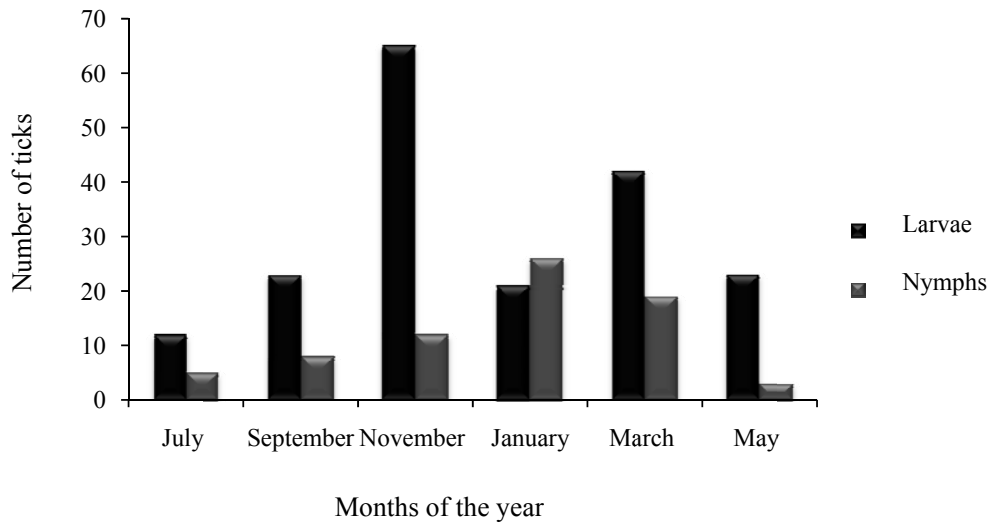
Fig. 9: Monthly rainfall at the Ann van Dyk Cheetah Breeding Centre – De Wildt/Brits from March 2008 to February 2009



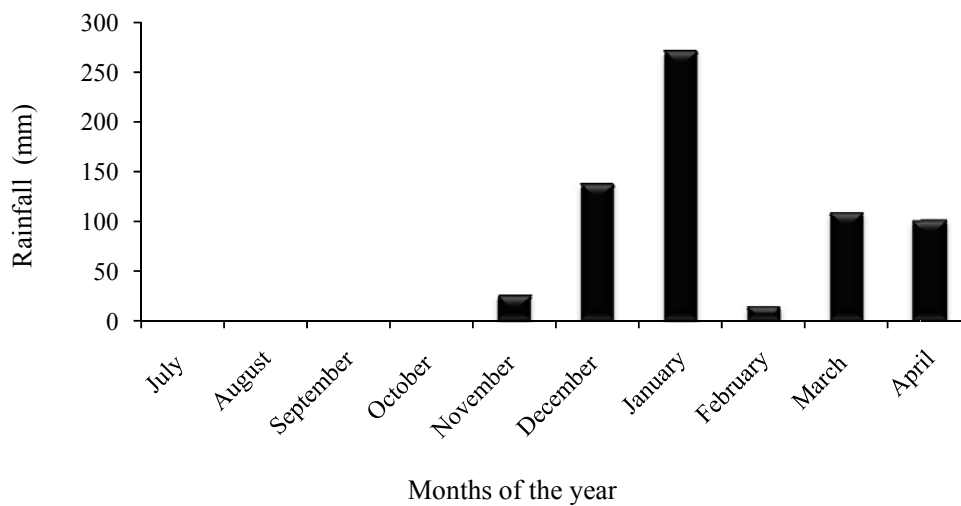
**Fig. 10 :** The number of mice trapped during each session on the census and control lines at the Ann van Dyk Cheetah Breeding Centre - De Wildt/Brits



**Fig. 11:** The number of ticks collected from the trapped mice at the Ann van Dyk Cheetah Breeding Centre - De Wildt/Brits (2010–2011)



**Fig. 12:** The number of ticks collected off the vegetation at the Ann van Dyk Cheetah Breeding Centre - De Wildt/Brits during the rodent trapping sessions (2010 – 2011)



**Fig. 13:** Rainfall during the study period at the Ann van Dyk Cheetah Breeding Centre - De Wildt/Brits (July 2010 – May 2011)

## Discussion

Most of the ticks recovered from the cheetahs in the study were undamaged, thus facilitating identification to genus and species level. Since the animals were temporarily physically restrained in a cage, it was only possible to collect engorged adult ticks which were slightly and/or fully palpable and visible. Hence the actual numbers recovered do not represent the total tick burden (Bryson *et al.*, 2000).

Factors such as climate and the vegetation are now generally considered as the broad-scale influences that potentially determine the extent of species ranges of ixodid ticks and also serve as possible predictors of tick diversity within a region (Walker, 1974; Norval, 1977; Cumming, 2002). Environmental factors, such as solar radiation, relative humidity, wind and soil moisture all influence the questing habits and activity of ticks, but not to the same extent as temperature (Harlan & Foster, 1990). Other studies have confirmed the influence of environmental temperature on the initiation and termination of questing (Daniels, Fish & Falco, 1989; Duffy & Campbell, 1994).

The rainfall at De Wildt varied considerably from month to month. The chart shows the rainfall values for the centre in each month during the survey (Fig. 8). The lowest rainfall occurred during the three months of winter 2008 and the highest in February 2009. During the rainy season and warm months of the year, drag-sampling showed an increase in the questing activity of ticks on vegetation, whereas, in the dry season and cold months of the year a decline was evident. However, the intensity of sunshine and rise in the midday temperature had a negative effect on questing activity, as few ticks were recovered from vegetation (Fig. 3, 4, 5 & 6). A rise in the incidence of infection with *Babesia* species corresponds with increased activity of vector ticks (Chapter 2, Fig. 5; Jacobson, Schoeman & Lobetti, 2000).

The various developmental stages in the life cycle of ixodid ticks in South Africa may exhibit their greatest rate of activity at different times of the day. In temperate climates, ticks are active throughout the day (Rechav, 1979) and year, provided that the environmental temperature is above that of the uncoordinated activity threshold temperature (a temperature below which a tick can no longer coordinate its host-seeking activity). More important is the activity threshold temperature at which all the

tick's activity will cease, thus determining the point at which the termination of tick-host contact occurs (Clark, 1995; Vail & Smith, 1998).

Following hatching or moulting and a period of quiescence, host-finding is assisted by orientation responses, which in ixodid ticks lead to a favourable distribution on the vegetation. Much of the behaviour of ticks seems to be dedicated to selecting optimal questing sites. Seasonal variation in terms of number of questing ticks can be the consequence of their developmental pattern (Randolph, 2002). Considering the microhabitat selection of ixodid ticks (achieved by responses to environmental cues such as gravity, light and humidity), many species habitually ascend vegetation to heights favourable for contact with their preferred hosts in the early morning and again in the evening (Rechav, 1979; Mehlhorn, 2008). Therefore, the time of day may certainly have an effect on microhabitat selection. Observation on the questing habits of various tick species highlights the possible importance of grass height in the questing behaviour of their immature stages (Londt & Whitehead, 1972; Spickett *et al.*, 1991).

The non-parasitic phases of ticks are the most critical period in their life cycles, and the ability of unfed ticks to survive when no hosts are available is crucial. The duration of this period of survival depends on atmospheric humidity (Knülle, 1966). At the equilibrium humidity, the tick's body weight will be constant by balancing water gain and loss. Body fluid homeostasis is one of the most important processes that influences tick survival in nature and the transmission of pathogens. The structure of the integument restricts the loss of water from the tick body and the loss of water may be compensated by uptake of water vapour from the atmosphere (Buczek, 1999). The sensitivity of ticks to desiccation is an important determinant of their geographical distribution.

Questing ticks have a daily probability of attaching to a host/s. Host-finding behaviour is habitually only demonstrated when the environmental and physiological conditions successfully sustain their survival (Mehlhorn & Armstrong, 2001). The ongoing process of alteration of the physical environment at De Wildt/Brits and Hoedspruit in the shape of building alterations and moving cheetahs from one pen to another could result in the developmental stages of a tick being forced to utilize available, but not necessarily optimum, microclimatic surroundings. Many ticks have

limited mobility (Rechav, 1979), therefore they follow an ambushing strategy, in which the parasites await hosts in their selected microhabitats in the vegetation, whereas, others, in particular certain *Hyalomma*, *Amblyomma*, *Ornithodoros* and *Dermacentor* species, seek their hosts by hunting, this implies that they actively move in the direction in which the host is seen or sensed. Each stage and state (questing, feeding and engorged) of the tick's life cycle is subject to a daily rate of mortality (Randolph, 2004).

Although the distribution of free-living ticks is not uniform within the host's habitat (Petney, Van Ark & Spickett, 1990), the diversity of on-host dispersion patterns of ticks includes the possibility that the presence of ticks on a particular host may attract more ticks to that host. Due to the low availability of a host pool in geographically isolated host populations, the parasite's exposure to different host species could be limited (Cumming, 1998). The cheetahs in their enclosures could be construed as a mini geographically isolated population and the host choice for adult ticks could be limited to these animals. Different tick instars can prefer different host species (Walker *et al.*, 2000; Walker, Bouattour, Camicas, Estrada-Peña, Horak, Latif, Pegram & Preston, 2003) and the murid rodents in and around the cheetah pens would be preferred hosts of the immature stages of some of the adult tick species infesting the cheetahs.

Adaptations acquired by parasites increase their chances of survival on their preferred host/s (Tompkins & Clayton, 1999; Dick & Patterson, 2007), hence their mean intensity and prevalence may be high on such hosts. The optimal height of grass can enhance the act of "pick up" of a questing instar by a passing host, and ixodid ticks can broadly be classified according to their preferences for a particular vegetation height (Londt & Whitehead, 1972). The optimal vegetation height may be related to the host's body size; it is, however, not clear as to how ticks would detect this parameter.

The monthly rainfall (July 2010-May 2011) at the Ann van Dyk Cheetah Breeding Centre ranged from 0 mm to 272 mm (Fig. 12). In comparison to the vegetation at Hoedspruit, the vegetation at De Wildt/Brits has been extensively disturbed by the activities of the cheetahs and humans and by a long dry season during 2010. Animals are kept in enclosures at De Wildt/Brits where the basal vegetational cover is

regularly mowed, besides being trampled by captive animals. These factors and the availability of a relatively large pool of hosts could have negatively influenced the numbers of ticks on the vegetation and on rodents.

The effect of removal of trapped rodents from the census line along the occupied enclosures was a major concern. As a consequence of poor vegetation cover and the possibility of predation pressure from cheetahs in the occupied enclosures, small mammal populations could be expected to be lower than in adjacent areas with good basal cover. The home ranges of South African small mammal species are poorly known, and it is not the objective of this study to elucidate that aspect *in situ*. However, it is reasonable to argue that there will be some migration of individuals from the lush environment of the unoccupied enclosures to the suboptimal environment of occupied enclosures. The primary objective of the control line was therefore to gauge the potential of the population in the unoccupied enclosures in order to provide for migration to the occupied enclosures where removal trapping was conducted, and thus compensate for removal trapping.

Individual trapped rodents were not sexed or weighed, as it was assumed that sampling would be random. The average mass for mouse species was taken from published data (Rautenbach, 1982; Skinner & Chimimba, 2005). The mass of a species is taken as a crude index of body size (Chimimba, 1998), indicating its capacity to accommodate a specific tick load (Gallivan & Horak, 1997). The number of traps per line was limited by the availability of uniform habitat within occupied and unoccupied enclosures. The basal cover of the occupied enclosures was sparse as a result of mowing and animal trampling, whereas the basal cover of the areas for the control lines was lush. Consequently it was anticipated that the control trap line along the unoccupied enclosures would yield a higher trapping success as a result of ample cover and nourishment compared to the conditions in the occupied enclosures.

The rationale for the trap line placing was the fact that habitat within the cheetah enclosures was suboptimal (cover and nourishment) and that the migration or exploratory movements of small mammals would be from the undisturbed areas towards the cheetah enclosures. The trapping success in the present study is not comparable with that reported by other researchers (Braack, Horak, Jordaan, Segerman & Louw, 1996) since throughout this study all traps were closed during the

day to avoid trapping yellow-footed squirrels. This procedure therefore excluded diurnal small mammals such as *Rhabdomys pumilio*, *Lemniscomys rosalia* and to some extent shrews such as *Crocidura hirta* and *Crocidura cyanea*.

*Mastomys natalensis* and *Mastomys coucha* are morphologically indistinguishable, and since the nature of this study excluded exhaustive genetic or morphometric identifications of sampled rodents, specimens of this genus were analysed generically and referred to as *Mastomys* species. The distribution ranges of these two species include both study sites (Skinner & Chimimba, 2005). *Aethomys chrysophilus* is absent at the De Wildt site, whereas both *A. chrysophilus* and *A. ineptus* apparently occur at the Hoedspruit Endangered Species Centre (Linzey, Kesner, Chimimba & Newberry, 2003; Skinner & Chimimba, 2005). As a consequence all specimens of this genus were also only considered at the generic level for the purposes of this study.

This study highlights the diversity of ectoparasite species associated with captive cheetah populations and their habitats at different localities in South Africa. The close relationship of various host species endorses the fact that ectoparasites may be shared amongst them and host density and host composition may also play important roles in parasite diversity and burdens at a locality.

### ***Amblyomma hebraeum***

The distribution of the South African bont tick, *A. hebraeum*, extends from the northern and north-western provinces into KwaZulu-Natal and the Eastern Cape Province of South Africa and also into Swaziland (Norval, 1977). Its climatic and vegetational requirements are similar to those of *Rhipicephalus appendiculatus* and consequently their distributions largely overlap within the borders of South Africa (Walker *et al.*, 2003; Horak, Nyangiwe, De Matos & Neves, 2009).

*Amblyomma hebraeum* prefers tall grassveld, adequate shrub and bush cover where rainfall exceeds 380 mm annually (Theiler, 1948; Theiler, 1969). Climate (day-length, temperature, rainfall and humidity) may affect the hatchability of the eggs of *A. hebraeum* and consequently the activity of its larvae (Norval, 1977). Populations of adult *A. hebraeum* reach a peak during the summer months (Norval, 1977; Knight & Rechav, 1978; Londt, Horak &

De Villiers, 1979; Horak, 1982). However, in the Lowveld regions of north-eastern KwaZulu-Natal, Mpumalanga and Limpopo provinces the occurrence of *A. hebraeum* appears to be non-seasonal. Large numbers of adults have been recovered from eland (*Taurotragus oryx*), giraffe (*Giraffa camelopardalis*) and African buffalo (*Syncerus caffer*) examined during the winter and spring (Horak, Potgieter, Walker, De Vos & Boomker, 1983), while the immature stages were present throughout the year (Horak, Boomker, Spickett & De Vos, 1992). Seasonal changes in the numbers of questing ticks can affect the intensity of infestation as well as the size of the tick burden. In this study, the low prevalence of *A. hebraeum* on the drag cloths was probably attributable to seasonality and habitat and host suitability (Fig. 2).

The larvae, and to a lesser extent the nymphs, of *A. hebraeum* infest a variety of small and large mammals, including carnivores, and also ground-frequenting birds (Horak *et al.*, 1987b). On the other hand, adults of *A. hebraeum* generally prefer animals with a large body mass (Gallivan & Horak, 1997) and consequently large numbers of adult ticks can be expected to be recovered from large hosts (Horak *et al.*, 1983; Horak *et al.*, 1987b). Impalas (*Aepyceros melampus*) and birds serve as hosts for *A. hebraeum* at the De Wildt/Brits Centre, while there are a number of antelope species, particularly elands, which maintain the *A. hebraeum* population at the Hoedspruit Centre and many birds that could disseminate its immature stages there.

### ***Amblyomma marmoreum***

*Amblyomma marmoreum* is widely distributed in South Africa and is known as the South African tortoise tick (Horak, McKay, Heyne & Spickett, 2006). Adults have a host preference for tortoises, and particularly leopard tortoises (*Geochelone pardalis*), but the immature stages often infest a variety of reptiles as well as mammals and birds (Horak *et al.*, 2006). The size of the host appears to have a significant effect on the magnitude of the adult tick burden. Leopard tortoises, the largest tortoise species in South Africa, harbour most adult *A. marmoreum* (Horak *et al.*, 2006). The presence of tortoises and birds at DeWildt/Shingwedzi probably account for the abundance of *A. marmoreum* at the centre. As no tortoises were examined in the present study,

no comparison between the tick burdens of the cheetahs and tortoises could be made.

### ***Haemaphysalis elliptica***

The three-host southern African yellow dog tick, *H. elliptica*, formerly incorrectly lumped with *H. leachi*, has recently been redescribed as a valid species (Apanaskevich, Horak & Camicas, 2007). For the past century it had taxonomically been grouped with *H. leachi* as a single species. Originally only the males were studied and were named *Rhipistoma ellipticum* (Koch, 1844). The distribution of *H. elliptica*, which is widespread in southern Africa, might to some extent overlap with that of *H. leachi* north of South Africa. All the earlier studies in South Africa, in which the tick was referred to as *H. leachi*, actually refer to *H. elliptica* since *H. leachi* apparently does not occur in this country.

Various wild and domestic carnivores are the preferred hosts of the adults of *H. elliptica*, amongst which it infests domestic dogs and cats as well as lions (*Panthera leo*), leopards (*Panthera pardus*) and cheetahs (Horak *et al.*, 1987a, 2000; Horak & Matthee, 2003; Apanaskevich *et al.*, 2007; Horak *et al.*, 2010). Various species of rodents as well as other small mammals are the preferred hosts of its immature stages (Hoogstraal, 1956; Petney *et al.*, 2004). Although Jacobs, Fourie and Horak (2004) demonstrated that this tick can complete more than one life cycle annually under laboratory conditions, they doubted whether this would occur in nature. *Haemaphysalis elliptica* (then referred to as *H. leachi*) has been proven to be the vector of *Babesia canis rossi*, the causative organism of canine babesiosis in domestic dogs in South Africa (Lewis, Penzhorn, Lopez-Rebollar & De Waal, 1996).

### ***Rhipicephalus (Boophilus) decoloratus***

This species is known as the blue tick by South African farmers and it commonly parasitizes both domestic and wild ungulates in South Africa with cattle and horses being the chief domestic animal hosts (Hoogstraal, 1956; Baker & Ducasse, 1967; Walker, 1991), wild ungulates such as greater kudu (*Tragelaphus strepsiceros*), Burchell's zebras (*Equus burchellii*) and impalas may also harbour large numbers of all developmental stages of this one-host

tick (Horak, De Vos & De Klerk, 1984; Horak *et al.*, 1992; Horak, Gallivan, Braack, Boomker & De Vos, 2003). In South Africa *R. (B.) decoloratus* occurs in all the provinces except the Northern Cape Province (Howell *et al.*, 1978). Nyalas (*Tragelaphus angasii*), kudus and impalas are present at the Hoedspruit centre and act as good hosts for this tick species there.

### ***Rhipicephalus simus***

*Rhipicephalus simus* has an extensive distribution in southern African (Walker *et al.*, 2000), and among domestic animals the adults of this tick species mostly parasitise domestic cattle and dogs (Horak *et al.*, 1987a; Walker *et al.*, 2000). The adults have also been recovered from many wild animals including wild felids (Norval & Mason, 1981; Horak *et al.*, 1983; Horak *et al.*, 1987a; Horak *et al.*, 2000). The immature stages usually prefer small burrow-dwelling rodents as hosts, particularly murid rodents (Hoogstraal, 1956; Norval & Mason, 1981; Braack *et al.*, 1996). With the exception of the dry regions of the Northern Cape Province in South Africa, *R. simus* is abundant virtually throughout the country, but it never occurs in really large numbers (Walker *et al.*, 2000).

### ***Rhipicephalus zambeziensis***

Walker, Norval and Corwin (1981) described all the developmental stages of *R. zambeziensis* and compared its morphology with that of *R. appendiculatus*. It is a tick species with a wide range of ruminant hosts, and all stages may use the same host species in order to complete its life cycle. The hosts of *R. zambeziensis* seem to range widely from impalas, bushbuck (*Tragelaphus scriptus*), nyalas, greater kudus, elands and African buffaloes to domestic cattle (Norval, Walker & Colborne, 1982; Horak *et al.*, 1983; Horak *et al.*, 1992, Walker *et al.*, 2000; Horak *et al.*, 2003). The distribution of this ixodid tick species is confined to the North West, Limpopo and Mpumalanga Provinces of South Africa (Norval *et al.*, 1982; Horak *et al.*, 1992; Horak *et al.*, 2003). The presence of a diversity of free-ranging antelopes such as impalas, bushbuck, nyalas, kudus and elands provided suitable hosts for *R. zambeziensis* to complete its life cycle at the De Wildt/Shingwedzi and the Hoedspruit centres.

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## Chapter 4:

### **Detection of *Babesia* species in captive cheetah (*Acinonyx jubatus*) populations, associated field-collected ticks (Acari: Ixodidae), mice and their related ticks in South Africa**

#### **Abstract**

The objective of this study was to make an inventory of the occurrence of *Babesia* species associated with captive cheetah populations in South Africa and to detect the *Babesia* parasites in associated ixodid tick species as potential vectors. A total of 143 cheetahs, 10,432 field-collected ticks of various species and 21 murid rodents were examined for babesial infection. Polymerase chain reaction (PCR) analysis of blood samples and tick specimens successfully amplified 500 bp fragments of the small subunit of the 18S rRNA gene of *Babesia* species. The PCR products were subjected to the Reverse Line Blot (RLB) hybridisation assay revealing that 48 (59.2%), 12 (54.5%), 5 (15.6%) and 8 (100%) of the cheetahs at The Ann van Dyk Cheetah breeding Centre-De Wildt/Brits, The Ann van Dyk Cheetah breeding Centre-De Wildt/Shingwedzi, The Hoedspruit Endangered Species and The Cheetah Outreach, respectively, were infected with *Babesia canis rossi*, *Babesia felis* and *Babesia lengau*, the latter being the dominant *Babesia* species. The RLB-F and RLB-R primers successfully amplified the V4 variable region of the small subunit of the 18S rRNA gene of *B. lengau* in unfed *Haemaphysalis elliptica* collected from the vegetation as well as the trapped rodents at the study sites.

## Introduction

Cheetah populations in Africa are divided into five zoogeographic subspecies, of which *Acinonyx jubatus jubatus* occurs in portions of South Africa (Bourlière, 1963; Hunter & Hamman, 2003). Cheetah distribution has been modified over historical times by modern man's colonization of the African continent. Cheetah range extends over south-eastern sub-Saharan Africa (Wilson & Reeder, 1993). In an electrophoretic survey of allozymes and cell proteins, it was discovered that the South African cheetah, *A. jubatus jubatus*, is unique among felids and also other mammals in having an extreme paucity of genetic diversity, in contrast to domestic cats (*Felis domesticus*) (Ó'Brien, Wildt, Goldman, Merrill & Bush, 1983) since the cheetah is a highly monomorphic species (O'Brien, Roelke, Marker, Newman, Winkler, Meltzer, Colly, Evermann, Bush & Wildt, 1985).

*Babesia* species, intra-erythrocytic haemoprotozoans with a wide range of wild target felid hosts in Africa (Penzhorn, 2006), are of great veterinary importance in regions where the specific tick vectors are present. Through the advent of molecular biology techniques such as polymerase chain reaction (PCR), recent studies on the characterizations of feline piroplasms of domestic and wild cats have revealed diversity of possible genetically distinct organisms (Davis, 1929; Carpano, 1934; Jackson & Dunning, 1937; Mudaliar, Achary & Alwar, 1950; Dennig, 1967; Dennig & Brocklesby, 1972; Penzhorn, Kjemtrup, López-Rebollar & Conrad, 2001; Baneth, Kenny, Tasker, Anug, Shkap, Levy & Shaw, 2004; Bosman, Venter & Penzhorn, 2007), leading to a discussion of reclassification within this group. Techniques for detection of these haemoparasites have been developed separately for use in each species.

Feline babesiosis is caused by multiple species of *Babesia*, most of which have been reported in wild felids (Ayoob, Prittie & Hancker, 2010). It is a relatively new clinical entity and little is known regarding its epidemiology and disease course. The felid piroplasms are informally divided into two forms (small and large) according to their morphology (Dennig & Brocklesby, 1972), the small form consisting of *Babesia felis* and *Babesia cati* and the large form comprising *Babesia herpailuri* and *Babesia pantherae*. Both forms (small and large) exhibit a worldwide distribution. Of various *Babesia* species, *Babesia cati* (India) and *Babesia felis* (South Africa) infect domestic

cats, while *Babesia herpailuri* (South America and Africa) and also *Babesia panthera* (Kenya) have been detected in wild felids (Schoeman & Leisewitz, 2006). The most and least virulent species in the felids are *B. felis* and *B. cati*, respectively (Ayoob *et al.*, 2010). *Babesia felis* occurs mostly in domestic cats but is believed to have a wide range of hosts within other member of the cat family (Levine, 1971). In the recent years, molecular study on the prevalence of *Babesia* species in felids (Bosman *et al.*, 2007) revealed the presence of a hitherto undescribed species in cheetahs, i.e. *Babesia lengau* (Bosman, Oosthuizen, Peirce, Venter & Penzhorn, 2010).

Tick-borne pathogens can co-exist in the same tick species (vector) and/or be transmitted by various tick species (De la Fuente, Estrada-Peña, Venzal, Kocan & Sonenshine, 2008; Kocan, de la Fuente & Blouin, 2008). Transmission of *Babesia* species occurs via blood feeding. Detecting and identifying a pathogen in a tick vector carrying a low level of infection is difficult. The introduction of molecular techniques greatly improved both specific and sensitive detection of the genomic DNA of various human and animal pathogens (Sparagano, Allsopp, Mank, Rijpkema, Figueroa & Jongejan, 1999). A chronic, asymptomatic carrier state of infection with *Babesia* species in domestic and wild animals has been recognized (Conrad, Thomford, Yamane, Whiting, Bosma, Uno, Holshuh & Shelly, 1991), and most information about the chronic babesial infections is from animal model studies. Animals can remain chronic carriers even after the resolution of the clinical signs (Figueroa *et al.*, 1992).

Transmission cycles of all babesial parasites are maintained via vector ticks and their vertebrate hosts (Homer, Aguilar-Delfin, Telford III, Krause & Pering, 2000). Wild rodents play an important role as reservoir hosts for many pathogens. The presence of *B. divergens*, *B. divergens*-like organisms, *B. hylomysci* and *B. microti* has been detected in rodents (Bafort, Timperman & Molyneux, 1970; Akinboade, Dipeolu, Oqunji & Adegoke, 1981; El Bahrawy, Nafei, Morsy, Farraq, 1993; Beck, Vojta, Ćurković, Mrljak, Margaletić & Habrun, 2011), especially by using various molecular techniques (Pressing, Mathiesen, Marshall, Telford, Spielman, Thomford & Conrad, 1992).

The literature on the means of transmission of piroplasms in felids as well as their potential vectors is scanty. The aim of this study was initially to investigate the occurrence and identity of *Babesia* species infecting cheetahs, using PCR and the Reverse Line Blot (RLB) hybridisation assay. The second objective was to detect *Babesia* species genomic DNA in field-collected (unfed) ixodid tick species to determine the potential tick vector, since components of the blood meal in engorged ticks can possibly inhibit the PCR reaction resulting in an underestimation of infection rate in ticks (Schwartz, Varde, Nadelman, Wormser & Fish, 1997), and the presence of *Babesia* DNA in engorged ticks does not necessarily imply that the tick is a vector of the *Babesia*. This field study was also conducted to detect possible *Babesia* species in local murid rodents at the Ann van Dyk Cheetah Breeding Centre and The Hoedspruit Endangered Species Centre. The attack frequency of tick species on restricted murid rodents indicates the risk of rodent babesiosis in the region and the necessity of precautionary measures to control the transmission of parasite to cheetahs.

## Materials and Methods

### *1. Survey localities and period*

This study was conducted between 2007 and 2011, during which 143 captive cheetahs resident at the four cheetah-breeding centres (Table 1) as described comprehensively in the Chapter 1, were examined in three different provinces in South Africa.

Table 1: Number of cheetahs examined in various localities

Localities	Number of cheetahs
The Ann Van Dyk Cheetah Breeding Centre - De Wildt/Brits	81
The Ann Van Dyk Cheetah Breeding Centre - De Wildt/Shingwedzi	22
The Hoedspruit Endangered Species Centre	32
The Cheetah Outreach	8

## **2. *Sample collection***

After restraining the cheetahs in a cage, 2 ml of blood was collected by the authorised veterinarian from the cephalic and/or saphenous vein using **BD Vacutainer™** tubes ([Franklin Lake, UDA](#)) containing EDTA. The blood tubes were labelled according to the animal code and placed in the a box, which was transported to the molecular biology laboratory in the Department of Veterinary Tropical Diseases, at the Faculty of Veterinary Science, where the tubes were placed in a refrigerator until further processing.

Various instars of ticks were collected by dragging the vegetation of various cheetah enclosures using a drag stick (the procedure was explained in more details in the [Chapter 3](#)). The ticks were removed from the flannel strips of the drag, placed in bottles containing 70% ethanol and transported to the ectoparasitology laboratory. After identification of the genus and species of the ticks under a stereoscopic microscope according to morphological features ([Walker, 1991](#); [Walker, Bouattour, Camicas, Estrada-Peña, Horak, Latif, Pegram & Preston, 2003](#)) and being recorded, the adult ticks were placed individually in labelled bottles, while the larvae and nymphs were pooled.

At each session of rodent trapping (as described in the [Chapter 3](#)), before the animals were processed for tick recovery, a small incision was made on the jugular vein and a few drops of blood were collected on labelled filter paper. The blood-stained papers were left in the laboratory for 24 hours to dry out. The dried filter papers were preserved at room temperature for further analysis.

## **3. *Preparation of blood smears***

Peripheral blood smears from cheetahs were randomly made on glass microscopic slides which were allowed to dry out in room temperature. The slides were fixed in 100% methanol for approximately 30 sec and rinsed off in tap water. The slides were dipped in a freshly-made solution of 10% Giemsa stain in distilled water for 30 sec. The slides were then rinsed off in tap water and dried out thoroughly using a hair drier. The slides were viewed under oil immersion with a 100x objective ([Voigt, 2000](#)) to observe parasites in the red blood cells and assess the parasitaemia.

#### **4. DNA isolation**

##### **4.1. Blood samples from cheetahs**

Genomic DNA was extracted from 200 µl of each blood sample using the [QIAamp<sup>®</sup> DNA Mini Extraction Kit](#) (Qiagen, Hilden, Germany), following the manufacturer's protocol:

The samples were initially equilibrated to the room temperature. A volume of 200 µl of the blood sample was pipetted into a 1.5 ml microcentrifuge test tube. Then 20 µl of Proteinase K and 200 µl of Buffer AL was added to the tube and mixed by pulse-vortexing for about 15 sec. The tube was incubated at 56°C for 10 min on a heating block. Afterwards, 200 µl of ethanol (96 - 100%) was added to the sample and mixed by pulse-vortexing for 15 sec. The mixture was applied to the [QIAamp Spin Column](#) followed by adding 500 µl of Buffer AW1. The mixture was subsequently centrifuged at full speed (20 200 xg) for 1 min. Buffer AW2 (500 µl) was added to the column, and then centrifuged at full speed (20 200 xg) for 3 min. The DNA was eluted by adding 100 µl of Buffer AE followed by incubation at room temperature for 1 min, then centrifuged at 11 543 xg for 1 min. The extracted genomic DNA collected in a 1.5 ml tube, labelled and stored at -20°C for further molecular analysis.

##### **4.2. Tick specimens**

Due to the costly, time-consuming procedures involved, only adult ticks were analysed individually, while larvae and nymphs of the same species were pooled in batches of 50 and 20, respectively. To achieve optimal homogenization of various tick samples, the genomic DNA of adult and immature ticks was extracted using the [MagNA Lyser Green Beads](#) (Roche, Germany) ([Brinkley, Nolskog, Golovljova, Lundkvist & Bergström, 2008](#)) prior to the [QIAamp<sup>®</sup> DNA Mini Extraction Kit](#) (Qiagen, Hilden, Germany), following the manufacturer's protocol:

The tick samples were initially cut into small pieces, which were transferred to a [MagNA Lyser Green Beads](#) tube and 180 µl of the ATL buffer (lysis buffer) was added. The tube was placed in the rotor ([MagNA Lyser Instrument, Roche Applied Science](#)) and spun at high speed (10 100 xg). The tube was cooled on ice prior to any enzymatic reaction to prevent any enzyme disruption. Subsequently, 20 µl of Proteinase K was pipetted into the tube followed by incubation at 56°C overnight, allowing comprehensive lysis of tick tissue. The tube was occasionally pulse-vortexed

for 15 sec during the incubation period. Then 200  $\mu$ l of Buffer AL was added to the tube, and mixed by pulse-vortexing for about 15 sec. The tube was incubated at 70°C for 10 min, followed by adding 200  $\mu$ l of ethanol (96-100%) to the tube and pulse-vortexing for 15 sec. The mixture was applied to the [QIAamp Spin Column](#) followed by adding 500  $\mu$ l of Buffer AW1 and centrifuging at full speed (20 200 xg) for 1 min. Subsequently, 500  $\mu$ l of Buffer AW2 was added to the column, which was then centrifuged at full speed (20 200 xg) for 3 min. The column was placed in a 1.5 ml tube and 100  $\mu$ l of Buffer AE was pipetted followed by incubation at room temperature for 1 min, then centrifuged (11 543 xg) for 1 min. The extracted genomic DNA was then stored at -20°C for supplementary analysis. Subsequently, 2  $\mu$ l of each DNA sample was submitted to the [NanoDrop ND-100 Spectrophotometer](#) (Wilmington, Delaware, USA) to assess the quality of the extracted DNA, in order to optimize the amount of DNA used as the template for PCR amplification.

Due to the expected relatively low level of parasite and DNA concentration, the genomic DNA was amplified prior to the PCR amplification, using the [GenomiPhi™ V2 DNA Amplification Kit](#) (GE Healthcare UK Ltd). For this purpose, 1  $\mu$ l (10 ng) of the genomic DNA was added to 9  $\mu$ l of the buffer sample in a PCR tube. The mixture was heated to 95°C (heating longer than this period can damage the genomic DNA) for 3 minutes in a thermocycler and immediately cooled to 4°C on ice. Then 1  $\mu$ l of reaction buffer and 1  $\mu$ l of enzyme mixture comprising the master mixture (containing the Phi29 DNA polymerase enzyme, additional random hexamers, nucleotidesalts and buffers) were added to the reaction tube which was followed by incubation at 30°C for 1.5 hrs and heat inactivation of the Phi29 DNA polymerase enzyme at 65°C for 10 minutes (heating is required to inactivate the exonuclease activity of DNA polymerase which may otherwise begin to degrade the amplification product). Ultimately, amplified genomic DNA sample was stored at -20°C for further utilization.

#### ***4.3. Blood samples from mice***

The genomic DNA was extracted from the mice' blood spots on the filter papers using the [QIAamp® DNA Mini Extraction Kit](#) (Qiagen, Hilden, Germany), following the manufacturer's protocol:

Three circles from a dried blood spot were punched out, placed in a 1.5 ml tube and 180 µl of Buffer ATL added. The tube was then incubated in the heating block at 85°C for 10 min. The tube was cooled on ice to prevent any enzyme disruption. After a brief centrifuge (18 757 xg, 5 sec), 20 µl of Protienase K was added to the tube prior to incubation at 56°C for an hour. Subsequently, 200 µl of AL was added and after a short vortexing (15 sec), the tube was incubated at 70°C for 10 min followed by adding 200 µl of 100% ethanol and vortexing.

The contents of the tube were transferred to a QIAamp mini column and centrifuged at full speed (20 200 xg) for 1 min. The flow in the collecting tube was discarded. The column was placed in a new 2 ml collecting tube and 500 µl of buffer AW1 was added. The tube was centrifuged at full speed (20 200 xg) for 1 min. The flow in the collecting tube was discarded and the column was placed in a new collecting tube followed by adding 500 µl of buffer AW2 and then centrifuging at full speed (20 200 xg) for 1 min. The flow was again discarded and the column was placed in a 1.5 ml tube. Finally, 100 µl of elution (AE) buffer was added to the column, which was left at room temperature for 1 min. The column was then centrifuged (11 543 xg, 1 min). The tube containing the elluted genomic DNA was labelled and stored at -20°C for further molecular analysis.

#### ***4.4. Tick specimens from the trapped mice***

The collected immature ixodid ticks from the trapped mice, following the procedure described in the [Chapter 3](#), were surveyed for the presence of *Babesia* species. The genomic DNA was extracted according to the procedure described above.

##### **1. PCR reactions**

For the purpose of PCR and reducing the risk of contamination through pipetting, a master mixture consisting of 0.75 U enzyme platinum<sup>®</sup> platinum Taq DNA polymerase, 0.5 U uracil deoxy-glycosade (UDG) ([Invitrogen, The Scientific Group, South Africa](#)) (12.5 µl), 0.1 µM of each RLB-F primer (5'-GAC ACA GGG AGG TAG TGA CAA G-3') (0.25 µl ) and RLB-R primer (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') ([Isogen, The Netherlands](#)) ([Gubbels, De Vos, Van Der Weide, Viseras, Schouls, De Vries & Jongejan, 1999; Matjila, Leisewitz, Jongejan & Penzhorn, 2008](#)), 3 mM MgCl<sub>2</sub>, 200 µM of dNTPs, and nuclease-free water (9.5 µl) to

a final volume of 22.5  $\mu$ l, was initially prepared per PCR reaction, to target the variable region of 18S rRNA gene of *Babesia*. Aliquots of 22.5  $\mu$ l were pipetted into PCR tubes where 2.5  $\mu$ l (~ 75 ng) of DNA template was then added to each tube to increase the reaction volume to 25  $\mu$ l. To validate the PCR, *Babesia*-positive blood samples from a cheetah and a lion, confirmed microscopically on blood smear, were used as positive controls and distilled water as negative control. One touch thermal cycler was used to amplify the 18S DNA of the *Babesia* parasite. The PCR condition was initiated at 94°C (10 min) followed by 2 cycles of 94°C (20 sec), 67°C (30 sec), 72°C (30 sec), 2 cycles of 94°C (20 sec), 65°C (30 sec), 72°C (30 sec), 2 cycles of 94°C (20 sec), 63°C (30 sec), 72°C (30 sec), 2 cycles of 94°C (20 sec), 61°C (30 sec), 72°C (30 sec), 2 cycles of 94°C (20 sec), 59°C (30sec), 72°C (30 sec), 40 cycles of 94°C (20 sec), 59°C (30sec), 72°C (30 sec) and subsequently an extension of 10 min at 65°C. The samples were eventually cooled down to 4°C.

## **2. Agarose gel electrophoresis**

A 2% agarose gel stained with 5  $\mu$ l of ethidium bromide was cast in TAE (Tris Acetate EDTA) buffer. A 100 base-pair DNA size marker ([Inqaba Biotechnical Industries Ltd, South Africa](#)) was used to identify a particular sequence of rRNA. A volume of 3  $\mu$ l of each PCR product was stained with 1  $\mu$ l of loading dye (which allowed monitoring the progress of the gel) ([Inqaba Biotechnical Industries Ltd, South Africa](#)) was loaded on the gel. The gel was run at 120 V for 30 min, to allow the DNA to move into the gel slowly and evenly. The gel was eventually analysed under the UV light for appropriate bands.

## **3. Reverse line blot (RLB) hybridisation assay**

### **7.1. Babesia species-specific probes**

A number of genus and species-specific oligonucleotide probes ([Table 2](#)) containing an N-terminal *N*-(trifluoroacetamido)hexylcyanoethyl, *N*, *N*-diisopropyl phosphoramidite [TFA]-C6 amino linker ([Applied biosystem, South Africa](#)) were covalently linked to a Biodyne C blotting membrane with different working concentrations (800 pmol) during the process of preparation of the RLB membrane.

**Table 2:** List of organisms and their corresponding probe sequences used to detect pathogen DNA in the RLB

Name of the organism	Sequence	Reference
<i>Babesia bigemina</i>	CGT TTT TTC CCT TTT GTT GG	Gubbels <i>et al.</i> , 1999
<i>Babesia canis canis</i>	TGC GTT GAC CGT TTG AC	Matjila <i>et al.</i> , 2004
<i>Babesia canis rossi</i>	CGG TTT GTT GCC TTT GTG	Matjila <i>et al.</i> , 2004
<i>Babesia felis</i>	TTA TGC TTT TTC CGA CTG GC	Bosman <i>et al.</i> , 2007
<i>Babesia</i> genus-specific I	ATT AGA GTG TTT CAA GCA GAC	Nijhof (unpublished)
<i>Babesia</i> genus-specific II	ACT AGA GTG TTT CAA ACA GGC	Nijhof (unpublished)
<i>Babesia gibsoni</i>	TAC TTG CCT TGT CTG GTT T	Matjila <i>et al.</i> , 2008
<i>Babesia lengau</i>	CTC CTG ATA GCA TTC	Bosman <i>et al.</i> , 2010
<i>Babesia leo</i>	ATC TTG TTG CCT TGC AGC T	Penzhorn <i>et al.</i> , 2001
<i>Babesia microti</i>	GRC TTG GCA TCW TCT GGA	Nijhof <i>et al.</i> , 2003
<i>Babesia vogeli</i>	AGC GTG TTC GAG TTT GCC	Matjila <i>et al.</i> , 2004
<i>Cytauxzoon felis</i>	CTG GTG ATT TTT CGG TAT	Nijhof (unpublished)
<i>Theileria</i> genus-specific	ATT AGA GTG CTC AAA GCA GGC	Gubbels <i>et al.</i> , 1999
<i>Theileria-Babesia</i> genus-specific	TAA TGG TTA ATA GGA RCR GTT G	Gubbels <i>et al.</i> , 1999

(Symbols indicate degenerate positions: R=A/G, W=A/T)

## 7.2. Preparation of the plasmid control

A novel plasmid control was used as an internal positive control to assure the proper attachment of *Babesia* species-specific oligonucleotides to the RLB membrane. The plasmids for *Theileria-Babesia* genus-specific were initially provided by [Isogen Life Science BV, the Netherlands](#). The genus and species-specific plasmids which was initially prepared from the full length of the suu 18S rRNA gene of *B. bicornis*, *B. bigemina*, *B. bovis*, *B. caballi*, *B. canis canis*, *B. canis rossi*, *B. divergens*, *B. felis*, *B. major*, *B. microti*, *B. vogeli* ([Matjila, Nijhof, Taoufik, Houwers, Teske, Penzhorn, De Lange & Jongejan, 2005](#)) was used to prepare the RLB plasmid control for this study. Prior to the RLB test, each extracted plasmid (~ 75 ng) was diluted 10 times and used as DNA templates. A 25 µl reaction volume composed of UDG (0.5 U), 20 pmol of T7 (0.5 µl) and SP6-biotin (0.5 µl) primers, 3 mM MgCl<sub>2</sub>, 200 µM of dNTPs, water (9 µl) and 2.5 µl of the DNA template was prepared. The amplification was performed in a thermal cycler as follows: initial denaturation at 94°C for 5 min, 35 cycles of 94°C for 30 sec, 50°C for 1 min, 72°C for 1 min and additional extension at

72°C for 7 min. The products were eventually cooled down to 4°C. A volume of 2 µl of PCR products of each above-mentioned *Babesia* species was added to a PCR tube as RLB plasmid control.

### **7.3. Preparation of the RLB membrane**

Biodyne C blotting membrane (Pall Biosupport, Ann Arbor, USA) has outstanding physical properties for multiple hybridisation and commercial applications, due to the high density of carboxyl groups on the surface (<http://www.pall.com>). To prepare a binding membrane, 8 µl of the selected *Babesia* species oligonucleotides were initially diluted in 142 µl of 0.5 M NaHCO<sub>3</sub> (pH 8.4) in PCR tubes in order to achieve a concentration of 800 pmol and promote the binding reactions of the probes to the membrane. The membrane was then activated by 10 min incubation in 10 ml freshly prepared 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) (Sigma-Aldrich, South Africa) at room temperature, followed by 2 min wash with demineralised water.

The membrane was then placed in a MN45 mini blotter (Immunetics, Cambridge, UK) and the slots were loaded with the already diluted probes, followed by 5 min incubation at room temperature to covalently link them to the membrane. The probe solution was removed by aspiration. The membrane was removed from the blotter and placed in a washing tray to inactivate it with 100 ml 100 mM freshly made NaOH for maximum 10 min, to denature the attached probes and to stop hybridization reaction of the probes. Subsequently, the membrane was washed in 100 ml 2XSSPE (360 mM NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM EDTA [pH 8.4]) containing 0.1% sodium dodecyl sulphate (SDS) at 60°C for 5 minutes, to dispose of the excess NaOH. The membrane was washed in 20 mM EDTA (pH 8), under gentle shaking, for 15 min at room temperature. Eventually, the membrane was stored in 20 mM EDTA, for prolonged storage at 4°C.

### **7.4. Hybridisation**

Hybridisation is defined as process in which a probe binds to a blot if the probe's DNA sequence and the DNA on the blot match, due to complementarities between the probes and target DNAs (Kong & Gilbert, 2007). For this purpose the membrane was initially incubated in 100 ml of 2XSSPE/0.1%SDS at room temperature for 5 minutes.

A volume of 20 µl of each PCR product was diluted in 130 µl of 2XSSPE/0.1%SDS in a PCR tube. The diluted PCR products were denatured at 96°C for 10 minutes followed by an immediately snap cooling on ice to avoid binding the two strands of DNA together.

The denatured PCR products were applied to the pre-loaded Biodyne C membrane placed in the mini blotter, containing *Babesia* and *Theileria* genus and species-specific probes. The products were hybridised by incubation at 42°C for 1 hour on a horizontal surface, after which the residual fluid was removed by aspiration. The membrane was removed from the mini blotter and washed twice in preheated 2XSSPE/0.5%SDS for 10 min at 50°C under gentle shaking to remove the PCR products that did not hybridise.

The membrane was then incubated for 30 minutes at 42°C in peroxidase-labeled streptavidin (Roche Diagnostic, South Africa), followed by a further two washing steps with 2XSSPE/0.5%SDS washing buffer at 42°C. The membrane was washed twice in 2XSSPE (360 mM NaCl, 20 mM NaH<sub>2</sub>PO<sub>4</sub>, 2 mM EDTA [pH 8.4]) for 5 min at room temperature. Usually, the detection of the probe-PCR-streptavidin complex is chemiluminescence-dependent. Therefore, the ECL detection reagents (AEC-Amersham, South Africa) were added to the membrane and then the membrane was exposed to an X-ray film (X-OMATTM Blue XB-1, Kodak, Separation Scientific, South Africa). The X-ray film was photographically developed using developer (X-Ray – Developer: African X-Ray Industrial and Medical (Pty) Ltd, South Africa) and fixer (X-Ray – Fixer: African X-Ray Industrial and Medical (Pty) Ltd, South Africa) to visualize any hybridisation complex. The black dots were considered as positive hybridisation. The results were eventually recorded.

After utilization, the membrane was stripped twice in 100 ml of pre-heated 1%SDS at 90°C for 30 min under gentle shaking, followed by a wash in 100 ml of 20mM EDTA for 15 min at room temperature. The membrane was eventually preserved in a plastic container, containing 20 mM EDTA [pH 8] at 4°C.

## Results

A total of 143 peripheral blood samples from the study localities in South Africa were examined (Table 1). Cubs (younger than 3 months) and pregnant cheetahs were excluded from the sample collection. Observation of random blood smears revealed the presence of merozoites in some red blood cells (Fig. 1). The initial amplification was carried out from genomic DNA, using the commercial forward and reverse RLB primers which successfully amplified a fragment of ~500 bp of DNA of the small subunit of the 18S rRNA gene spanning the V4 hypervariable region corresponding to that of *Babesia* species (Fig. 2). No DNA contamination was observed in the negative control. The amplifications performed with serial dilutions of DNA from a *Babesia* species-positive cheetah using the universal RLB primers showed the sensitivity of hybridization. The capability of PCR reaction in detecting the templates was  $10^2$  (Fig 3) whereas the absolute limit of detection for the combined PCR and subsequent RLB procedures using 18S rRNA gene as templates was  $10^4$  (Fig 4).

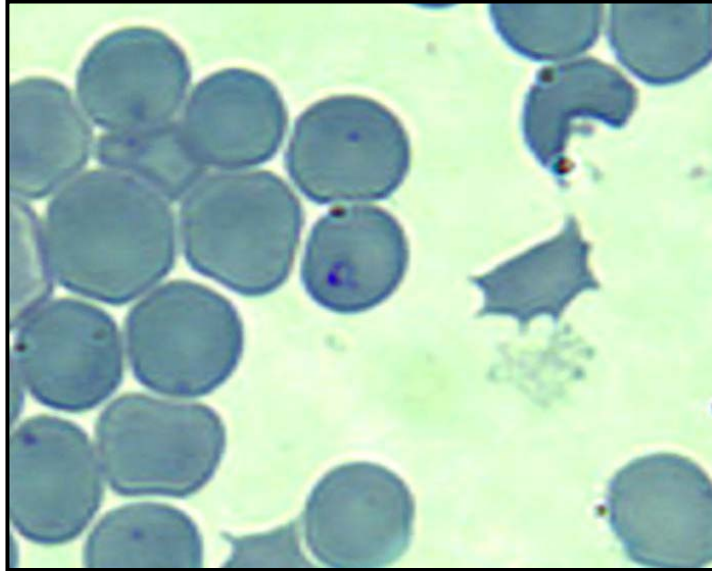
The various *Babesia* probes in the RLB assay successfully differentiated between *Babesia* species infecting the cheetahs. Screening of the PCR products with the RLB showed that 48 (59.2%), 12 (54.5%), 5 (15.6%) and 8 (100%) of the cheetahs were infected with three different *Babesia* species at De Wildt (Brits), De Wildt (Shingwedzi), the Hoedspruit Endangered Species Centre and the Cheetah Outreach, respectively, with De Wildt (Brits) having the highest rate of infection (Table 3). Generally, 2.4%, 3.7% and 53.1% of the *Babesia*-positive cheetahs were infected with *Babesia canis rossi*, *Babesia felis* and *Babesia lengau* (the newly indentified *Babesia* species in cheetahs), correspondingly (Fig. 6). No mixed infection was observed on the RLB X-ray film.

A total number of 10,432 ixodid ticks of six species, namely *Amblyomma hebraeum*, *Amblyomma marmoreum*, *Haemaphysalis elliptica*, *Rhipicephalus (Boophilus) decoloratus*, *Rhipicephalus simus* and *Rhipicephalus zambeziensis*, were examined for the presence of *Babesia* species. Numbers and instars of the various tick species collected at each study site are shown in Tables 4 and 5. The RLB-F and RLB-R primers successfully amplified the V4 hypervariable region of the small subunit of the 18S rRNA gene of *Babesia* parasites (Fig. 5), and unlike for other tick species, ~500 bp bands were only visible for the *Babesia*-positive tick samples of *H. elliptica*. All

the nucleotide probes used for hybridisation assays gave positive results with their corresponding genotypes and did not show any cross-reaction with other non-cheetah *Theileria* or *Babesia* species tested: *B. canis*, *B. rossi*, *B. felis* and *B. leo* (Fig. 7).

The majority of unfed-tick samples of various species collected at the study sites were uninfected since there was no hybridisation reaction with any *Babesia* probes pre-hybridised on the C Biodyne membrane. Negative control (distilled water) showed no DNA contamination since no signals were observed. The genus-specific oligonucleotide probe described elsewhere to specifically detect the 18S rRNA genes of the genera *Theileria* and *Babesia* was found to hybridise in all the cases where one or more species/genotype was present. The PCR/RLB hybridisation assay detected the presence of *B. lengau* in the immature (larvae and nymphs) and adult ticks of *H. elliptica*: one pool of larvae, four pools of nymphs, and four adults were positive. No mixed infection was detected (Fig. 7).

A total of 17 trapped mice, identified as *Aethomys* sp. (n=8), *Graphiurus murinus* (n=1), *Mastomys* sp. (n=8), trapped at De Wildt (Brits) as well as 5 trapped mice, identified as *Mastomys* sp. (n=1) and *Saccostomus caepstris* (n=4) at the Hoedspruit Endangered Species Centre were screened for the presence of cheetah-associated *Babesia* species. Amplification of the V4 hypervariable region of the parasite 18S rRNA gene was assessed by submitting the PCR products to the gel electrophoresis as ~500 bp bands were visible for 5 mice including *Aethomys* sp. (n=4) and *Mastomys* sp. (n=1) at De Wildt (Brits) and 2 including *Mastomys* sp. (n=1) and *Saccostomus caepstris* (n=1) at the Hoedspruit Endangered Species Centre (Fig 8). All seven specimens produced ~500 bp bands on the gel agarose electrophoresis at the end of the PCR process, indicating the presence of *Babesia* species. The RLB hybridisation assay showed the hybridisation with genus (*Theileria* and *Babesia* genus-specific) and species-specific (*B. lengau*) oligonucleotides (Fig 9). The number and species of mice which were infected with *Babesia* species are shown in Table 6. The genomic DNA of the tick specimens collected from the mice testing positive for *Babesia* was tested for the presence of cheetah-associated *Babesia* species (Table 7).



**Fig. 1:** A pleomorphic trophozoite (arrow) in an erythrocyte. Giemsa-stained blood smear from a cheetah (The Ann van Dyk-DeWildt Cheetah Breeding Centre)

**Table 3:** The RLB results indicating the prevalence of *Babesia* infection in captive cheetahs

Number of cheetahs	<i>Babesia</i> species		
	<i>B. canis rossi</i>	<i>B. felis</i>	<i>B. lengau</i>
<b>The De Wildt (Brits)</b>			
81	2 (2.4%)	3 (3.7%)	43 (53.1%)
<b>The De Wildt (Shingwedzi)</b>			
22	0	0	12 (54.5%)
<b>The Hoedspruit Endangered Species Centre</b>			
32	0	0	5 (15.6%)
<b>The Cheetah Outreach</b>			
8	5 (62.5%)	0	3 (37.5%)

**Table 4:** Number of unfed ixodid ticks examined

Tick species	LL	NN	Ad	Total
<i>Amblyomma hebraeum</i>	1200	80	15	1295
<i>Amblyomma marmoreum</i>	37	0	0	37
<i>Haemaphysalis elliptica</i>	6600	840	150	7590
<i>Rhipicephalus (B.) decoloratus</i>	1002	0	0	1002
<i>Rhipicephalus simus</i>	320	0	0	320
<i>Rhipicephalus zambeziensis</i>	150	40	0	190
<b>Total number of ticks examined</b>				<b>10432</b>

Ad = Adult, LL = Larva, NN = Nymph

**Table 5:** Number and species of unfed ixodid ticks examined at each study site

Number and species of ticks		Localities		
		The De Wildt Centre		The Hoedspruit Centre
		Brits	Shingwedzi	
<i>Amblyomma hebraeum</i>	LL	1107	0	93
	NN	52	0	28
	Ad	0	0	15
<i>Amblyomma marmoreum</i>	LL	0	37	0
<i>Haemaphysalis elliptica</i>	LL	4330	228	2042
	NN	571	34	235
	Ad	114	15	21
<i>Rhipicephalus (B.) decoloratus</i>	LL	0	0	1000
<i>Rhipicephalus simus</i>	LL	320	0	0
<i>Rhipicephalus zambeziensis</i>	LL	0	54	96
	NN	0	0	40
<b>Total</b>		<b>6494</b>	<b>368</b>	<b>3570</b>

Ad = Adult, LL = Larva, NN = Nymph

**Table 6:** Number and species of mice infected with *Babesia* species at the cheetah breeding centres

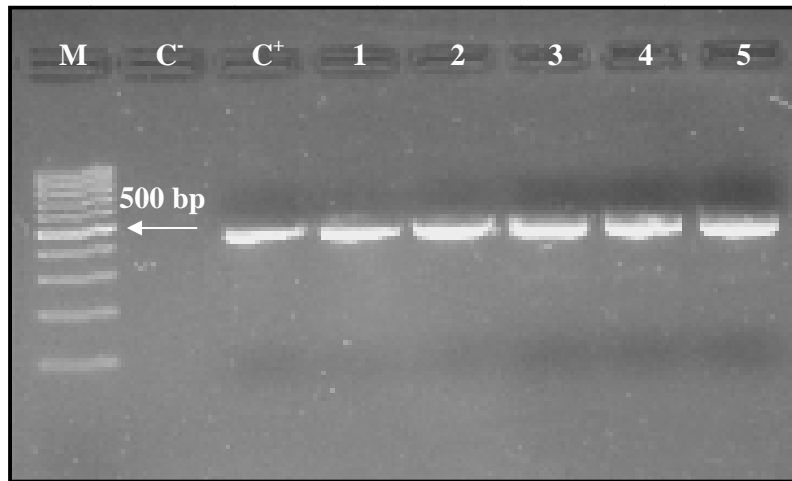
Mouse species	<i>Babesia</i> species		
	<i>B. felis</i>	<i>B. lengau</i>	<i>B. rossi</i>
<i>Aethomys</i> sp. (n=8)	0	4 (50%)	0
<i>Graphiurus murinus</i> (n=1)	0	0	0
<i>Mastomys</i> sp. (n=9)	0	2 (22.2%)	0
<i>Saccostomus capestris</i> (n=4)	0	1(25%)	0

n = number of mice tested for babesial infection

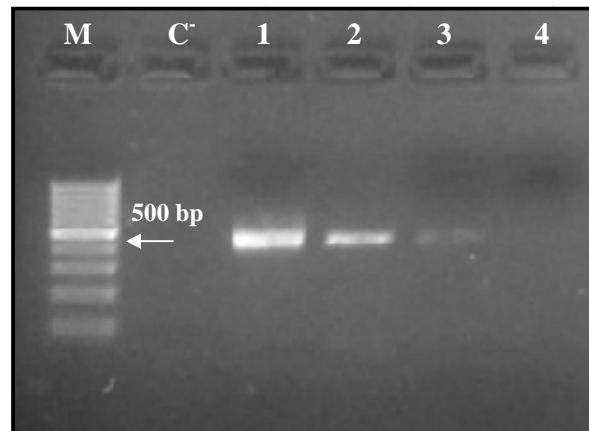
**Table 7:** Number and species of ticks, which were collected from trapped mice, tested for *Babesia* species

Mouse species	Tick species							
	<i>H. elliptica</i>		<i>R. (B.) decoloratus</i>		<i>R. simus</i>		<i>R. zambeziensis</i>	
	LL	NN	LL	NN	LL	NN	LL	NN
<i>Aethomys</i> sp.	45	13	0	0	0	0	0	0
<i>Aethomys</i> sp.	62	3	0	0	0	0	0	0
<i>Aethomys</i> sp.	12	2	0	0	7	0	0	0
<i>Aethomys</i> sp.	25	0	0	0	2	0	0	0
<i>Mastomys</i> sp.	1	0	2	0	0	2	2	0
<i>Mastomys</i> sp.	14	1	0	0	0	0	0	0
<i>Saccostomus capestris</i>	35	1	0	0	0	0	0	0

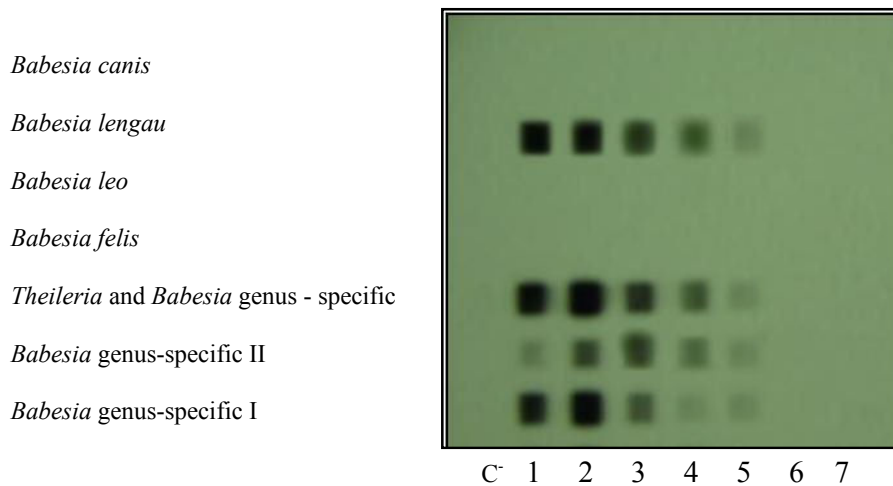
n = number of mice tested for babesial infection



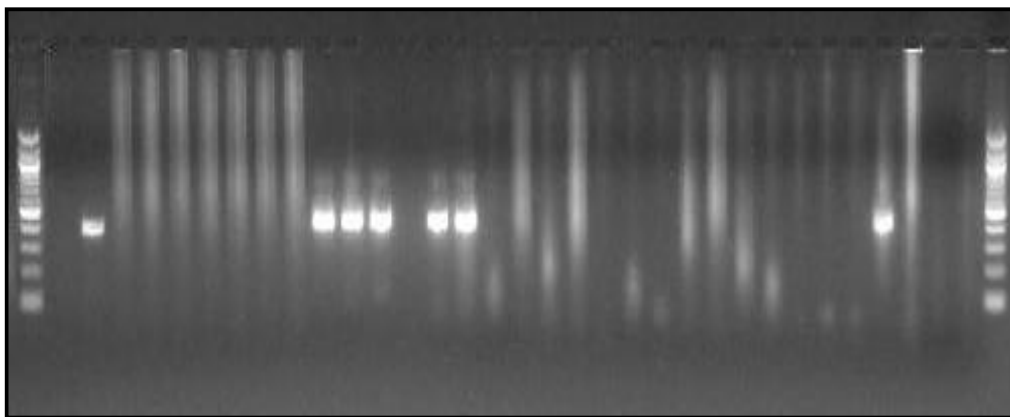
**Fig. 2:** PCR assay performed on representative control and study blood samples. Lane M: 100 bp DNA ladder (Fermantas). Lane C<sup>-</sup>: negative control (H<sub>2</sub>O). Lane C<sup>+</sup>: positive-control sample (microscopically visualised *Babesia* species on the blood smear) from cheetah. Lane 1 – 5: study blood samples collected from cheetahs at the study sites



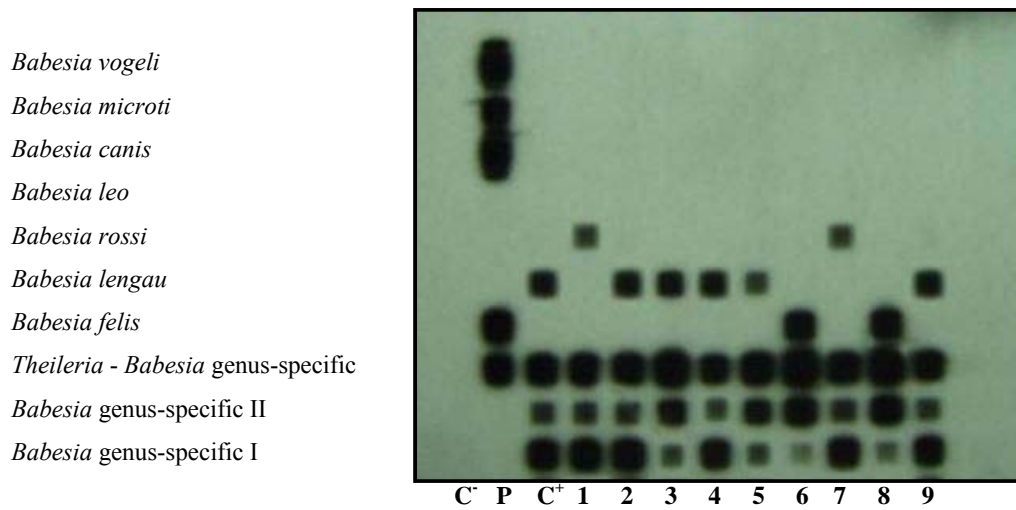
**Fig. 3:** Agarose gel showing the effect of reducing the amount of template DNA by serial dilutions in standard PCR amplifications performed with the universal RLB primers. Lane M: molecular marker. Lane C<sup>-</sup>: negative control (H<sub>2</sub>O). Lanes 1 – 4: 1, 10, 10<sup>2</sup>, 10<sup>3</sup> serial dilutions



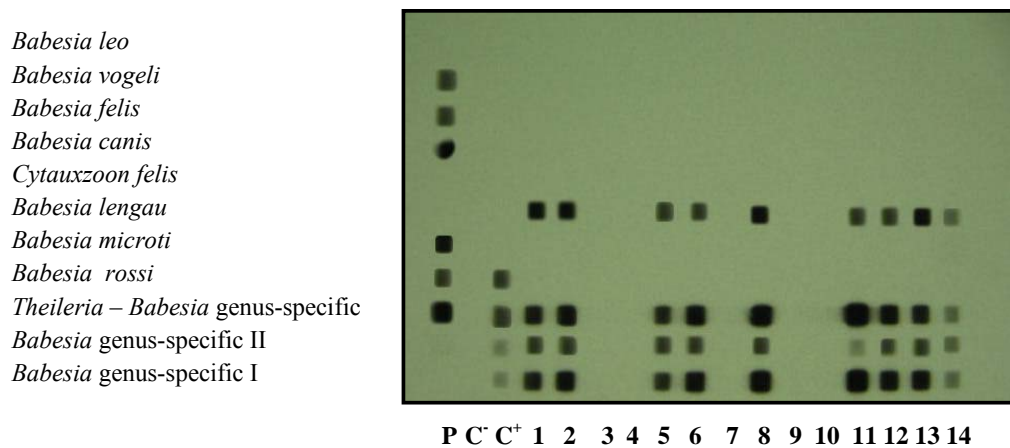
**Fig. 4:** RLB hybridisation assay confirming the detection level of the amplified DNA after serial dilutions on the membrane. Lane C<sup>-</sup>: negative control (H<sub>2</sub>O). Lane C<sup>+</sup>: positive control sample. Lanes 1 – 7 represent the serial dilutions of the DNA template (1, 0.1, 0.01, 0.001, 0.0001, 0.00001, 0.000001)



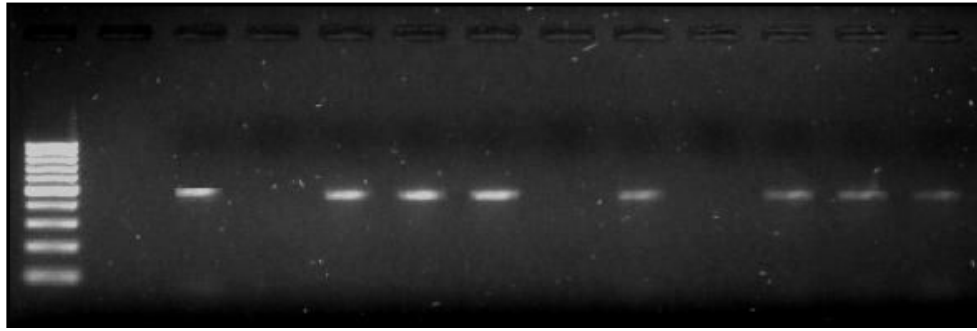
**Fig. 5:** Standard PCR analysis showing the presence of *Babesia* species in ticks. Lane M: 100 bp DNA Ladder; Lane C<sup>-</sup>: water (negative control); Lane C<sup>+</sup>: piroplasm DNA obtained from infected cheetah blood (positive control); Lanes L, N and A: individual infected tick samples (*Haemaphysalis elliptica*). Lanes with smears represent individual non-infected tick samples



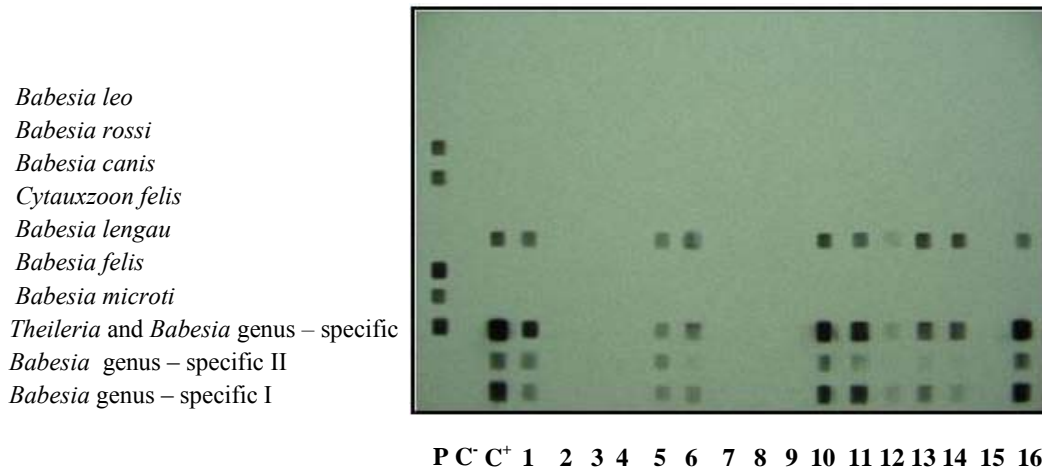
**Fig. 6:** RLB results showing species-specific oligonucleotides of the 18S rRNA gene in the horizontal lanes and PCR products in the vertical lanes. A *Babesia*-positive (*Babesia*-positive blood sample from a cheetah) control (C<sup>+</sup>), RLB plasmid control (P) and water as a negative control (C<sup>-</sup>) were included. Lanes 1-9 represent the blood samples from cheetahs



**Fig. 7:** RLB hybridisation assay demonstrating the positive hybridisation of DNA with the *Babesia* genus and species-specific probes. Lanes represent RLB plasmid control (P), negative control (C<sup>-</sup>), positive control (C<sup>+</sup>) (blood sample from a domestic cat, diagnosed positive for *Babesia* via RLB hybridisation assay), larvae (lane 1), nymphs (lanes 2-8) and adults (lanes 9-14) of *Haemaphysalis elliptica*, respectively



**Fig. 8:** Standard gel electrophoresis showing the presence of *Babesia* DNA in blood samples from mice and their associated ticks. Amplification was performed using the universal RLB primers specific for *Babesia* and *Theileria* species. Lane M: 100 bp molecular marker; Lane C<sup>-</sup>: negative control (water); Lane C<sup>+</sup>: positive control; Lanes 1-7 and 8-10 represent the mice's blood samples and their associated ticks, respectively



**Fig. 9:** RLB hybridisation assay demonstrating the positive hybridisation of DNA samples with *Babesia* probes in mice and their associated ticks. Lanes P, C<sup>-</sup> and C<sup>+</sup>, 1-11 and 12-16 represent RLB plasmid control, negative control, positive control (blood sample from a *Babesia*-positive cheetah), the mouse blood samples and tick specimens collected from the mice, respectively

## Discussion

Molecular biology is an invaluable tool in studying *Babesia* species in wild animals. Due to the sensitivity of PCR, there is always the concern of false positives due to contaminating DNA. In all instances, the putative negative controls were negative, indicating that positive PCR reactions did not result from contaminating DNA. The combination of PCR and RLB permitted us to identify the *Babesia* species infecting the cheetahs at those localities, using oligonucleotide probes whose specificity has been previously determined (Gubbels *et al.*, 1999; Matjila *et al.*, 2005).

It was initially uncertain if the cheetahs were infected with any *Babesia* species at the breeding centres. Thus for the purpose of this study, *Babesia lengau* in cheetahs was chosen as the target species. However, microscopic examination of randomly selected blood smears showed to be positive which was then confirmed by molecular techniques (Fig. 2). Based on the number of positive samples, the failure in the past to detect and identify *Babesia* species in felids was most probably due to low parasitaemia, difficulties in morphological differentiation between the species and not the absence of the species. In contrast, the ease and certainty by which PCR differentiated between *Babesia* species underscores the comparative usefulness of molecular tools for species identification.

All *Babesia*-positive samples showed hybridisation reactions with the genus-specific as well as the species-specific probes. Four probes, namely *B. felis*, *B. rossi*, *B. lengau* and *B. leo*, were only hybridised on the RLB membrane to detect the haemoparasite *Babesia* in the cheetahs. Very few data are available for comparison with those presented here. *Babesia*-like parasites were observed in cheetah blood smears (Averbeck, Bjork, Packer & Herbst, 1990). In a later study, a number genus-specific hybridisation signals on RLB hybridisation assay indicated a taxonomically different *Babesia* parasite with the 18S rRNA gene dissimilar to other species (Bosman *et al.*, 2007). This was later named, *B. lengau*, (Bosman *et al.*, 2010).

This study showed that a number of DNA samples unexpectedly reacted with *B. canis rossi* and *B. felis* probes indicating that *Babesia* species may have broadened the range of their host specificities. *B. felis* is usually associated with domestic cats along the mesic eastern and southern coastal regions and eastern escarpment of South Africa, with the vector still unknown

(Jacobson, Schoeman & Lobetti, 2000; Penzhorn, Stylianides, Coetzee, Viljoen & Lewis, 1999), whereas *B. rossi* which occurs only in sub-Saharan Africa (Lewis, Penzhorn, Lopez-Rebollar & De Waal, 1996) was originally detected in a side-striped jackal (*Canis adustus*) in East Africa (Nuttall, 1910), and is the main causative agent of canine babesiosis in South Africa (Collett, 2000), with *H. elliptica* (previously lumped with *Haemaphysalis leachi*; Apanaskevich, Horak & Camicas, 2007) being the potential vector. Close contact with other animal species and the tick vector/s in small enclosures could possibly contribute to mixed infections becoming established. A previous molecular study showed that 6% of the wild dogs at Ann Van Dyk Cheetah Breeding Centre-De Wildt/Brits are infected with *B. canis rossi* (Matjila *et al.*, 2008), which could have contributed to our detection of *B. rossi* in cheetahs. It was earlier observed that cheetahs could be infected with other *Babesia* species as studies showed infections with *B. felis* and *B. leo* which are of domestic cats and lions (*Panthera leo*), respectively (Penzhorn, 2006; Bosman *et al.*, 2007).

The prevalence of *Babesia* species in captive cheetahs is higher than in free-ranging ones (Bosman *et al.*, 2007), presumably due to a higher probability of close contact with infected ticks. The observation that so many *Babesia* species infect so many vertebrates without any apparent disease manifestations begs the question of whether there might be some selective advantage conferred on the carrier. Although the cheetahs at the centres showed no associated clinical manifestations of babesiosis, the results showed that they were sub-clinically infected with *Babesia* species. Relatively little is known about the chronic carrier state in cheetahs. With the advent of PCR, however, surveys on blood samples have shown that the chronic carrier state can last for months to years (Krause, Spielman, Telford, Sikand, McKay, Christianson, Pollack, Brassard, Magera, Ryan & Persing, 1998). PCR in conjunction with the RLB assay described here is a useful tool for the detection and identification of *Babesia* species in clinically healthy animals and should be considered for the diagnosis and control of feline babesiosis.

All the ticks collected at the study sites were three-host species (Walker, 1991). The presence of *B. lengau* DNA in unfed *H. elliptica* larvae indicates transovarial transmission, while its presence in unfed nymphs and adults may indicate transstadial transmission. De Waal and Potgieter (1987) showed that *Rhipicephalus evertsi evertsi* can transmit *Babesia caballi* transstadially, whereas *Hyalomma truncatum* transmits the same parasite transovarially (De Waal, 1990). On

the other hand, the role of the tick sex in parasite transmission was previously studied as such no significant association between the rate of *Theileria* transmission and tick sex was found (Sangwan, Chhabra & Samantaray, 1989). However in our study, the sex of the ticks was not taken into consideration. The possession of a hard chitinous exoskeleton by ticks often makes DNA extraction problematic (Mauel, Carlton & Mather, 1999; Halos, Jamal, Vial, Maillard, Suau, Le Menach, Boulouis, Vayssier-Taussat, 2004) since it has to be disrupted before the extraction procedure takes place. For unknown reasons, however, the extracted genomic DNA from ticks is unstable and is frequently subjected to rapid degradation (Hubbard, Cann & Wright, 1995; Hill & Gutierrez, 2003). In addition, possible presence of enzymatic inhibitors has been associated with both unfed and engorged ticks (Hubbard *et al.*, 1995; Schwartz *et al.*, 1997). These factors can have a major impact in reducing the efficacy of PCR amplification, resulting in a negative impact on the rate of micro-parasite infection in ticks.

Developing an understanding of the basis for the pathogen development and the prevalence of tick-borne pathogens in potential vector ticks of the region is crucial for defining the epidemiology of tick-borne diseases and the interaction between the tick and the pathogen (de la Fuente *et al.*, 2008; Kocan, *et al.*, 2008). Recent advances in these areas have shown the complexity and complications of these interactions. In a survey done by Kirvar, Ilhan, Katzer, Wilkie, Hooshmand-Rad and Brown (1998), the advantage of PCR technique in terms of sensitivity and specificity over the conventional methods of detecting the definitive and intermediate hosts infected with *Babesia* species was demonstrated. This PCR assay followed by RLB hybridisation assay detected *Babesia* species parasites, despite of very low level, in ethanol-fixed ticks. Primers used in the present study were previously designed and determined to be specific for amplification of *Theileria* and *Babesia* species (Gubbels *et al.*, 1999). We showed that approximately 500 bp fragments of 18S rRNA genes of *Babesia* species can be amplified from blood samples obtained from carrier cheetahs in captivity. No inhibition was observed when PCR was performed on infected and non-infected tick species.

Although *Babesia* species are generally transmitted by ixodid ticks, the mode of transmission of feline-associated *Babesia* species is still unknown (Jacobson *et al.*, 2000; Penzhorn *et al.*, 1999). However, it is argued that animals with subclinical form of babesiosis may be sources for ticks since they carry the piroplasms (Neitz, 1956; De Waal & Potgieter, 1987; Homer *et al.*, 2000;

Hunfeld & Hildebrandt & Gray, 2008), as a result detection and discrimination of these parasites in their natural and definitive host are consequently crucial for understanding the epidemiology of the disease. There is a paucity of information on the epidemiology of babesiosis in wild and domestic felids in South Africa. Therefore, our study which was based on determination of the pattern of *Babesia* challenge in the field through tick studies can be a vital approach to cheetah-associated *Babesia* epidemiology in South Africa.

The homology of the 18S rRNA gene, which is relatively conserved among *Babesia* species (Gubbels *et al.*, 1999), to the known cheetah *Babesia* species was a significant finding in rodents. Since adult ticks do not feed on rodents and we managed to detect babesial infections among unfed immature stages of *H. elliptica*. *Babesia* circulation might be dependent on these developmental stages feeding on infected rodents and in turn on the infection of new rodents by feeding infected immature ticks. The conclusion that rodents in our study sites do not carry the zoonotic strain of *B. microti* is strengthened by the total absence of hybridisation reactions with the *B. microti* species-specific probe. We cannot totally rule out the possible absence of the zoonotic strain in the regions.

In conclusion, the results presented in this study have demonstrated the biological survival of cheetah-associated *Babesia* species by its presence in the trapped rodents as well as in *H. elliptica* ticks collected from the vegetation and the rodents at cheetah breeding centres and the risk of cheetahs to contract babesiosis. Finding *B. lengau* in unfed instars of this tick species suggests that it may be a vector, although more study is required. Our results show the competence of *H. elliptica* tick as a possible vector and reservoir of *B. lengau* in the centre. Therefore, *H. elliptica* may play an important role in the field as a natural vector of *Babesia* species.

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## Chapter 5:

### **Phylogeny of *Babesia* species detected in captive cheetahs and *Haemaphysalis elliptica* (Acari: Ixodidae) in South Africa**

#### **Abstract**

Three *Babesia* species, namely *Babesia canis rossi*, *Babesia felis* and *Babesia lengau* were detected in blood samples collected from cheetahs at the various cheetah breeding centers in South Africa. Unfed developmental stages of *Haemaphysalis elliptica* tick (Acari: Ixodidae) amongst other tick species infesting the vegetation at the centers were examined for *Babesia* species. The phylogenetic relationships of the detected *Babesia* species with other *Theileria* and *Babesia* species infecting other hosts, was determined based on 18S rRNA gene sequence analysis. The full-length of 18S rRNA gene of the parasite in cheetahs and *Babesia*-positive ticks, previously detected on Reverse Line Blot (RLB) hybridization assay, were amplified, cloned and sequenced. Sequences were aligned with published sequences of related species and phylogenetic trees were constructed. The BLASTn algorithm was used to compare the obtained sequences with sequences deposited in GenBank. The analyses indicated similarity of the sequences with *Babesia canis rossi* (100%), *Babesia felis* (100%) and *Babesia lengau* (99%), respectively. This study aimed at the characterization of the *Babesia* species in captive cheetahs, and their associated ticks, from the Cheetah Breeding Centers in South Africa.

## Introduction

Piroplasms are tick-borne parasitic protozoa of the genera *Theileria* and *Babesia* (Levine, 1971). Some *Babesia* species are infective for the cat family (Schoeman, Lobetti, Jacobson & Penzhorn, 2001; Bosman, Venter & Penzhorn, 2007; Bosman, Oosthuizen, Peirce, Venter & Penzhorn, 2010). The majority of studies and case series documenting both natural and experimental infection originate from South Africa. In South Africa, the strip along the coast is identified as endemic area for *Babesia felis* (Jacobson, Schoeman, & Lobetti, 2000; Taboada & Lobetti, 2005), probably due to the distribution of the potential vector. The African continent with its suitable environmental and climatic conditions remains one of the few relatively uncharted regions of the world and hence it hosts a wide range of tick-borne protozoas with genetic variations in domestic and wild animals (Jongejan & Uilenberg, 2005).

Tick-borne conditions may share the same clinical presentation, pathogenicity, therapeutic response and vector in a region (Goddard, 2008), however, co-infection may account for the diverse clinical signs some patients exhibit (Kordick, Breitschwerdt, Hegarty, Southwick, Colitz, Hancock, Bradley, Rumbough, Mcpherson, MacCormack, 1999). A definite laboratory diagnosis of the haemotropic organism *Babesia* is traditionally based on microscopic examination of the peripheral or central blood smears (Purnell, 1981; Ristic, 1981). The advent of molecular biology field has provided the opportunity to perform genetic analysis of the parasite DNA to differentiate haemoparasite genus and/or species and to identify genetically distinct populations (Figueroa & Buening, 1995).

More recently, an 18S rRNA gene (rDNA)-based PCR method was developed to provide greater sensitivity and specificity in terms of detection and differentiation of *Babesia* species (Brikenheuer, Levy & Breitschwerdt, 2003). The ssu18S RNA genes have successfully been applied to identify and classify several previously unknown *Theileria* and *Babesia* species (Persing, Kobayashi, Juranek & Conrad, 1993, Persing, Herwaldt, Glaser, Lane, Thomford, Mathiesen, Krause, Phillip & Conrad, 1995; Quick, Herwaldt, Thomford, Garnett, Eberhard, Wilson, Spach, Dickerson, Telford, Steingart, Pollock, Persing, Kobayashi, Juranek & Conrad, 1993; Thomford, Conrad, Telford, Mathiesen, Bowman, Spielmann, Eberhard, Herwaldt, Quick & Persing, 1994; Herwaldt, Kjemtrup, Conrad, Barnes, Wilson, McCarthy, Sayers & Eberhard,

1997; Katzer, Mckellar, Kirvar & Shiels, 1998; Gubbels, Yin, Bai, Liu, Nijman & Jongejan, 2002).

The comparison of small well-chosen gene sequences has therefore become a particularly powerful tool for establishing evolutionary relationships (phylogeny) between prokaryotes. The arrival of molecular diagnosis methods has led to the discovery of some new piroplasmids. The phylogenetic classification of cat-infecting piroplasms by the analysis and comparison of 18S rRNA genes has been shown to correspond with traditional taxonomy and provides additional refined information on their evolutionary relationship (Penzhorn, Kjemtrup, López-Rebollar & Conrad, 2001).

Previously *Babesia* species, namely *B. felis*, *B. leo* were detected in cheetahs in Africa (Bosman *et al.*, 2007) and recently *B. lengau* have been found to infect cheetahs (Bosman *et al.*, 2007; Bosman *et al.*, 2010) with unknown etiology and distribution. *Babesia canis* is divided into three subspecies, *B. canis canis*, *B. canis vogeli*, and *B. canis rossi* depending on vector specificity, pathogenicity and antigenic properties (Uilenberg, Franssen & Spanjer, 1989; Hauschild, Shayan & Schein, 1995). *Babesia canis rossi* cause a major clinical problem (canine babesiosis) in domestic dogs in South Africa (Collett, 2000). By using these molecular methods, the diagnosis of *Babesia* infection is easily performed at the subspecies level. In South Africa, *B. rossi* infection was found in African wild dogs (Matjila *et al.*, 2008). Being a vector-specific parasite transmitted by *Haemaphysalis elliptica* (previously lumped with *Haemaphysalis leachi*; Apanaskevich, Horak & Camicas, 2007), *B. canis rossi* has a sub-Saharan distribution in Africa (Lewis *et al.*, 1996). The geographical distribution of the causative agent and thus the occurrence of babesiosis are largely dependent on the habitat of relevant vector tick species. The mode of transmission of felid babesias with its coastal distribution is unknown in South Africa (Jacobson *et al.*, 2000; Penzhorn *et al.*, 2004).

The objective of the study was to characterize the *Babesia* species found in captive cheetahs, and their associated ticks, from the Ann van Dyk Cheetah Breeding Centre-De Wildt/Brits, the Ann van Dyk Cheetah Breeding Centre-De Wildt/Shingwedzi, the Hoedspruit Endangered Species Centre and the Cheetah Outreach by using 18S rRNA gene sequence analysis.

## Materials and methods

### 1. DNA samples

The DNA extracted from cheetahs blood samples and *H. elliptica* ticks collected (Table 1) which previously showed a positive hybridization reaction with *B. canis rossi*, *B. felis* and/or *B. lengau* RLB probes (Chapter 4), were chosen for sequencing and phylogenetic analysis.

Table 1: Origin of samples received for this study

Samples	Location	Blood/Tick	RLB results	Phylogenetic position
F4	De Wildt/Brits	Blood	T/B genus-specific, <i>B. felis</i>	<i>Babesia felis</i>
G2	De Wildt/Brits	Blood	T/B genus-specific, <i>B. felis</i>	<i>Babesia felis</i>
H7	Cheetah outreach	Blood	T/B genus-specific, <i>Babesia rossi</i>	<i>Babesia canis rossi</i>
J5	Cheetah outreach	Blood	T/B genus-specific, <i>Babesia rossi</i>	<i>Babesia canis rossi</i>
C6	Cheetah outreach	Blood	T/B genus-specific, <i>Babesia rossi</i>	<i>Babesia canis rossi</i>
B12	De wildt/Brits	Blood	T/B genus-specific, <i>Babesia rossi</i>	<i>Babesia canis rossi</i>
M13	De wildt/Brits	Blood	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
103F	De wildt/Brits	Blood	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
98F	De wildt/Brits	Blood	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
32F	De wildt/shingwedzi	Blood	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
23M	Hoedspruit	Blood	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
34F	Cheetah outreach	Blood	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
L1	De Wildt/Brits	Tick (Larvae)	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
N1	De Wildt/Brits	Tick (Nymph)	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
Ad1	De Wildt/Brits	Tick (Adult)	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
Ad2	De Wildt/Brits	Tick (Adult)	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
Ad3	De Wildt/Brits	Tick (Adult)	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>
Ad4	De Wildt/Brits	Tick (Adult)	T/B genus-specific, <i>Babesia lengau</i>	<i>Babesia lengau</i>

## 2. PCR reaction and PCR product purification

For this purpose, 20 pmol of the two universal primers P-F: (5'-AAC CTG GTT GAT CCT GCC AGT AGT CAT-3'), P-R: (5'-GAT CCT TCT GCA GGT TCA CCT AC-3') (Liu, Zhao, Zhou, Liu, Yao, Fu, 2005) were used to amplify the full-length of 18S rRNA complete gene sequence of *Babesia* species. A master mixture containing Takara Ex Taq (1.5 U) (Takara Bio Inc. Japan), dNTPs (0.4 mM), 10X buffer (2.5 µl), H<sub>2</sub>O (16.7 µl) was prepared per PCR reaction. DNA template (~ 67 ng) was added to each aliquot of the master mixture. The PCR reaction volumes were 25 µl, protocol as following: initial denaturation 3 min at 96°C, 32 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, extension at 72°C for 2 min (Criado-Fornelio, Martines-Marcos, Buling-Sarana & Barba-Carretero, 2003), subsequently an extension of 10 min at 72°C and 5 min at 15°C. The PCR products were eventually cooled down to 4°C. Blood samples from cheetahs and lions, in which the *Babesia* parasites were microscopically diagnosed, were used as positive controls.

Subsequently, 3 µl of the PCR products was stained with loading dye and submitted to the 1.5% agarose gel electrophoresis, run for 30 min at 120V, after which an specific band size were surveyed. Assuring the achievement of correct size of the DNA fragments (~1800 bp), four more 25 µl reactions with the same condition were set up and eventually all five reactions were pooled together and analyzed on the agarose gel under UV light. The PCR products were purified with aid of a commercial QIAquick® PCR purification kit (Qiagen, Hilden, Germany), following the below protocol:

To bind the DNA, 500 µl of buffer PBI was added to the PCR reaction and mixed. The entire contents of the tube was transferred to a QIAquick column placed in 2 ml collecting tube, centrifuged for 30-60 s. The flow-through was discarded and a column was placed back into the same tube. Washing buffer PE (750 µl) was then added to the QIAquick column and centrifuged for 30 – 60 s. The flow-through was discarded and a column was replaced. The PCR product was eluted by adding 50 µl of the eluting buffer EB (10 mM Tris.Cl, pH 8.5) to the QIAquick membrane, and then the column was centrifuged for 1 min. The purified DNA was analysed on the gel and the concentration was assessed using the NanoDrop 2000 Fluorospectrometer (Thermo Scientific, USA).

### 3. Cloning and plasmid extraction

The purified PCR products were cloned using the commercial pGEM-<sup>®</sup>T and pGEM-<sup>®</sup>T easy Vector System kit (Promega, USA) and transformed into *E. coli* and JM109 cells. For DNA ligation, a master mixture containing 2X Rapid Ligation Buffer, T4 DNA Ligase (5 µl), T4 DNA Ligase (3 U), pGEM-<sup>®</sup>T and pGEM-<sup>®</sup>T easy Vector (50 ng) and purified PCR products (~ 52 ng) with total reaction volume of 10 µl was prepared per reaction, followed by an incubation of 1-3 hrs at room temperature. A volume of 2 µl of the master mixture was used for transformation following the manufacturer's protocol:

A volume of 2 µl of the ligation reaction (the other 8 µl was stored if the procedure was not successful, we would be repeat) was added to a chilled 1.5 ml tube. Then, 30 µl of the JM109 high efficacy component cells was added to the culture tube and mixed very gently, followed by 20 min incubation on the ice. The cells underwent a heat-shock for 45-50 s in a water bath at 42°C, followed by immediate ice incubation for 2 min.

Then, 900 ml of room temperature SOC medium (Super Optimal Broth with Catabolite repression) (Invitrogen, South Africa) was added to the ligation reaction transformation. The tube was incubated by shaking for 1.5 hours at 37°C. For the purpose of plating, 100 µl of the transformation mixture was poured onto duplicate Lactose Broth/ampicillin/IPTG/X-Gal (Invitrogen, South Africa) plates. The plates were then incubated at 37°C overnight. White colonies which contain inserts, were selected with a pipet tip which was then dropped into the bottles containing immedia<sup>®</sup> Amp Liquid. The culture media were incubated at 37°C for 16 hrs and ones which were cloudy were regarded as positive, indicating bacterial growth.

Plasmids was subsequently extracted using the High Pure Plasmid Isolation Kit (Roche Applied Science, Germany) in steps followed as; 4 ml of the culture media was pelleted via centrifuging at 6000 x g for 30 s. The supernatant was discarded. The pellet was resuspended in 250 µl of the suspension buffer/RNase. Lysis buffer (250 µl) was then added and mixed gently. The tube was incubated at room temperature for 5 min followed by centrifuging at maximum speed for 10 min. The supernatant was transferred to a High Pure Filter tube, which was then centrifuged at maximum speed for 60 s.

Then, 500 µl of washing buffer I was added, followed by centrifuging at 13000 xg for 1 min. Washing buffer II (700 µl) was added, followed by centrifuging at 13000 xg for 1 min. Eventually, the plasmid was eluted in 100 µl of the elution buffer. The tube was centrifuged at 13000 xg for 1 min. The plasmid DNA was stored at -20°C for further utilization. After the completion of the plasmid extraction procedure, the positive plasmids containing the target DNA were confirmed by restriction enzyme, using *EcoR*I enzyme (Takara Bio Inc. Japan) (2 U), 10X H Buffer (2 µl), plasmid (1 µl) and water (16.7 µl) as a master mixture per reaction as well as colony PCR following the PCR protocol described by Nene, Musoke, Gobright, and Morzaria, (1996). The mixture (20 µl) was then incubated at 37°C for one hour, followed by analysis on gel electrophoresis for the presence of specific bands. Subsequently, three positive clones (350 ng of each positive plasmid) and 2 pmol of each P-F and P-R were randomly chosen and sent to the Inqaba Biotechnical Industries Ltd, South Africa, for sequencing, using the ABI BigDye™ terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystem, USA).

#### **4. Sequencing analysis**

The DNA sequences were assembled and analysed with the Staden package (version 1.6.0 for windows) (Bonfield, Smith & Staden, 1995; Staden, 1996). The obtained sequences were subjected to a BLASTn homology search (<http://www.ncbi.nlm.nih.gov>) to verify the highest-species identity. The sequences as well as closely related sequences from the GenBank (Table 2) were aligned using Clustal X (Thompson, Higgins & Gibson, 1994; Jeanmougin, Thompson, Gouy, Higgins, Gibson, 1998).

**Table 2:** Genbank accession numbers for all *Babesia* and *Theileria* species whose 18S rRNA gene were examined.

<b>Taxon</b>	<b>GenBank accession number</b>
<i>Babesia bigemina</i>	X56605
<i>Babesia bovis</i>	L31922
<i>Babesia caballi</i>	Z15104
<i>Babesia canis canis</i>	AY072926
<i>Babesia canis rossi</i>	DQ111760
<i>Babesia canis vogeli</i>	AY371198
<i>Babesia conradae</i>	AF158702
<i>Babesia felis</i>	AF244912
<i>Babesia divergens</i>	AY789076
<i>Babesia gibsoni</i>	AB118032
<i>Babesia lengau</i>	GQ411405
<i>Babesia leo</i>	AF244911
<i>Babesia microti</i>	U09833
<i>Babesia motasi</i>	AY260179
<i>Babesia occultans</i>	EU376017
<i>Babesia orientalis</i>	AY596279
<i>Babesia ovis</i>	AY260178
<i>Babesia ovata</i>	AY603400
<i>Babesia</i> sp. (sable)	EU376016
<i>Babesia</i> sp. (Xinjiang)	DQ159073
<i>Isospora felis</i> *	L76471
<i>Theileria annulata</i>	M64243
<i>Theileria mutans</i>	AF078815
<i>Theileria parva</i>	L02366
<i>Theileria separata</i>	AY260175
<i>Theileria taurotragi</i>	L19082
<i>Theileria velifera</i>	AF097993

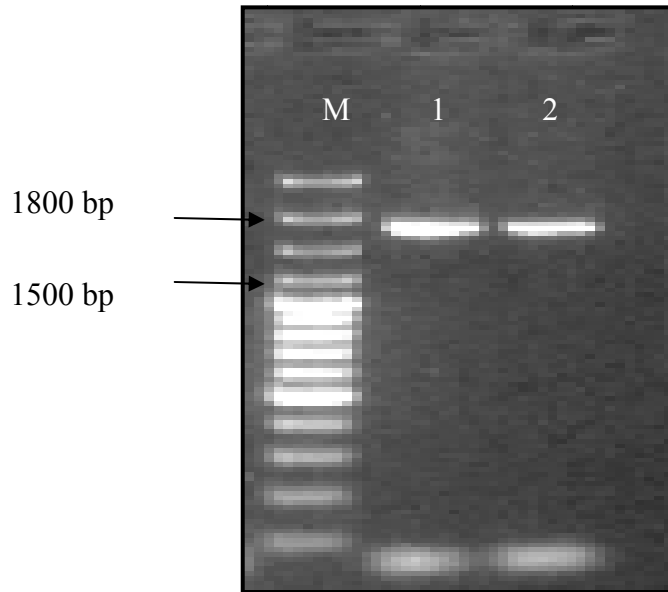
\* *Isospora felis* is not a piroplasm

## 5. Phylogenetic tree construction

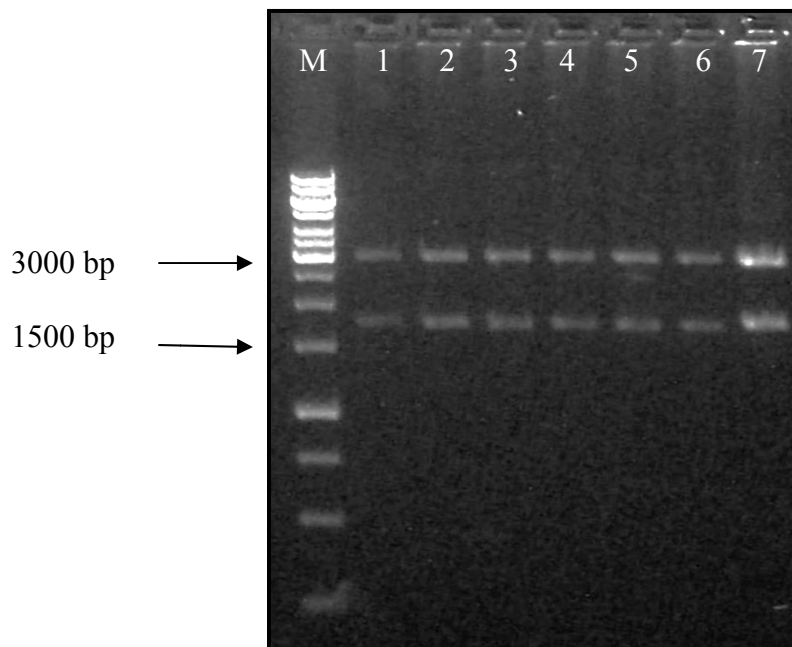
The sequences were truncated to the shortest length and trees were constructed using maximum likelihood, maximum parsimony, neighbour-joining and MrBayes analysis. The model used for 18S rRNA was TrN+I+G. The obtained sequences were compared with the complete 18S rRNA gene sequences of the other *Babesia* and *Theileria* species (Table 2) which were published in GenBank. The 18S rRNA of *Isospora felis* (L76471) was used as the outgroup. The model of nucleotide substitution was determined by the JModeltest 0.1.1 program (Guindon & Gascuel, 2003; Posada, 2008), selected by AIC calculations. Substitution mode was used in PAUP\* v4b10 (Swofford, 2002) to explore different trees. The Bayesian phylogeny was explored by MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck, 2003). The phylogenetic trees were visualized and subsequently edited in MEGA v4.0.2 (Tamura, Dudley, Nei & Kumar, 2007; Kumar, Nei, Dudley & Tamura, 2008).

## Results

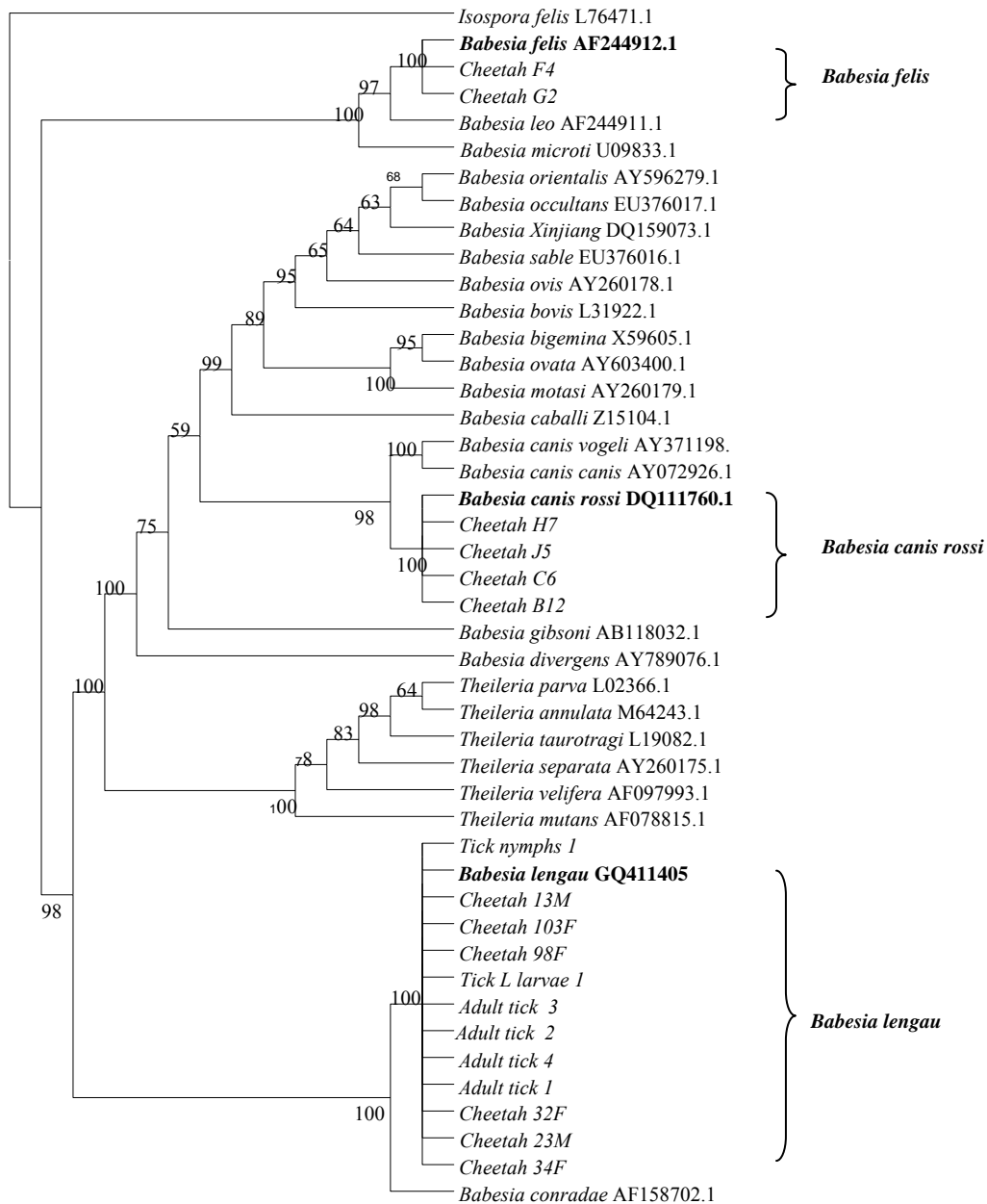
The full-length of 18S rRNA gene (~1800 bp) of the parasite was amplified using primers F and R (Fig. 1). After cloning recombinant plasmids were selected using enzyme restriction digest and the resultant agarose gel showed two different sizes of DNA fragments, indicating that the T-vectors (3000 bp) were carrying the target ssu18S rRNA gene (~ 1800 bp) (Fig. 2). The 18S rRNA sequences were assembled, edited and aligned with related *Theileria* and *Babesia* sequences. The alignment of the 18S rRNA gene sequences of *Babesia* species in blood and ticks resulted in about 1550 characters including the gaps. The BLASTn homology search indicated that 12 out of 18 gene sequences obtained from cheetah blood and *H. elliptica* were 99% identical to *B. lengau* (accession number: GQ411405), with the exception of one nucleotide difference, which could be due to a PCR error. The sequences of the *Babesia* species obtained from cheetas that tested positive for *Babesia* were blasted and the results showed 100% similarity with *B. felis* (accession number: AF244912) and *B. rossi* (accession number: DQ111760). The phylogenetic analysis using the distance analysis, neighbour joining, parsimony, maximum-likelihood and Bayesian placed all *Babesia* species from blood and ticks in the clades with *B. canis rossi* and *B. felis* and *B. lengau* trees with approximately identical topologies (100% reliability) and nodal support values (Figs. 3, 4, 5, 6 & 7).



**Fig 1:** Gel electrophoresis indicating the amplification of complete 18S rRNA gene (1800 bp) of *Babesia* species in cheetah blood. Lane M: 100 bp molecular marker (100 bp). Lanes 1 and 2: blood samples collected from cheetahs



**Fig. 2:** Restriction enzyme assay showing the recombinant plasmids, the T-vector (3000 bp) and the 18S rRNA gene (1800 bp) as the target gene. Lane M; 100 bp molecular marker. Lanes 1-7: cheetah blood samples and tick specimens



**Fig. 3:** Phylogenetic relationship of suu18S rRNA genes of *Babesia* species with *Isospora felis* as an out group. The tree was constructed and analysed with the parsimony method with 1000 bootstrap replicates. Percentage of reliability of each branch of the tree was indicated as numbers at the nodes.

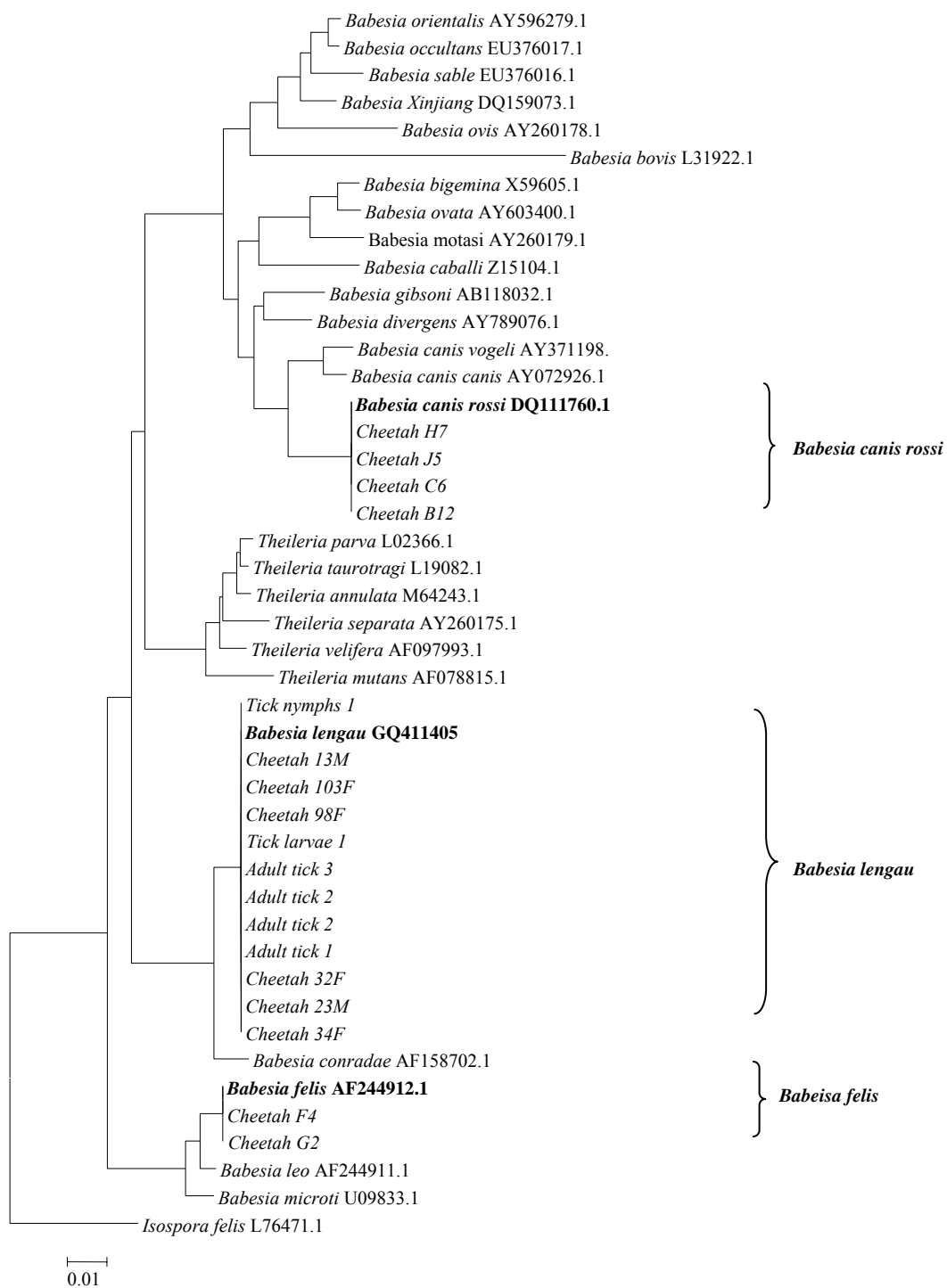


Fig. 4: Phylogenetic relationship of suu18S rRNA genes of *Babesia* species with *Isospora felis* as an out group. The tree was constructed and analysed with the Maximum likelihood

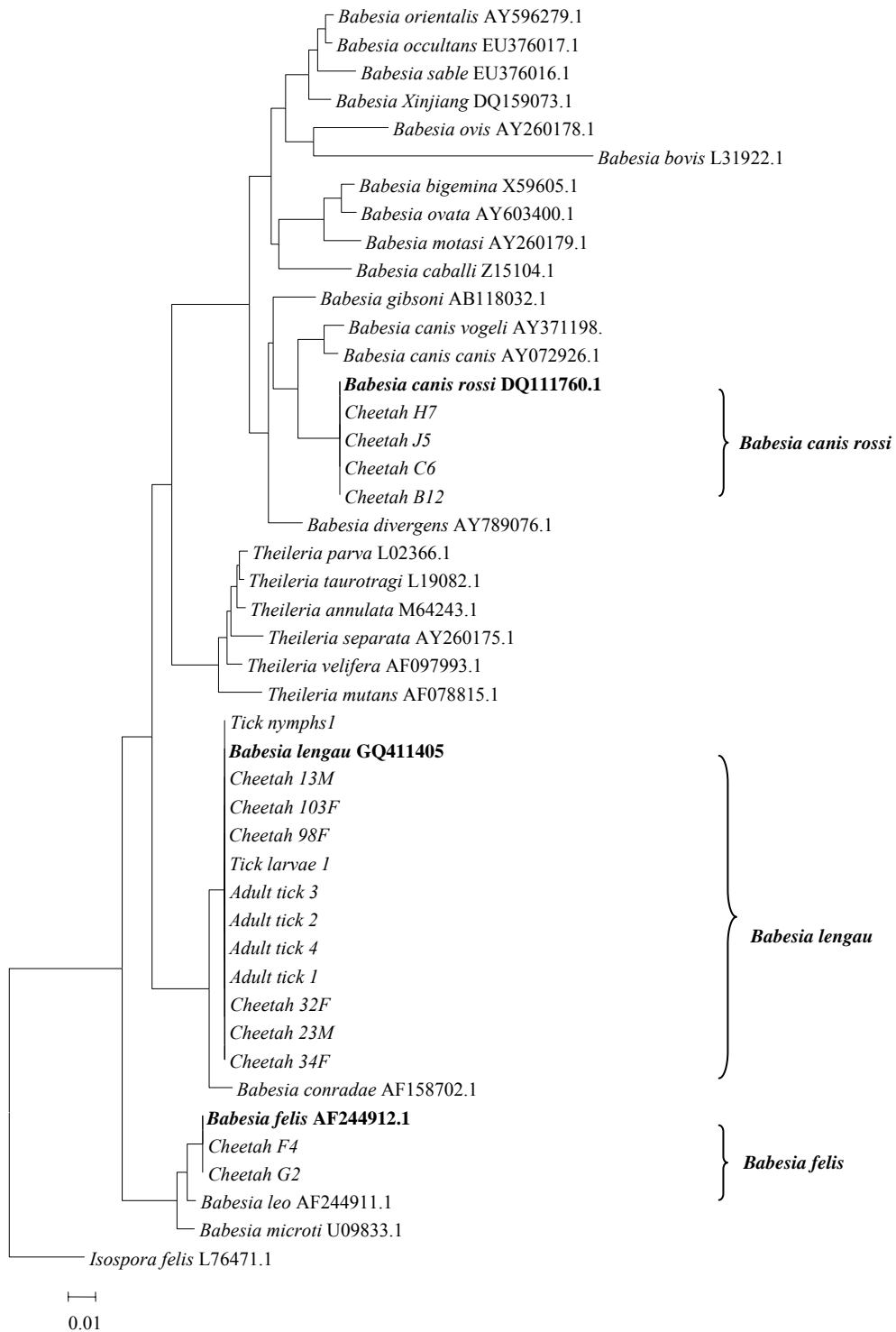


Fig. 5: Phylogenetic tree based on a sequence distance analysis constructed from the sequencing results of suu 18S rRNA genes of *Babesia* species

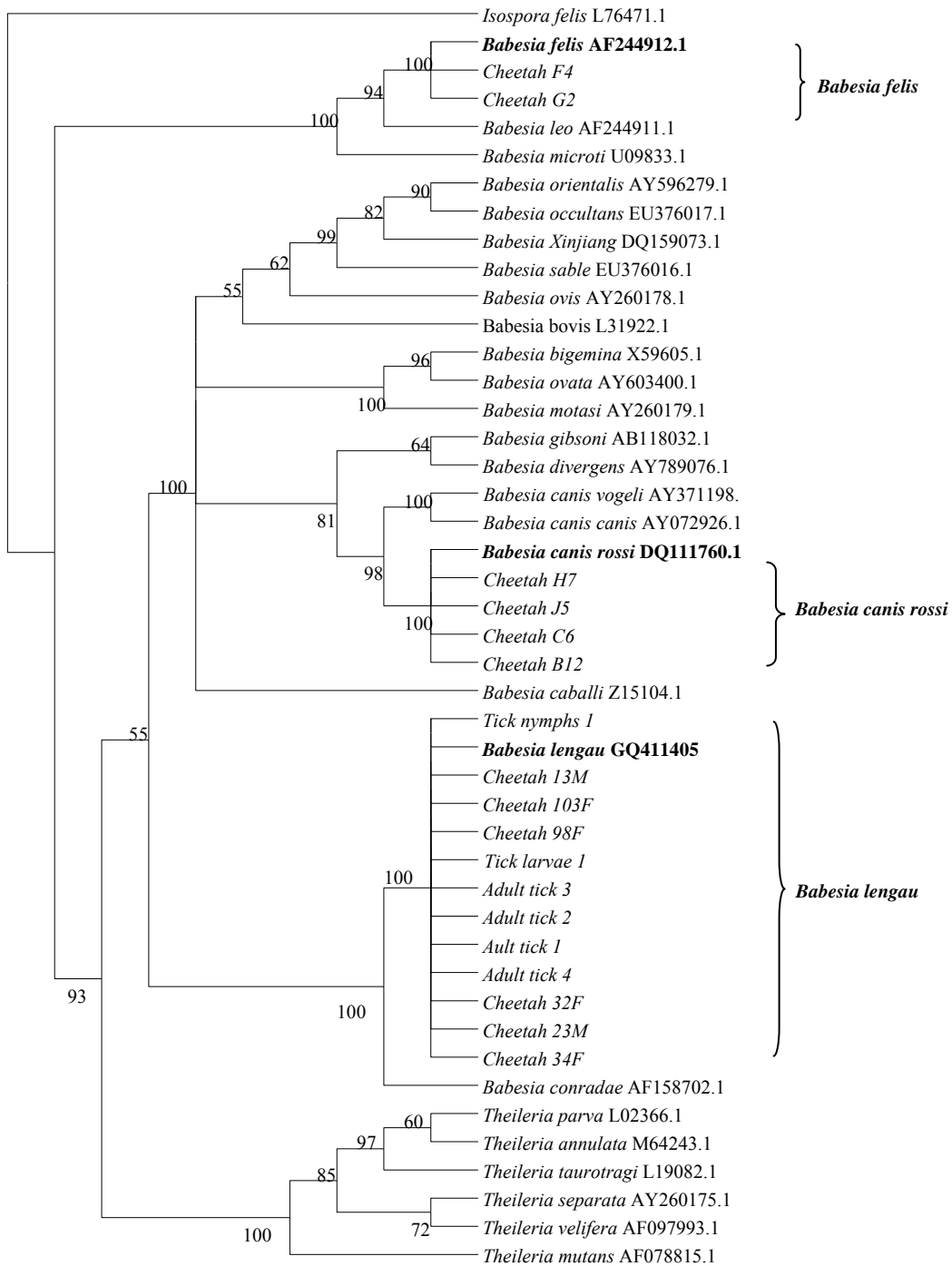


Fig. 6: Phylogenetic constructed using neighbor joining from the sequencing results of suu 18S rRNA genes of *Babesia* species

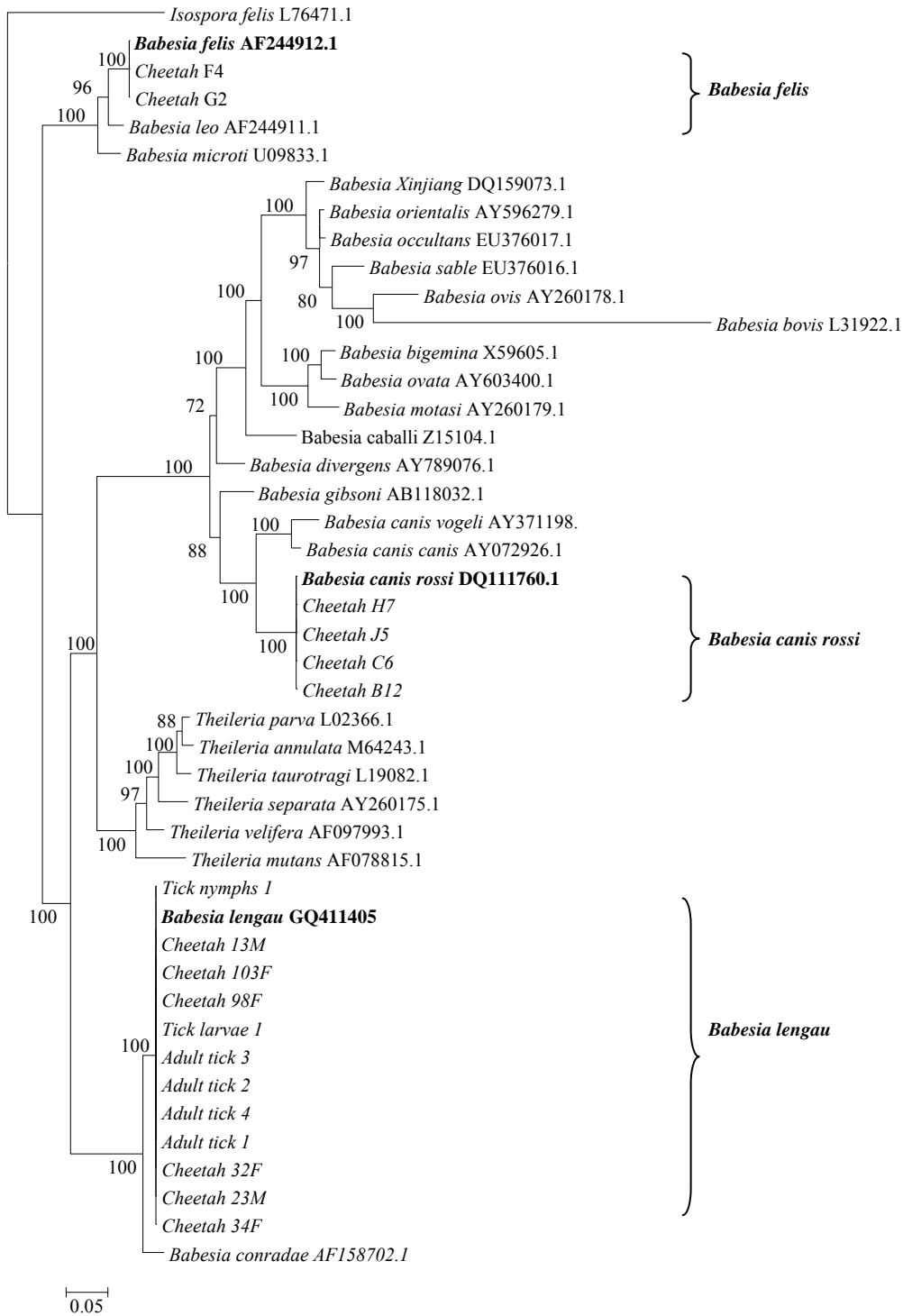


Fig. 7: Phylogenetic tree of suu 18S rRNA genes of *Babesia* species, with *Isospora felis* as an out group constructed with Bayesian analysis

## Discussion

In the past, *Babesia* species have been described on the basis of their morphology and animal hosts. More recently, genetic and antigenic analyses have enhanced taxonomic studies. The phylogenetic relationships among *Babesia* species were inferred through the molecular data obtained from 18S rDNA sequence analysis which is one of the most commonly used genes for elucidating the phylogenetic relationships among families. The small subunit ribosomal RNA of 18S gene possesses several characteristics (conserved and variable regions that provide unequivocal sequence alignment and phylogenetic discrimination, respectively) that as a result it is extensively used for the assignment of organisms to a particular genus (Allsopp & Allsopp, 2006). Those ssu18S rRNA gene sequences which consistently behaved as a single operational unit were combined in clusters/subclusters, as listed in Table 2. The 18S rRNA gene was used in this study because it has been used successfully to resolve phylogenies of *Babesia* parasites (Craido-Fernelio *et al.*, 2006). For reasons of clarity, representative selected 18S rRNA gene sequences are shown in several clades of the phylogenetic tree. The affiliation of an isolate to a piroplasm species based on the segregation of its ssu18S RNA gene sequence within a phylogenetic tree is somewhat subjective. The *Theileria* and *Babesia* ssu18S rRNA sequence clusters, as presented, corresponded to clusters/clades as it was described in other surveys on *Theileria* and *Babesia* 18S RNA sequences (Allsopp, Cavalier-Smith, De Waal & Allsopp, 1994; Gubbels *et al.*, 2002; Homer, Aguilar-Delfin, Telford, Krause & Persing, 2000).

Previously, *Babesia rossi* was detected on the RLB hybridization assay to infect wild dogs at the Ann van Dyk Cheetah Breeding centre (Matjila *et al.*, 2008), however, no clinical manifestations of babesiosis has ever been reported, although the parasite generally causes major mortality in domestic dogs (Jacobson, 2006). The sequence identity of the 18S rRNA genes of *B. canis rossi* isolates from the De Wildt/Brits and the Cheetah Outreach corresponded to the isolate obtained from the GenBank (accession number: DQ111760.1). The phylogenetic analyses grouped *B. canis rossi* with its other subspecies, *B. canis* and *B. vogeli* in one clade (Uilenberg *et al.*, 1989). Based on our current results, it would appear that in South Africa, *Babesia rossi* is not confined to domestic and wild dog populations. The only known vector of *B. rossi*, *H. elliptica*, has been recorded from domestic and wild dogs (Horak, 1995). In as far as we can ascertain, there is no

physical contact between cheetahs and dogs (domestic and wild) at the De Wildt/Brits and the cheetah Outreach.

*Babesia* species display some biological characteristics concerning tick and vertebrate host specificity, morphological similarities and similar pathogenicity and can currently be distinguished by molecular approaches (Gubbels, De Vos, Van Der Weide, Viseras, Schouls, De Vries & Jongejan, 1999). The 18S rRNA gene sequence analyses were distinct from those of other felid babesias, such as *B. felis* and *B. leo*. The association of cheetahs with *B. felis* and *B. leo* was shown in South Africa (Bosman *et al.*, 2007). In a phylogenetic study, felid-associated *Babesia* species were compared to other related *Babesia* and *Theileria* species. The analysis grouped *B. felis* with *B. leo* and *B. microti*, suggesting that these species may share the same mode of transmission by tick vectors (Penzhorn, Kjemtrup, López-Rebollar & Conrad, 2001).

The cheetah-associated small *Babesia* species, *B. lengau*, which was detected in cheetahs and *H. elliptica* ticks clustered separately from *B. felis*, *B. leo* and *B. rossi* and was grouped with the only other genotypically canine-related *B. conradae* (a novel species which was described from a dog in California by Kjemtrup, Wainwright, Miller, Penzhorn and Carreno in 2006). This may indicate that *B. lengau* is classified under the previously described “western clade” of piroplasms, comprising of *B. conradae*, *B. duncani*, and the piroplasms isolated from both wildlife and humans from the western United States (Bosman *et al.*, 2010).

In conclusion, 18S rRNA gene sequence similarity and phylogenetic analysis support our results regarding the presence of *Babesia* species in captive cheetahs at the various cheetah breeding centers in South Africa.

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## Chapter 6:

### **Phylogeny of *Haemaphysalis elliptica* (Acari: Ixodidae) using mitochondrial 12S and 16S rRNA gene sequence analysis**

#### **Abstract**

The genetic identity of *Haemaphysalis elliptica* (Koch, 1844), as ticks of carnivores in tropical and subtropical regions of East and Southern Africa, was determined. Morphological and phylogenetic data sets were examined separately. For this purpose, six tick specimens were collected from three study sites, namely The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Brits, The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Shingwedzi and The Hoedspruit Endangered Species Centre in South Africa. The domain III region of the mitochondrial 12S (420 bp) and 16S rRNA (460 bp) gene were amplified and directly sequenced. The DNA sequences were assembled and edited using the Staden package. The 12S and 16S rRNA genes were aligned with sequences obtained from Genbank. No variation between the examined *H. elliptica* tick species was detected. Molecular data were analysed by maximum parsimony, maximum likelihood, and neighbor-joining methods using PAUP\* v4b10. The phylogenetic trees were constructed separately and compared. No variation in the topology of the trees was detected. The phylogenetic tree grouped *H. elliptica* and *H. leachi* ticks together indicating the correspondence of the genetic diversity with their morphology. This study has made the first sequences of 12S and 16S rRNA genes of *H. elliptica* available in the Genbank.

## Introduction

Ticks (Order Parasitiformes, Suborder Ixodida) as blood-feeders with body size ranging from 2 to 30 mm and specialised mouthparts for attachment are viewed as a group of mites (Subclass Acari) parasitising a number of animals (Roberts, 1970) and transmitting a variety of pathogens (de la Fuente, Estrada-Peña, Venzal, Kocan & Sonenshine, 2008). They express various habitat and feeding associations with their vertebrate hosts, based on global reports (Hoogstraal & Aeschlimann, 1982; Ntiamoa-Baidu, Carr-Saunders, Matthews, Preston & Walker, 2004). The 899 named tick species have been divided into three main families (Ixodidae, Argasidae and Nuttalliellidae) which have been further divided into subfamilies, genera and species. The genus *Ixodes* (subfamily Ixodinae) in the family Ixodidae, represent typical “hard ticks” which possess specialized secondary biological and structural development (Hoogstraal & Kim, 1985). The genus *Haemaphysalis* (Koch, 1844; Nuttall & Warburton, 1915) is represented by about 160 known species world-wide. Various *Haemaphysalis* species have been listed according to their occurrence and distribution in South Africa (Walker, 1991).

*Haemaphysalis elliptica* (Koch, 1844), a South African carnivore haemaphysalid, which was formerly lumped with *Haemaphysalis (Rhipistoma) leachi* (Audouin, 1826) (a species found commonly in Central Africa and known as the yellow dog tick) has recently been redescribed morphologically as a species in South Africa (Apanaskevich, Horak & Camicas, 2007), where it commonly infests domestic and wild canids and felids. The *H. elliptica* male was originally described by Koch (1844), who named it *Rhipistoma elliptica*; for a century, this was considered a junior synonym for *H. (R.) leachi*. The distribution of *H. elliptica* is limited to East and Southern Africa, whereas *H. leachi* occurs in North and East Africa, as far south as northern Zimbabwe (Apanaskevich *et al.*, 2007). Morphological and molecular characters among tick subfamilies reflect their phylogenetic relationships (Black, Klompen & Keirans, 1997). Genetic analysis can target differences at various levels, such as inter-species, inter-strain within a species and inter-individuals (Grant, 1994). Various phylogenetic trees of ticks have recently been published: Hoogstraal and Aeschlimann (1982) for the Ixodida, as well as Klompen (1992) and Klompen and Oliver (1993) for Argasidae. Unlike the tree of Hoogstraal and Aeschlimann (1982) which was based on the biology of ticks and their host specificity, the trees of other

researchers (Klompen, 1992; Klompen, 1999; Klompen & Oliver, 1993) were inferred from cladistic analysis.

Phylogenies were initially based on morphology of tick species (Walker, Keirans & Horak, 2000; Walker, Bouattour, Camicas, Estrada-Peña, Horak, Latif, Pegram & Preston, 2003). The recent application of polymerase chain reaction (PCR) and direct sequencing technology revolutionised the study of tick genetics and has allowed the genotypes of a number of individual ticks to be determined. Nucleotide sequence data have widely been used to determine phylogenetic relationships among tick species, genera and subfamilies. Analyses of the sequence variation in the 18S rDNA substantially confirmed the phylogenetic relationship among tick taxa which was originally proposed. However, 18S rRNA gene based phylogeny differs from the mitochondrial 16S rRNA based phylogeny in various respects (Black *et al.*, 1997). The mitochondrial 12S and 16S rRNA gene sequences of various tick species demonstrate many of the same features as the mitochondrial ribosomal genes of arthropods do (Simon, Frat, Bechenbach, Crespi, Liu & Flook, 1994). The sequence variation from approximately 460 bp of the 3' end of the 16S mitochondrial rDNA gene indicated five broad-spectrum divergences (Black & Piesman, 1994). In fact, they explained that members of the Amblyomminae were not monophyletic and also that members of the Haemaphysalinae arose within the Amblyomminae. This finding was not only well supported by the 16S-based phylogeny, but also by sequence analysis of both 12S and 16S mitochondrial rDNA.

The detection of cheetah-associated *Babesia* species in instars of *H. elliptica* (Chapter 4) was a significant finding. Since the distribution of *H. elliptica* and *H. leachi* overlap in southern Africa, establishing the integrity of *H. elliptica* as a potential vector for *Babesia* species in cheetahs was investigated. We examined the phylogenetic history of *H. elliptica* and determined its closest relation with other tick species in the same genus and other genera. The domain III region of both mitochondrial 12S and 16S rRNA genes of six *H. elliptica* ticks collected from various study localities in South Africa were partially sequenced and compared genetically.

## Materials and Methods

### 1. *Sample collection and localities*

Two adult ticks (males or females) from each study site, namely The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Brits and The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Shingwedzi as well as The Hoedspruit Endangered Species Centre, previously identified to species level on the basis of their morphological characteristics, were randomly chosen from the set of the tick samples collected by dragging the vegetation (Table 1).

Table 1: Sources of *H. elliptica* tick specimens

Sample code	Location
B1	The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Brits
B2	The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Brits
H1	The Hoedspruit Endangered Species Centre
H2	The Hoedspruit Endangered Species Centre
S1	The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Shingwedzi
S2	The Ann Van Dyk Cheetah Breeding Centre-De Wildt/Shingwedzi

### 2. *Genomic DNA extraction*

The genomic DNA of adult ticks was extracted using the MagNA Lyser Green Beads (Roche, Germany) for mechanical crushing of the polysaccharide chains of the chitin of the tick exoskeleton and tearing of the ticks to very fine pieces (Halos, Jamal, Vial, Maillard, Suau, Le Menach, Boulouis, Vayssier-Taussat, 2004) and the QIAamp<sup>®</sup> DNA Mini DNA Extraction Kit (Qiagen, Hilden, Germany) for enzymatic protein digestion, according to the manufacturer's protocol as described in Chapter 5.

### 3. *PCR amplification and purification*

Two specific pairs of forward and reverse primers (Table 2) were used to amplify 420 bp and 460 bp of the third domain region of the mitochondrial 12S rRNA and 16S rRNA genes, respectively (Norris, Klompen, Keirans & Black, 1996; Norris, Klompen & Black, 1999). The

12S rRNA fragment extends from stem 31 to stem 32 (Hickson, Simon, Cooper, Spicer, Sullivan & Penny, 1996), whereas the 16S rRNA fragment was from stem 68 to stem 90 (Larsen, 1992; Gutell, Larsen & Woese, 1994).

**Table 2:** Primers used for amplification and sequencing of the 12S and 16S RNA genes in ticks

Primer name	Primer sequence	Use*
12S – F	5'-TACTATGTTACGACTTA-3'	P, S
12S – R	5'-AAACTAGGATTAGATACCC-3'	P, S
16S – F	5'-CCGGTCTGAACTCAGATCAAGT-3'	P, S
16S – R	5'-CTGCTCAATGATTTTTTAAATTGCTGTGG-3'	P, S

\* P, used in PCR; S, used in sequencing

Amplification was initially accomplished using a 20 µl master mixture per reaction with the components of **Takara Ex Taq kit™** (Takara Bio Inc, Japan) (1.5 U), 10X buffer (2.5 µl), dNTPs (0.4 mM), P-F (2 pmol), P-R (2 pmol), H<sub>2</sub>O (15.7 µl), in a 1.5 ml tube. Tick template DNA (~46 ng) was then added to each aliquot of the reaction mixture in PCR tubes. Negative controls (no template) were always run simultaneously and reaction mixtures were discarded when no band appeared, confirming that the controls were negative. The tubes were placed in a thermal cycler providing optimised conditions (94°C for 2 min, 40 cycle of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min, followed by extension at 72°C for 1 min and hold at 4°C) for amplification (Black & Piesman, 1994). The accomplished PCR reaction and the predicted product size were evaluated by electrophoresis on 1.5% agarose gel under UV light. In order to show the true integrity of tick DNA regardless of the blood parasites, the genomic DNA of four positive ticks along with four negative ticks was amplified and the PCR products were analysed by agarose gel electrophoresis.

The commercial **QIAquick® PCR purification kit** (Qiagen, Hilden, Germany) was used for purification of the PCR products, by the following protocol:

Initially, 500 µl of buffer PBI was added to the PCR reaction and mixed to bind the DNA. The contents of the tube was transferred to a **QIAquick** column placed in 2 ml collecting tube,

centrifuged for 30-60 s. The flow-through was discarded and a column was placed back into the same tube. Washing buffer PE (750  $\mu$ l) was then added to the [QIAquick](#) column and centrifuged for 30-60 s. The flow-through was then discarded and a column was replaced. The PCR product was eluted by adding 30  $\mu$ l of the eluting buffer EB (10 mM Tris.Cl, pH 8.5) to the [QIAquick](#) membrane, and then the column was centrifuged for 1 min. The purified DNA was analysed on the gel and the concentration was assessed using the [NanoDrop 2000 Fluorospectrometer](#) ([Thermo Scientific, USA](#)).

#### **4. Sequencing and alignment**

For each tick specimen, four more 25  $\mu$ l reactions were set up and all the products were eventually pooled (to minimise the risk of contamination) and analysed by electrophoresis. All PCR products were purified using [Qiagen® DNA purification Kit](#) ([Qiagen, Hilden, Germany](#)), following the manufacturer's protocols ([Chapter 5](#)). Eventually, 20  $\mu$ l of each PCR product together with 20 pmol of each forward and reverse 12S and 16S primer was sent to the [Inquba Biotechnical Industries \(Pty\) Ltd](#) in South Africa, to perform the sequencing reaction, using the [ABI BigDye terminator™ Cycle Sequencing Ready Reaction Kit](#) ([PE Applied Biosystem, USA](#)).

The DNA sequences were assembled and edited using the [Staden](#) package ([Staden, 1996](#)). The sequences obtained were subjected to a BLASTn homology search. For all data sets, all sequences were truncated 50-60 bases downstream and upstream of the forward and reverse primers, ensuring that the sequences would begin and end at the same position. The mitochondrial 12S and 16S r RNA sequences from the tick specimens were aligned with related sequences obtained from Genbank ([Table 3 & 4](#)) using the [MAFFT 6](#) ([Katoh, Misawa, Kuma & Miyata, 2002; Katoh, Kuma, Toh & Miyata, 2005](#)) and were manually edited using [BioEdit 7.0.9.0](#) ([Hall, 1999](#)) software.

**Table 3:** Genbank accession numbers for all ticks whose mitochondrial 12S rRNA gene were examined

<b>Taxon</b>	<b>GenBank accession number</b>
<i>Amblyomma americanum</i>	U95849
<i>Amblyomma cajennense</i>	U95850
<i>Amblyomma darwini</i>	U95851
<i>Amblyomma maculatum</i>	U95854
<i>Amblyomma tuberculatum</i>	U95855
<i>Amblyomma variegatum</i>	U95849
<i>Aponema glebopalma</i>	U95858
<i>Boophilus annulatus</i>	U95866
<i>Boophilus microplus</i>	U95867
<i>Dermacentor andersoni</i>	EU711343
<i>Dermacentor variabilis</i>	U95869
<i>Haemaphysalis cretica</i>	U95870
<i>Haemaphysalis inermis</i>	U95871
<i>Haemaphysalis leachi</i>	AF150035
<i>Haemaphysalis leporispalustris</i>	U95873
<i>Haemaphysalis punctata</i>	AF150032
<i>Hyalomma dromedarii</i>	U95874
<i>Hyalomma rufipes</i>	U95875
<i>Ornithodoros turicata</i> *	U95912
<i>Rhipicephalus appendiculatus</i>	U95914
<i>Rhipicentor bicornis</i>	U95917
<i>Rhipicephalus decoloratus</i>	EU921774
<i>Rhipicephalus haemaphysaloides</i>	DQ003006
<i>Rhipicephalus sanguineus</i>	AY559843
<i>Rhipicephalus sanguineus</i>	DQ003004
<i>Rhipicephalus turanicus</i>	U95916

\* *Ornithodoros turicata* is an argasid tick

**Table 4:** Genbank accession numbers for all ticks whose mitochondrial 16S rRNA gene were examined

<b>Taxon</b>	<b>GenBank accession number</b>
<i>Amblyomma dubitatum</i>	DQ858954
<i>Amblyomma glauerti</i>	U95852
<i>Amblyomma maculatum</i>	L34318
<i>Amblyomma tigrinum</i>	FJ965339
<i>Amblyomma tuberculatum</i>	U95856
<i>Amblyomma variegatum</i>	L34315
<i>Aponema glebopalma</i>	U95859
<i>Aponema varanensis</i>	L34320
<i>Dermanyssus gallinae</i> *	L34326
<i>Haemaphysalis cretica</i>	L34308
<i>Haemaphysalis qinghaiensis</i>	EF605264
<i>Haemaphysalis inermis</i>	U95872
<i>Haemaphysalis juxtakochi</i>	AY762324
<i>Haemaphysalis juxtakochi</i>	AY762323
<i>Haemaphysalis leporispalustris</i>	L34309
<i>Repicentor bicornis</i>	L34304

\* *Dermanyssus gallinae* is a mite

## 5. Phylogenetic analysis

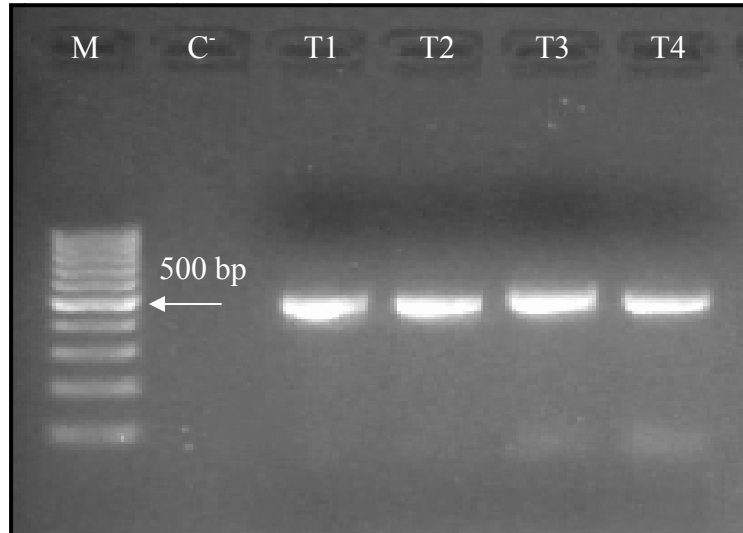
The sequences obtained were compared with the 12S and 16S rRNA gene sequences of all species listed in table 3 and 4. The 12S rRNA of *Ornithodoros turicata* (U95912) and the 16S rRNA of *Dermanyssus gallinae* (L34326) were used as outgroups. The best-fit model of nucleotide substitution was determined by JModeltest 0.1.1 (Guindon, Lethiec, Duroux, Gascuel, 2005; Posada, 2008) selected by AIC calculations. The models used for 12S and 16S mDNA were TPM1uf+G and TVM+G, respectively. Substitution mode was used in PAUP\* v4b10 (Swofford, 2002) to explore distance analysis, neighbour-joining, parsimony and maximum likelihood methods. MrBayes v3.1.2 (Huelsenbeck & Ronquist 2001; Ronquist & Huelsenbeck, 2003) was used to explore Bayesian phylogeny. The consensus trees were subsequently edited in MEGA v4.0.2 (Kumar, Dudley, Nei & Tamura, 2008; Tamura, Dudley, Nei & Kumar, 2007).

## Results

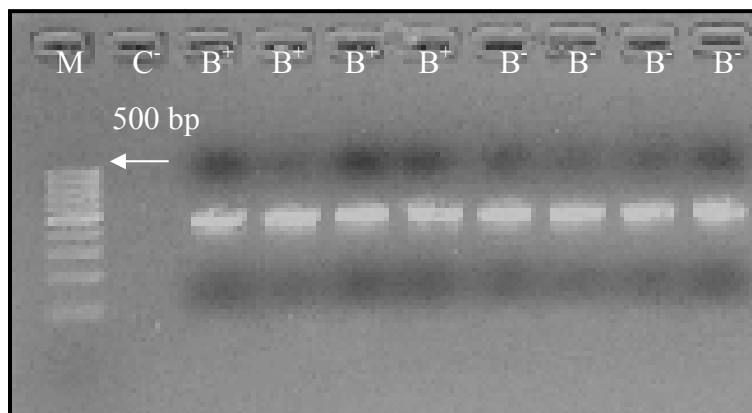
The average length of the amplified 12S and 16S domain III regions were 420 bp and 460 bp, respectively. A single amplicon was resolved for each PCR reaction, indicating successful amplification in all specimens, but no bands were visible in the negative (i.e. no gDNA) control (Fig. 1). For all these ticks, there was no detectable intraspecific variation in the size of amplicons. The result of agarose gel electrophoresis also showed the true integrity of tick DNA when smaller than 500 bp bands were observed on the gel for all *Babesia* positive and negative *H. elliptica* ticks (Fig. 2). The sequences obtained were assembled, edited and incorporated in GenBank under the accession numbers shown in Table 5. The alignment of the mitochondrial 12S and 16S rDNA sequences of the 6 tick specimens resulted in a total of 350 and 415 characters including the gaps. The sequences were aligned and compared with those species obtained from GenBank. The average nucleotide composition of the mitochondrial 12S rRNA in *H. elliptica*, excluding gaps, was 37.4% adenine, 8.3% cytosine, 13.4% guanine, and 34.8% thymine. However, the average nucleotide composition of the mitochondrial 16S rRNA, excluding gaps, was 38.8% adenine, 6.9% cytosine, 14.4% guanine, and 35.9% thymine. A bias toward adenine and thymine in 12S rRNA and 16S rRNA was 72.2% and 74.7%, respectively. This was consistent with the base composition of arthropod mitochondrial DNA (Simon *et al.*, 1994).

The matrix distance determined the number of base differences per sequence from analysis between sequences of the third domain of mitochondrial 12S rRNA (Table 6) and 16 rRNA (Table 7) of *H. elliptica*, based on the pairwise analysis of 31 and 23 sequences, respectively. Analyses were conducted in MEGA v4.0.2. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were 291 and 364 positions in the final dataset. The sequence alignment of 12s rRNA of *H. elliptica* detected 4 nucleotide difference with that of *H. leachi* (Fig. 3). The phylogenetic analysis using the distance analysis, neighbour joining, parsimony, maximum-likelihood and Bayesian inference all yielded trees with almost identical topologies and nodal support values (Figs. 4, 5, 6, 7, 8, 9, 10, 11, 12 & 13). They indicated that *H. elliptica* is more closely related to *H. leachi* than to the other *Haemaphysalis* species. The analysis showed no variation between the examined *H. elliptica* specimens from

various localities. Despite nucleotide difference between 12S rRNA gene sequences of *H. elliptica* and *H. leachi*, they were grouped in one clade in the phylogenetic tree.



**Fig 1:** Agarose gel electrophoresis of the domain III region of mitochondrial 12S and 16S rRNA gene. Lane M: 100 bp DNA ladder as a size marker. Lane C<sup>-</sup>: Negative control (water). Lane T1 - T4: DNA samples from tick specimens



**Fig. 2:** Comparison of 12S rRNA gene of *Babesia* positive and negative *H. elliptica* ticks. Lane M: molecular marker (100 bp); Lane C<sup>-</sup>: negative control; Lane B<sup>+</sup>: *Babesia* positive ticks; Lane B<sup>-</sup>: *Babesia* negative ticks

**Table 5:** Genbank accession numbers for tick specimens examined

<b>Taxon</b>	<b>Length</b>	<b>GenBank accession number</b>
<b>12S rRNA gene sequence</b>		
<i>Haemaphysalis elliptica</i> - 12S rRNA partial sequence – B1	410 bp	HM068950
<i>Haemaphysalis elliptica</i> - 12S rRNA partial sequence – B2	385 bp	HM068951
<i>Haemaphysalis elliptica</i> - 12S rRNA partial sequence – H1	406 bp	HM068952
<i>Haemaphysalis elliptica</i> - 12S rRNA partial sequence – H2	408 bp	HM068953
<i>Haemaphysalis elliptica</i> - 12S rRNA partial sequence – S1	359 bp	HM068954
<i>Haemaphysalis elliptica</i> - 12S rRNA partial sequence – S2	360 bp	HM068955
<b>16S rRNA gene sequence</b>		
<i>Haemaphysalis elliptica</i> - 16S rRNA partial sequence – B1	447 bp	HM068956
<i>Haemaphysalis elliptica</i> - 16S rRNA partial sequence – B2	446 bp	HM068957
<i>Haemaphysalis elliptica</i> - 16S rRNA partial sequence – H1	445 bp	HM068958
<i>Haemaphysalis elliptica</i> - 16S rRNA partial sequence – H2	403 bp	HM068959
<i>Haemaphysalis elliptica</i> - 16S rRNA partial sequence – S1	418 bp	HM068960
<i>Haemaphysalis elliptica</i> - 16S rRNA partial sequence – S2	449 bp	HM068961

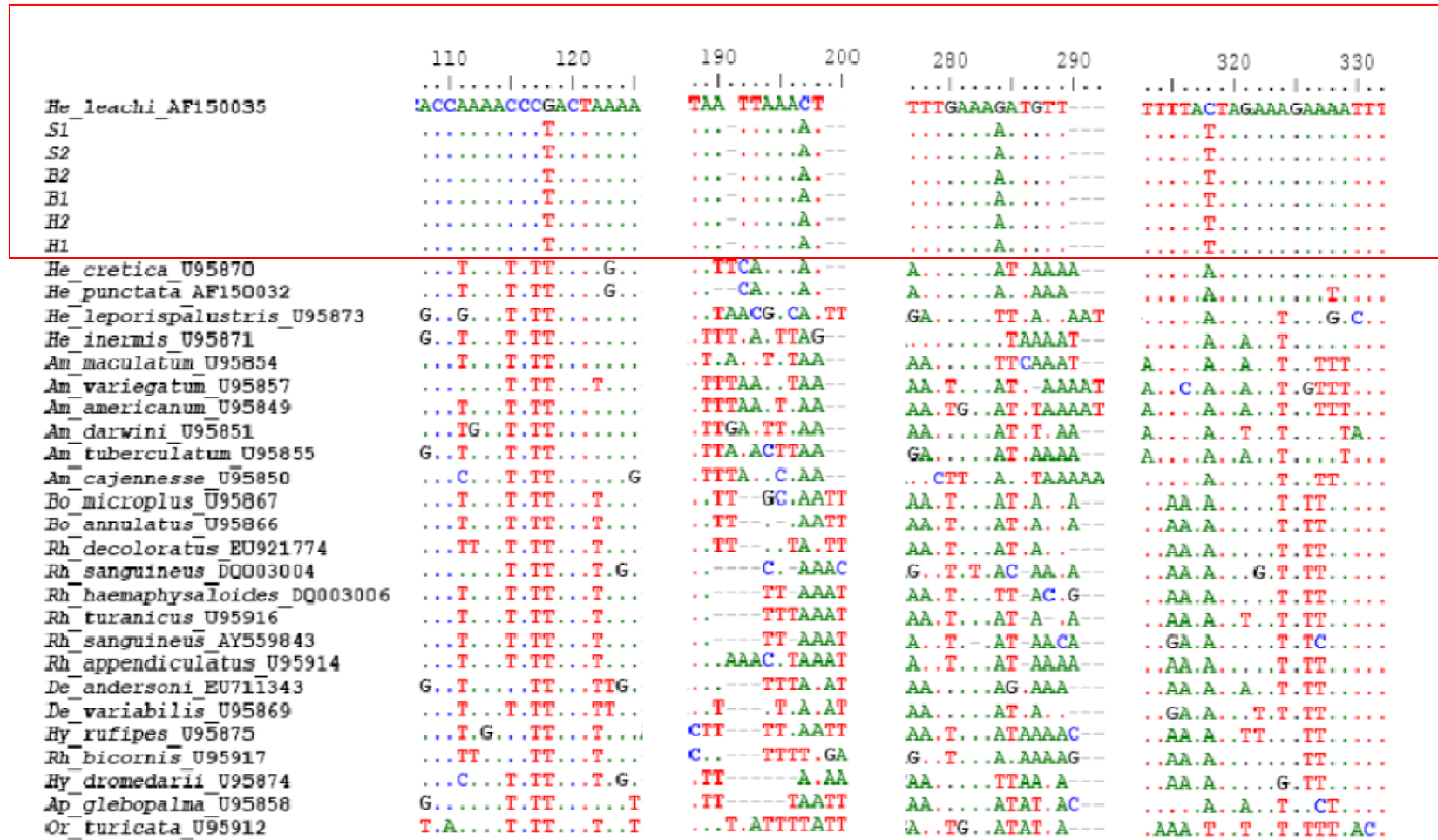
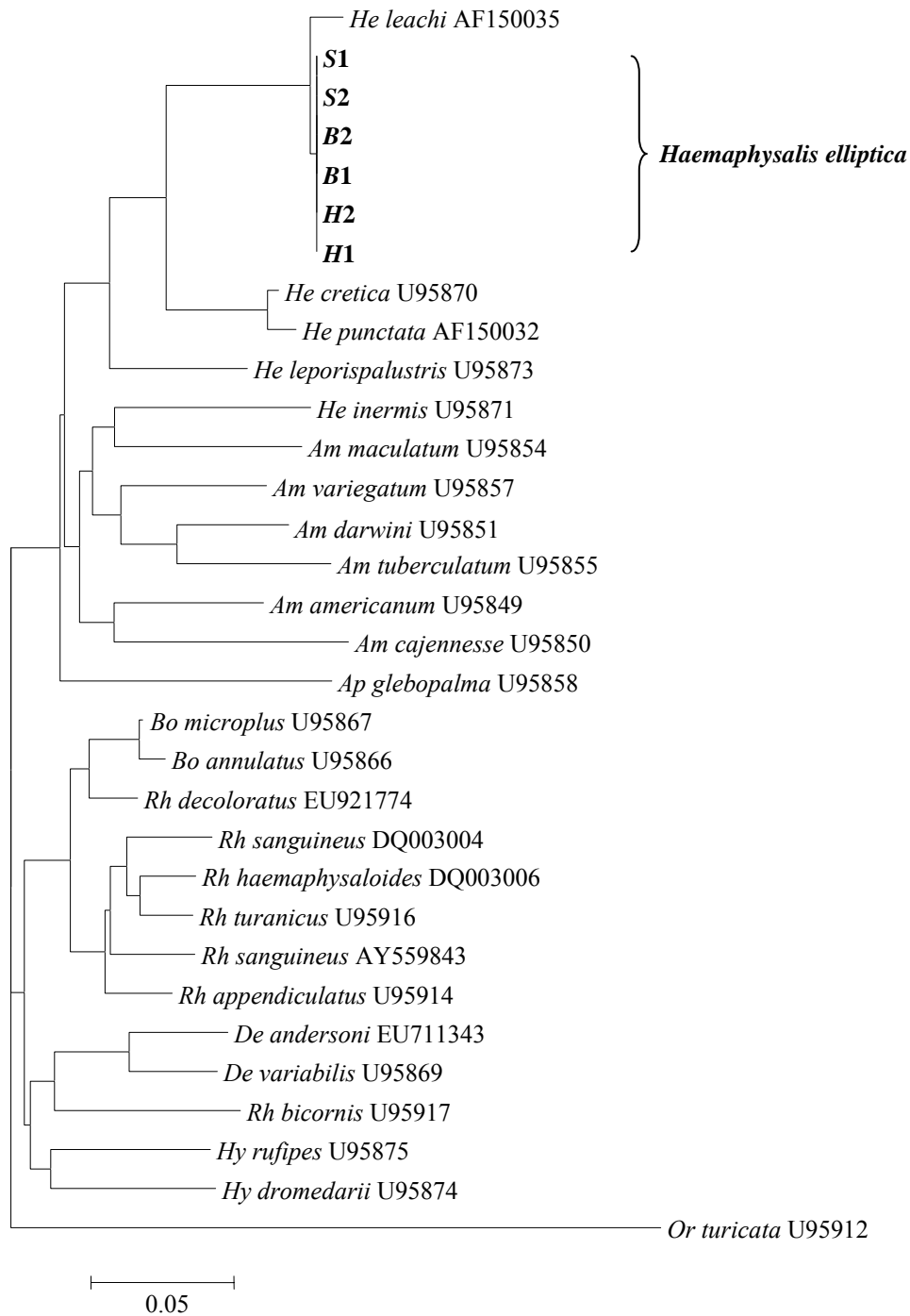


Fig. 3: Nucleotide differences found in the sequence alignment of the mitochondrial 12S rRNA genes of *H. elliptica*. Numbers (to be read in the horizontal) refer to positions in the alignment. Hyphens indicate alignment gaps whereas letters indicate the nucleotide differences of *H. elliptica* with *H. leachi*

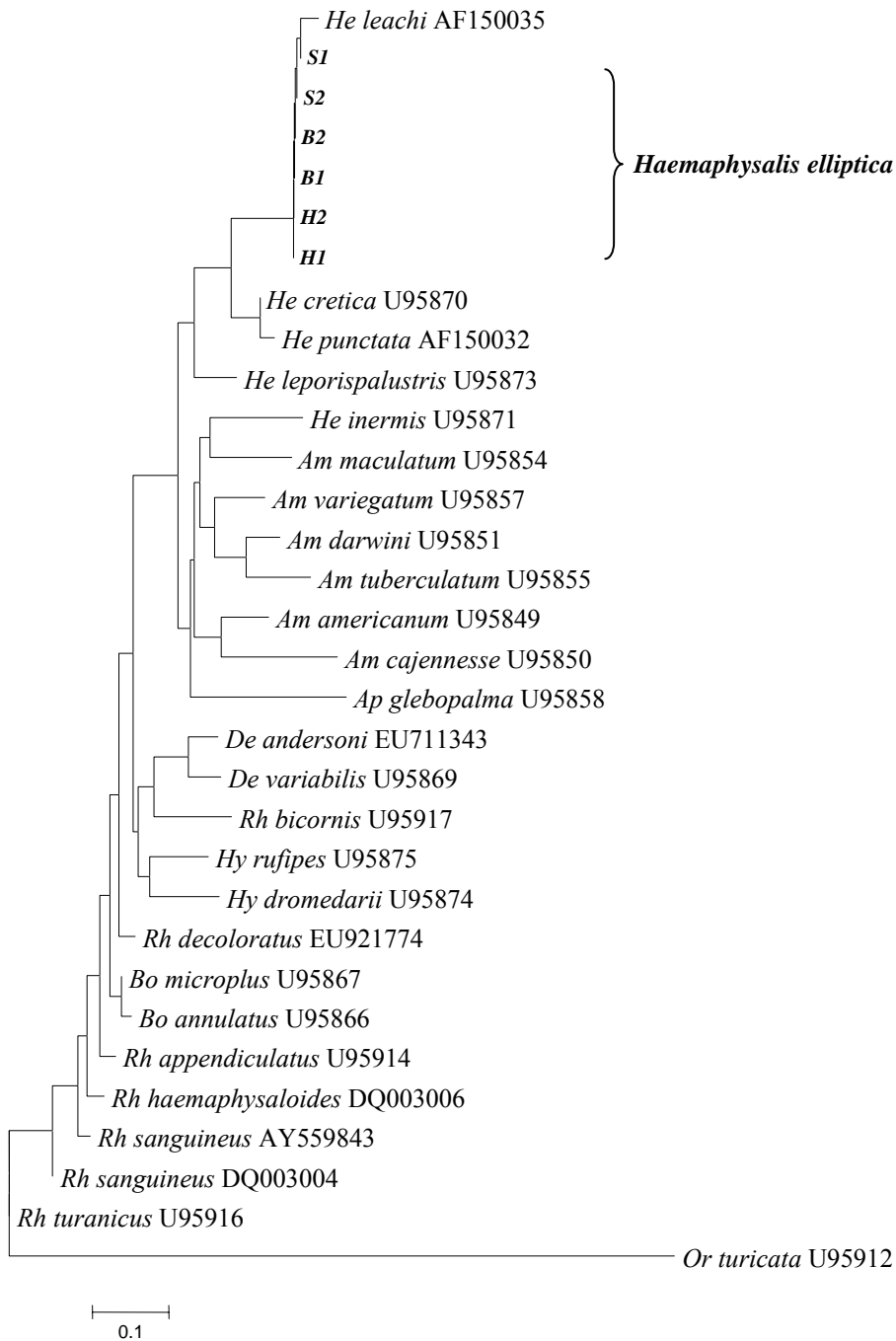


**Table 7:** Sequence pair distances between mitochondrial 16S rRNA gene sequences. The absolute nucleotide differences on pairwise comparisons of the 16S mitochondrial rRNA gene for 23 tick species. The nucleotide differences are shown in the lower left matrix.

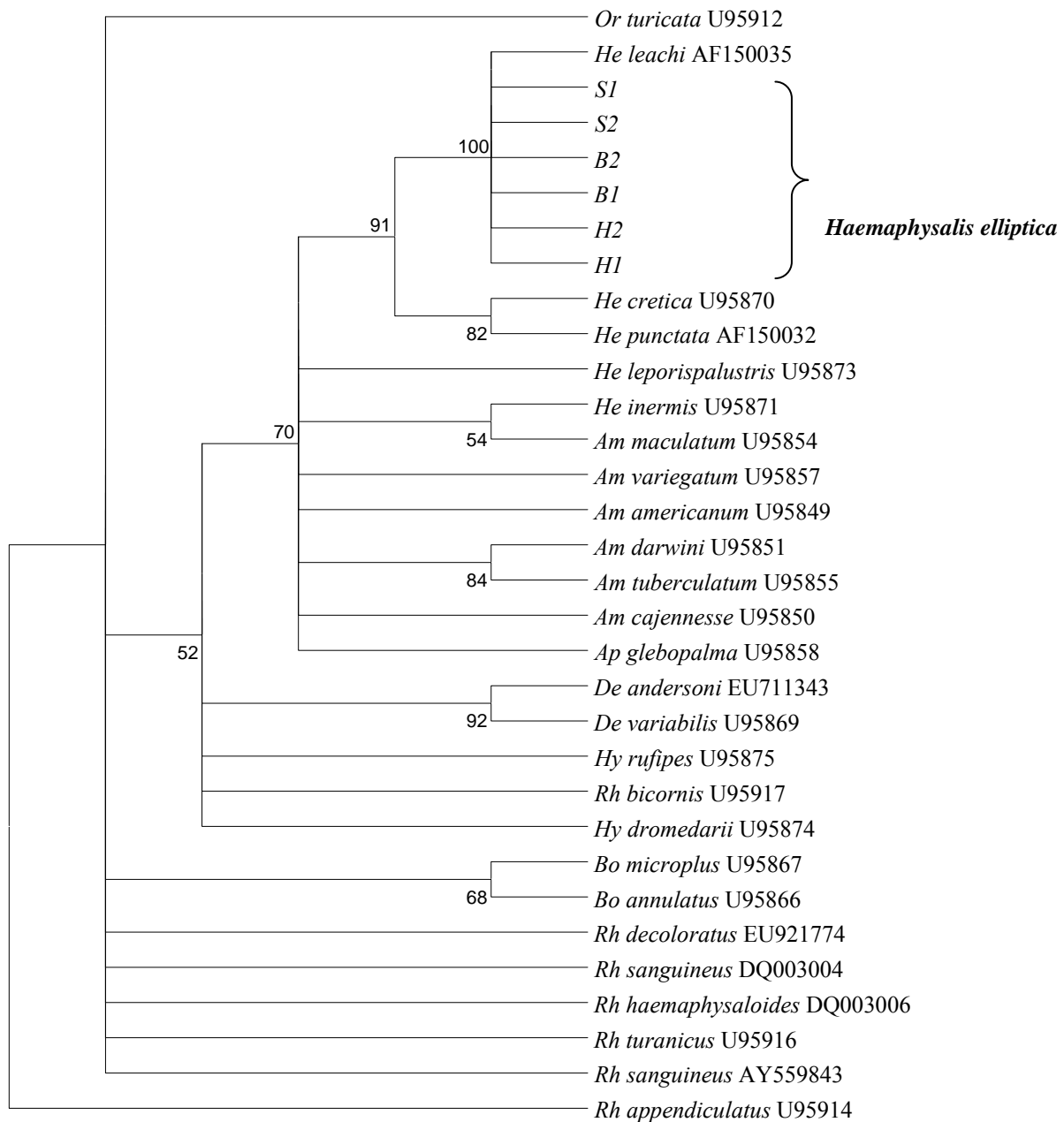
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	17	18	19	20	21	22	23	
1 <i>H2</i>																							
2 <i>SI</i>	0																						
3 <i>B2</i>	0	0																					
4 <i>H1</i>	0	0	0																				
5 <i>B1</i>	0	0	0	0																			
6 <i>S2</i>	0	0	0	0	0																		
7 <i>He cretica</i> L34308	35	35	35	35	35	35																	
8 <i>He intermis</i> U95872	31	31	31	31	31	31	34																
9 <i>Ap glebopalma</i> U95859	35	35	35	35	35	35	40	32															
10 <i>He juxtakochi</i> AY762323	43	43	43	43	43	43	46	46	52														
11 <i>He juxtakochi</i> AY762324	43	43	43	43	43	43	47	47	53	1													
12 <i>He ginghaiensis</i> EF605264	40	40	40	40	40	40	45	43	53	30	31												
13 <i>He leporispalustris</i> L34309	42	42	42	42	42	42	42	43	46	30	31	34											
14 <i>Am glauerti</i> U95852	46	46	46	46	46	46	44	44	53	50	51	45	48										
15 <i>Am tigrinum</i> FJ965339	44	44	44	44	44	44	39	46	46	45	46	41	44	36									
16 <i>Am maculatum</i> L34318	48	48	48	48	48	48	37	47	53	45	46	45	48	37	14								
17 <i>Am goeldii</i> GQ891950	51	51	51	51	51	51	47	54	57	54	55	48	54	46	32	36							
18 <i>Am dubitatum</i> DQ858954	46	46	46	46	46	46	43	47	51	52	53	47	52	43	30	31	28						
19 <i>Am variegatum</i> L34315	42	42	42	42	42	42	39	44	48	52	51	51	53	51	36	36	36	38					
20 <i>Ap varanesis</i> L34320	49	49	49	49	49	49	49	46	50	53	54	50	52	48	41	39	51	47	36				
21 <i>Am tuberculatum</i> U95856	50	50	50	50	50	50	47	48	48	58	59	56	55	47	49	49	52	43	43	44			
22 <i>R bicornis</i> L34304	47	47	47	47	47	47	54	44	49	54	53	53	60	57	50	51	56	58	48	49	57		
23 <i>D gallinae</i> L34326	94	94	94	94	94	94	97	92	97	106	107	100	102	97	93	95	96	97	87	92	101	82	



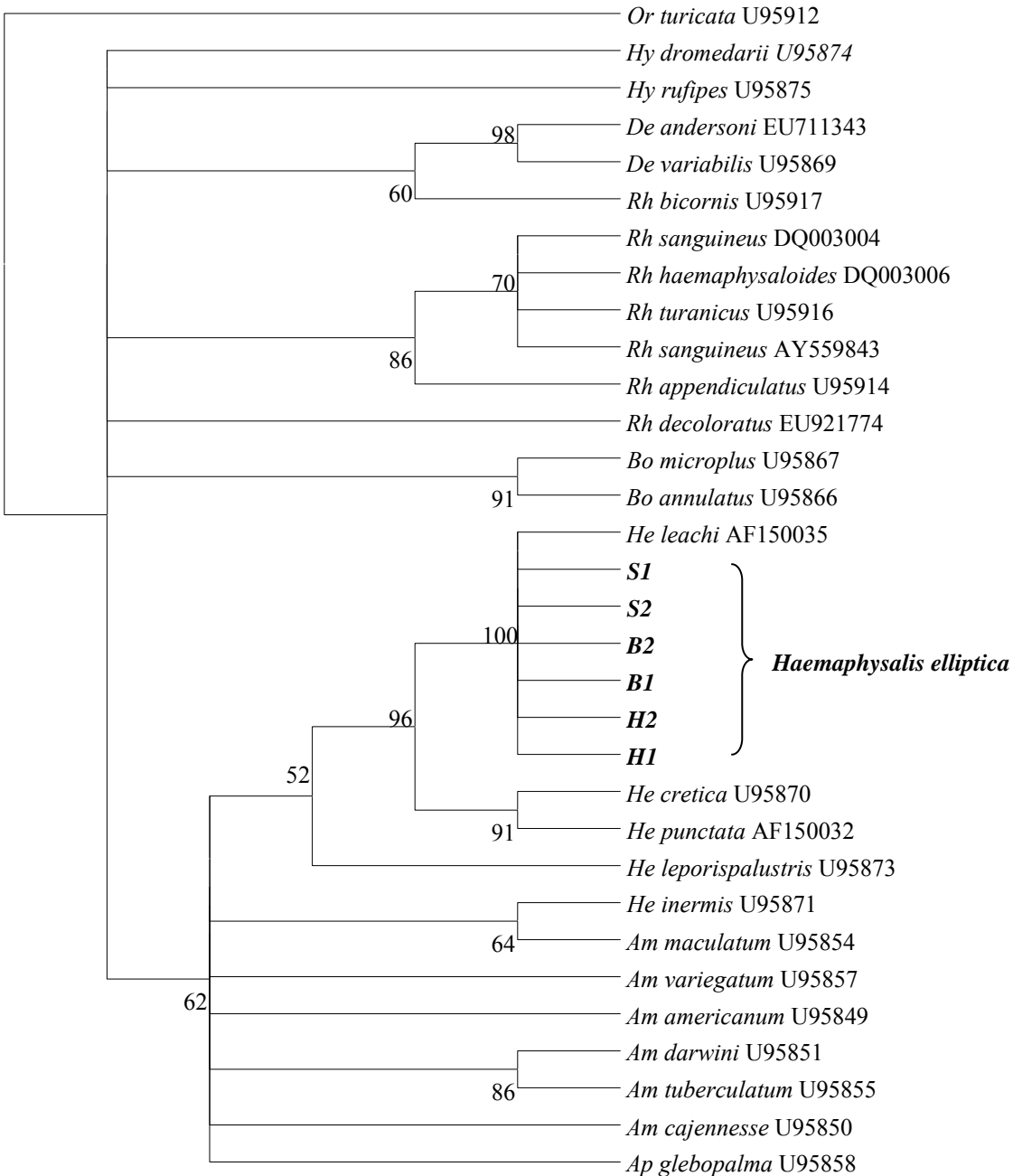
**Fig. 4:** Phylogenetic relationship of mitochondrial 12S rDNA genes of *H. elliptica*, with *Ornithodoros turicata* as an out-group. The tree was constructed and analysed with the Maximum likelihood



**Fig. 5:** Phylogenetic tree based on distance analysis constructed from the sequencing results of mitochondrial 12S rDNA genes of *H. elliptica*, with *Ornithodoros turicata* as an out-group



**Fig. 6:** Phylogenetic tree based on neighbour joining constructed from the sequencing results of mitochondrial 12S rDNA genes of *H. elliptica*, with *Ornithodoros turicata* as an out-group



**Fig. 7:** Phylogenetic relationship of mitochondrial 12S rDNA genes of *H. elliptica*, with *Ornythodoros turicata* as an out-group. The tree was constructed and analysed with the parsimony method with 1000 bootstrap replicates. Percentage of reliability of each branch of the tree was indicated as numbers at the nodes. Branch lengths are drawn proportional to the estimated sequence divergence

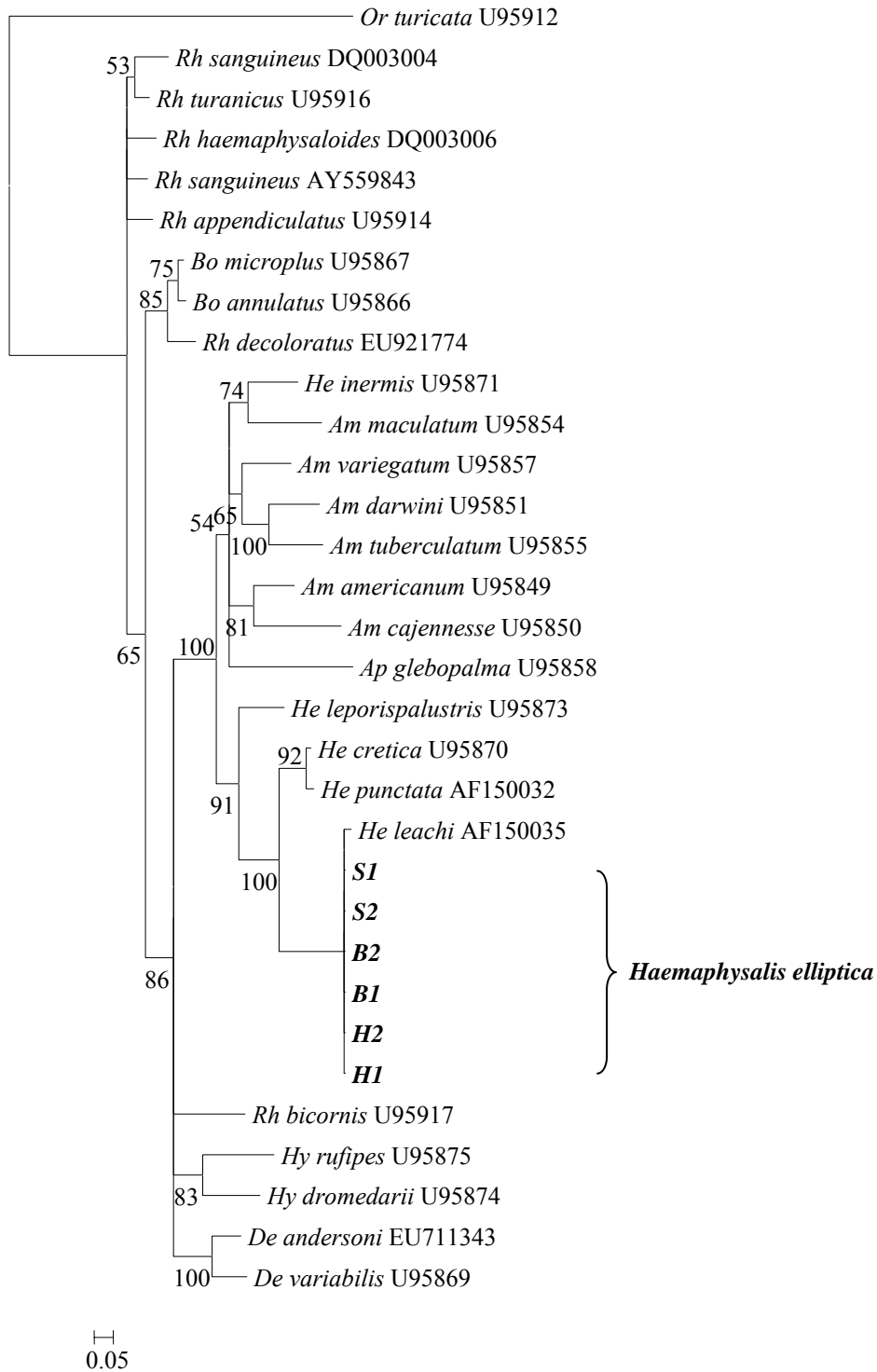
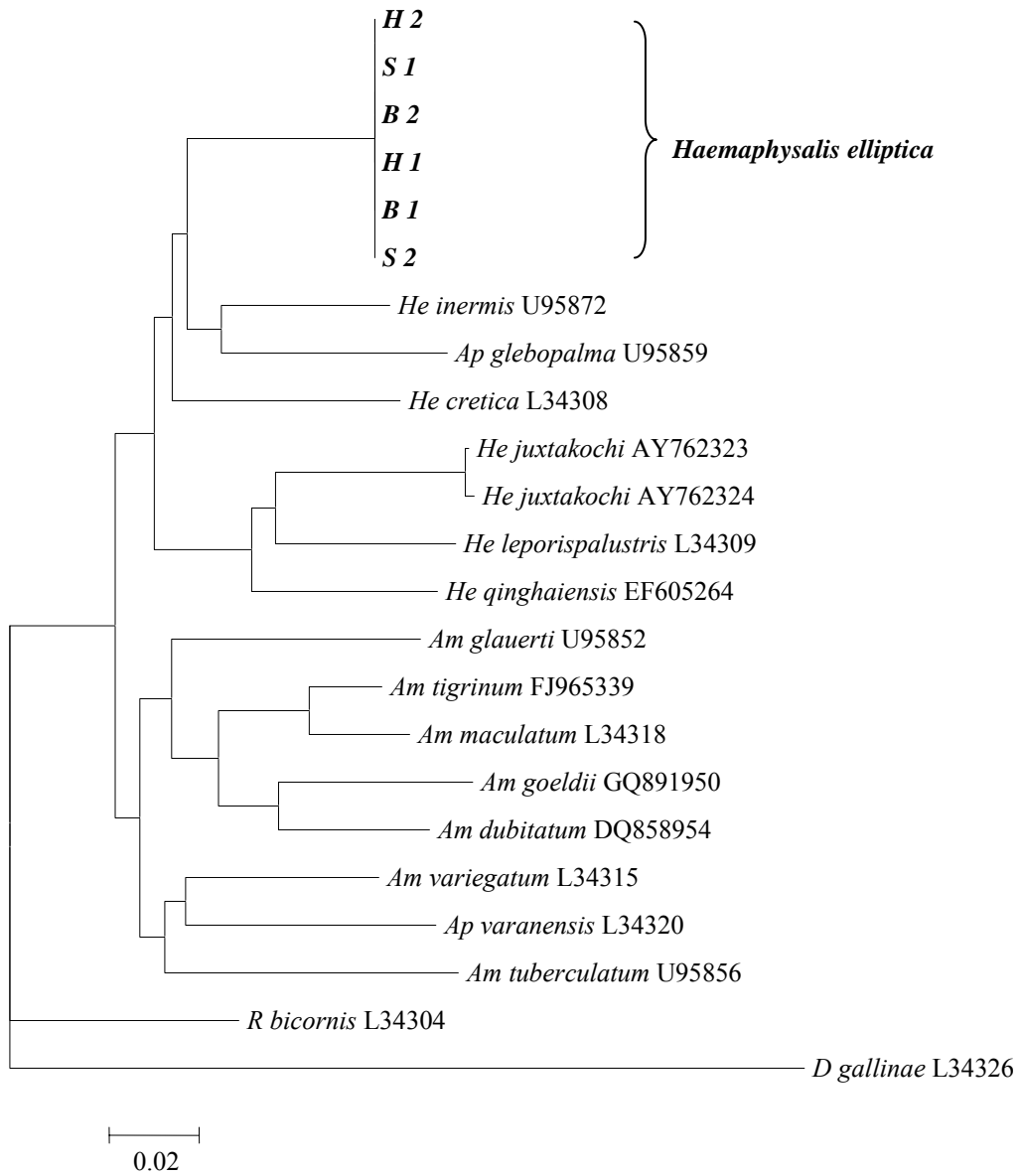
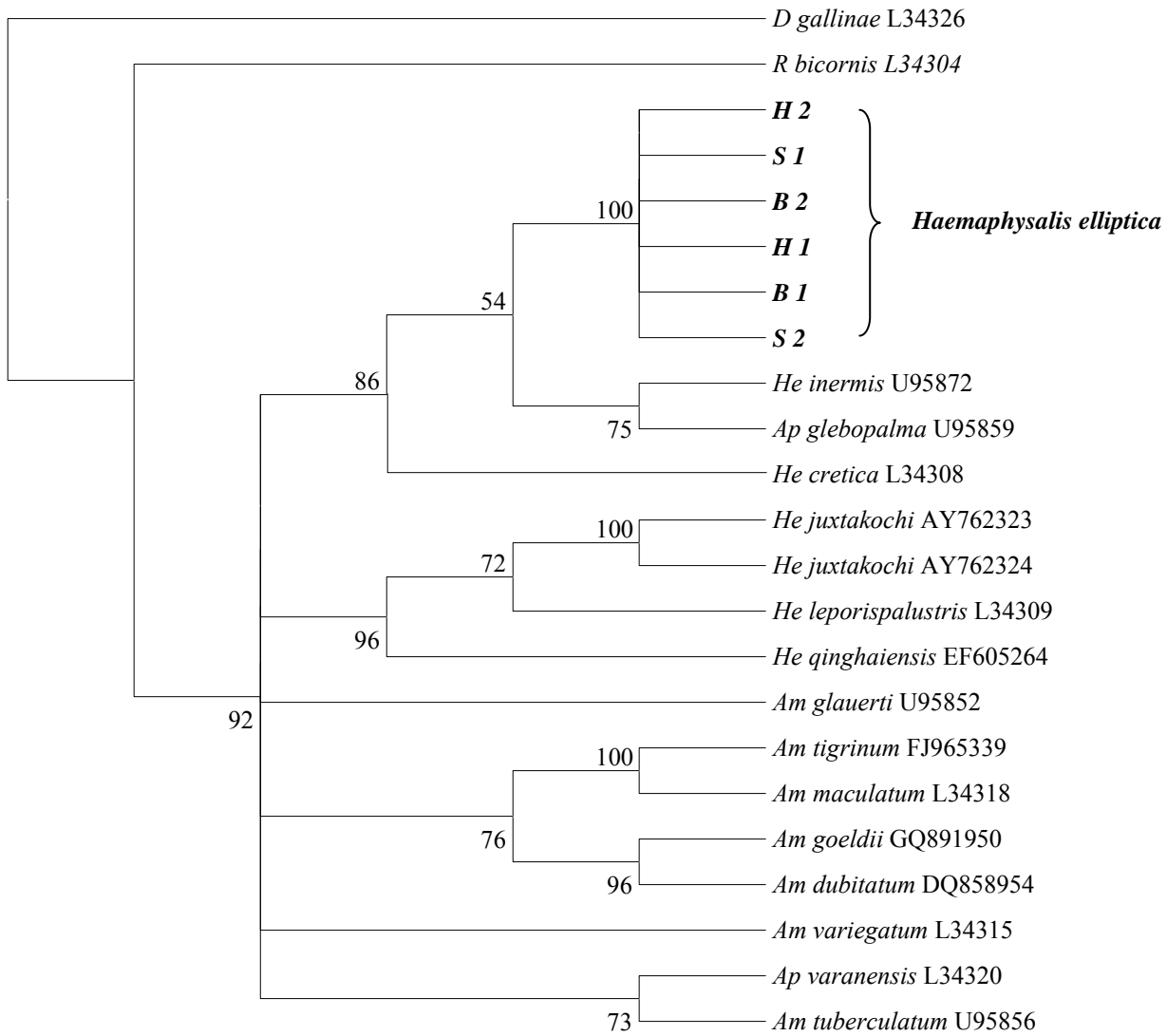


Fig. 8: Phylogenetic tree of mitochondrial 12S rDNA genes of *H. elliptica*, with *Ornithodoros turicata* as an out-group constructed with Bayesian analysis



**Fig. 9:** Phylogenetic relationship of mitochondrial 16S rDNA genes of *H. elliptica*, with *Dermatomyssus gallinae* as an out-group was analysed with Maximum likelihood tree



**Fig. 10:** Phylogenetic relationship of mitochondrial 16S rDNA genes of *H. elliptica*, with *Dermanyssus gallinae* as an out-group. The tree was constructed and analysed with the parsimony method with 1000 bootstrap replicates. Percentage of reliability of each branch of the tree was indicated as numbers at the nodes. Branch lengths are drawn proportional to the estimated sequence divergence

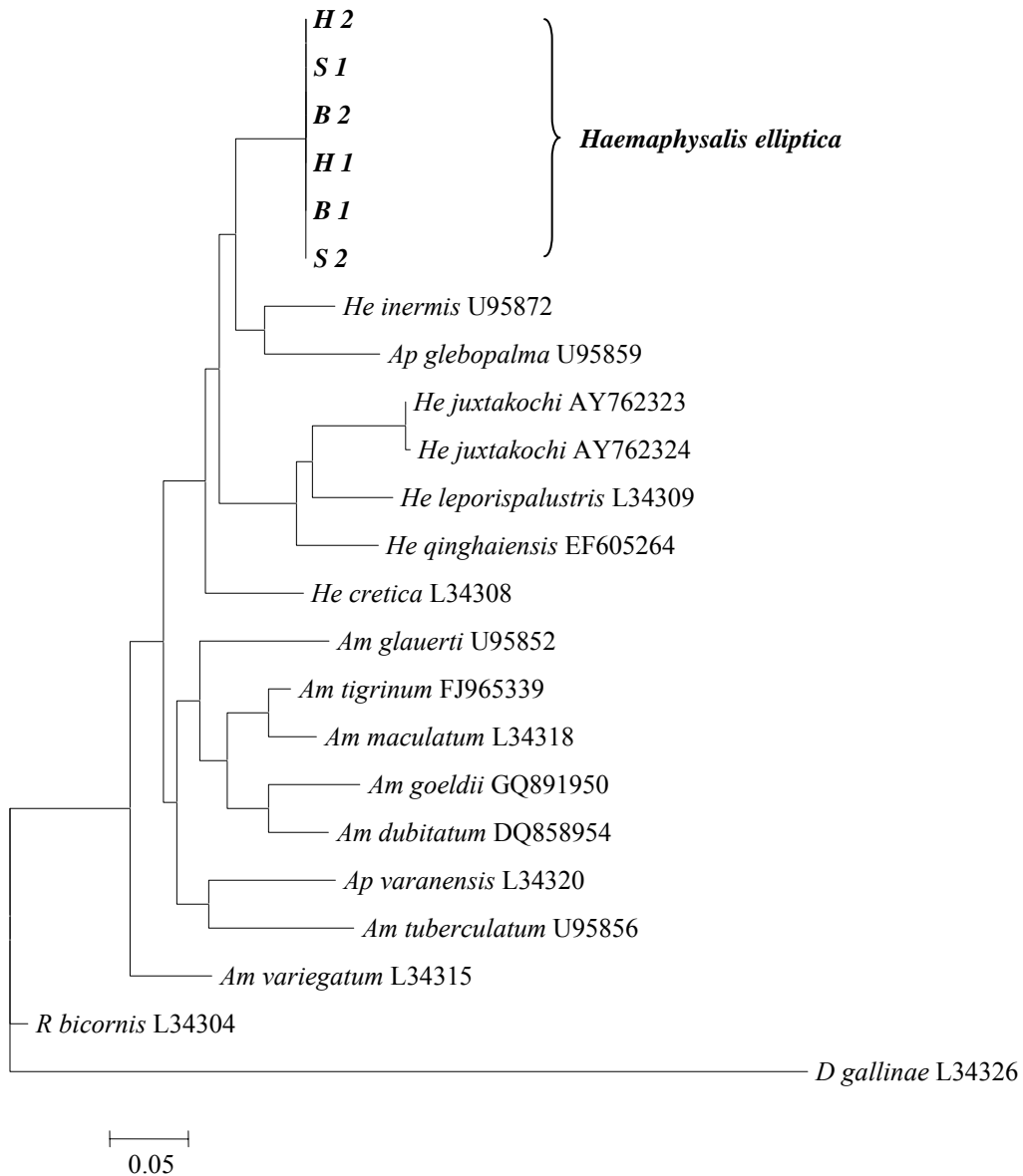


Fig. 11: Phylogenetic tree based on a sequence distance analysis constructed from the sequencing results of mitochondrial 16S rDNA genes of *H. elliptica*

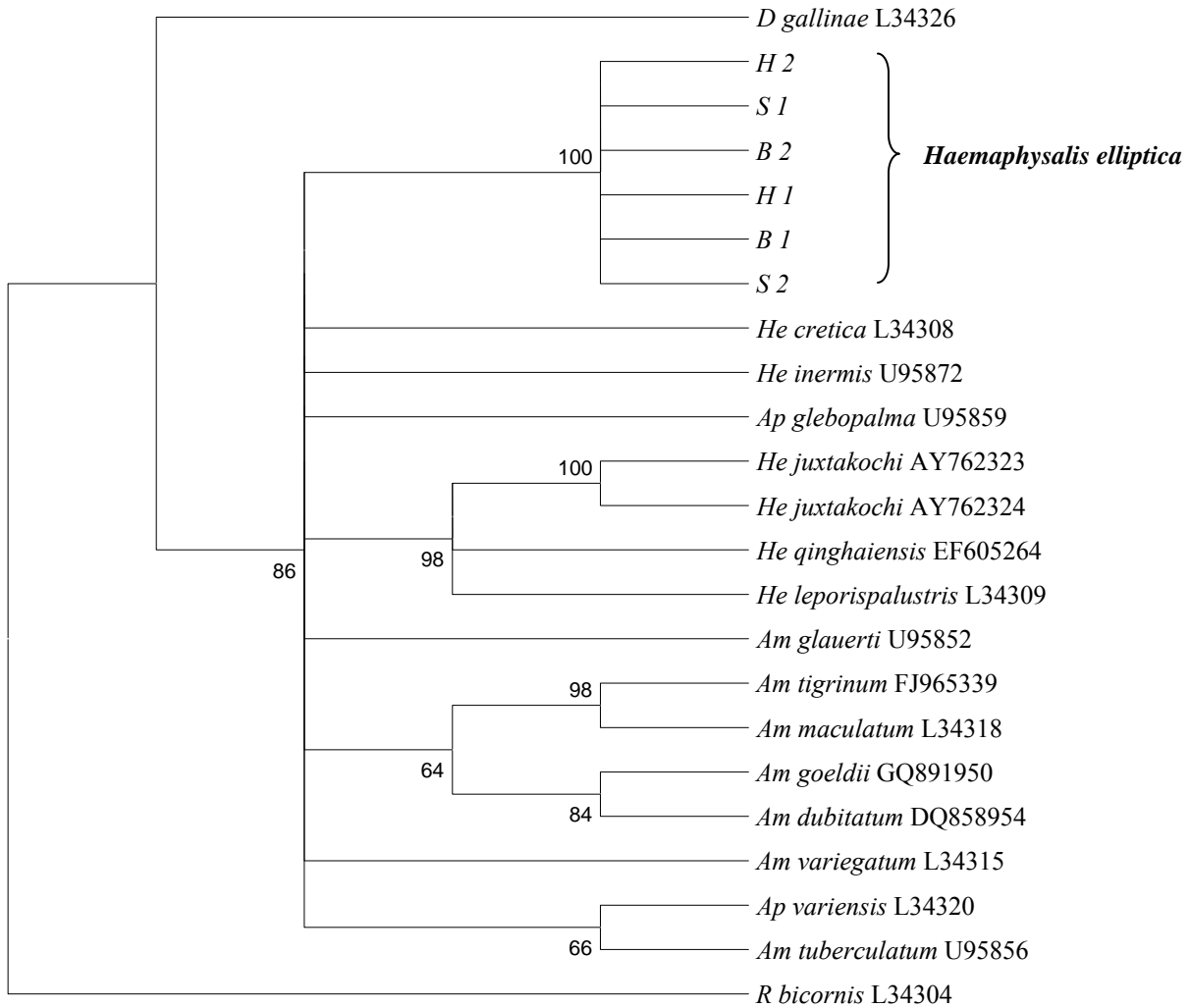


Fig. 12: Phylogenetic tree based on a sequence neighbour joining constructed from the sequencing results of mitochondrial 16S rDNA genes of *H. elliptica*, with *Dermanyssus gallinae* as an out-group

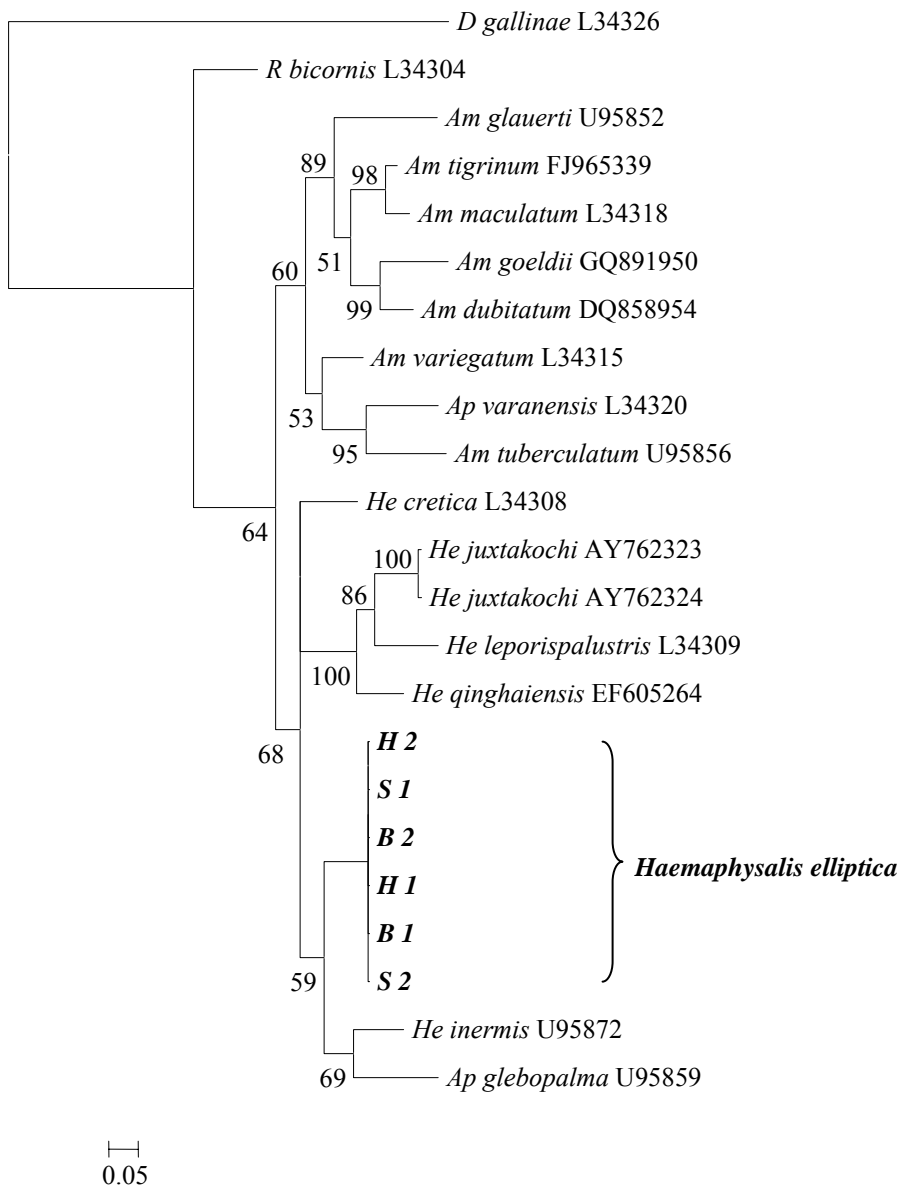


Fig. 13: Phylogenetic tree of mitochondrial 16S rDNA genes of *H. elliptica*, with *Dermanyssus gallinae* as an out-group constructed with Bayesian analysis

## Discussion

The phylogeny of *H. elliptica* was inferred from the subfamilies Haemaphysalinae using 12S and 16S rRNA mitochondrial gene sequences. This study describes the first genetic classification of the mitochondrial 12S and 16S ribosomal genes among *H. elliptica* ticks collected at various cheetah breeding centers. The support of the bootstrap values presented here showed that both above-mentioned genes contain phylogenetic information that determines parts of the phylogeny of the ixodid tick genus.

The phylogeny of the Ixodida has been studied in recent years (Balashov, 1994; Klompen, Black, Keirans & Norris, 2000; Barker & Murrell, 2004). The phylogenetic relationships in *Ixodes* ticks can be determined by analysing the sequence heterogeneity of the mitochondrial 12S and 16S rRNA genes (Caporale, Rich, Spielman, Telford & Kocher, 1995; Rich, Caporale, Telford, Kocher, Hartl & Spielman, 1995). Among Metastriata ticks, these relationships are similar to those obtained using mitochondrial and nuclear rRNA genes in previous studies (Black & Piesman, 1994; Black *et al.*, 1997). Being genetically highly conserved, the mitochondrial 12S as well as 16S sequences provide a reliable and convenient tool for detecting the lineages of ixodid tick populations. Due to the high homoplasy in evolving genes (Simon *et al.*, 1994), the mitochondrial 16S rRNA gene is recognised as an outstanding marker for studying the genetic relationship among closely related tick species (Black & Piesman, 1994; Norris *et al.*, 1996; Norris *et al.*, 1999).

The Amblyomminae and Haemaphysalinae were initially not grouped together, as there was a preference for putting Haemaphysalinae in a lineage with Hyalomminae and Rhipicephalinae, based on the variations in the morphological features (Hoogstraal & Aeschlimann, 1982). Most *Haemaphysalis* ticks, like *Rhipicephalus* ticks, possess short palps with femur on the lateral margin of the capitulum, whereas *Amblyomma* and *Hyalomma* tick species have long palps. Studies on the 12S and 16S rDNA genes revealed a monophyletic relationship among species in the subfamily Amblyomminae. The phylogenetic studies based on morphology, life history and host association showed that *Rhipicephalus* and *Hyalomma* species shared a common branch on the tree (Black & Piesman, 1994).

The distribution of *Haemaphysalis* species was previously shown to be basal to that of the Hyalomminae-Rhipicephalinae group (Mangold, Bargues & Mas-Coma, 1998). However, the Haemaphysaline species which we studied fell into one well-supported clade in each phylogenetic tree, supporting the findings that *H. elliptica* can be grouped with other *Haemaphysalis* species according to the closeness of its relationship with other species within the genus. Our combined analysis of these gene sequences indicated that, similar to *Rhipicephalus* (Murrell, Campbell & Barker, 2000), the genus *Haemaphysalis* is paraphyletic.

The phylogenetic trees constructed by parsimony analysis showed no discrimination among the ticks collected from the various localities, suggesting absence of different lineages. The possibility of genetic variation was not ruled out in this study, however. Despite four nucleotide differences observed in the aligned sequences of *H. leachi* and *H. elliptica*, the parsimony tree grouped them together with 100% reliability. Distribution of *H. leachi* overlaps with that of *H. elliptica* from East Africa to northern Zimbabwe (Apanaskevich *et al.*, 2007). The partial sequence of mitochondrial 12S ribosomal DNA gene of *H. (R.) leachi* was initially obtained by Beati and Keirans (2001). It is not confirmed whether the specimen was identified correctly as true *H. leachi*. In case of possible misidentification, however, the close grouping can explain an inter-species variation. On the other hand, the phylogenetic analysis of 12S and 16S ribosomal DNA genes showed *Haemaphysalis inermis* grouped with the genera *Amblyomma* and *Aponemma* in a clade, indicating possible misidentification of this species.

In conclusion, our comparison of the mitochondrial 12S and 16S rDNA sequences of *H. elliptica* ticks permitted quantitative assessment of their relatedness to other tick species. This study provided the first genetic characterization of the mitochondrial DNA sequences of *H. elliptica* in South Africa, since mDNA contributes a suitable means for classifying ticks regarding the geographic variation and reproductive compatibility. Based on the sequence divergence of the examined genes, all the *H. elliptica* ticks at various cheetah breeding centers were genetically related and formed a monophyletic group.

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## Chapter 7:

### Risk factors for infection with *Babesia* species at various cheetah breeding centres in South Africa

#### Abstract

A total of 103 cheetahs were studied in terms of characterization associated with the animals and groups (prevalence of *Babesia* species infection, location, gender, age and tick burdens) at two sites belonging to the De Wildt Cheetah Breeding Centre in South Africa. The V4 hypervariable region of the 18S rRNA gene was amplified by PCR. A large number (58%) of cheetah blood samples tested positive for different *Babesia* species. They produced 500 bp DNA fragments specific for cheetah-associated *Babesia* species gene as well as hybridization signals with relevant *Babesia* probes on the RLB assay which indicated a high risk of *Babesia* species in cheetahs. A total of 1,137 adult ticks identified as *Haemaphysalis elliptica* were recovered from cheetahs at the two localities. Multiple logistic regression model revealed that cheetahs with high tick burdens were at far greater risk, compared to those with low tick burdens (odds ratio = 32;  $P < 0.001$ ) and those with medium tick burdens (odds ratio = 12;  $P = 0.002$ ). Regarding the tick burden and locality, the risk of infection with *Babesia* species was significantly higher as the cheetahs aged ( $P = 0.039$ ). There were no significant effects of gender or locality on risk of harbouring *Babesia* species. The Hosmer-Lemeshow goodness-of-fit test statistic indicated adequate fit of the model. The results strongly emphasize that cheetah-associated babesiosis has a tick-borne integrity.

## Introduction

With increasing pressure to understand transmissible agents, redescription of infectious pathogens causing diseases is taking place. A multitude of tick-transmitted pathogens, with worldwide distribution, represents a growing risk to animal populations and may pose important problems for health and management (George, Davey & Pound, 2002). These pathogens are maintained in invertebrate vectors, and also cycle in vertebrate hosts. The relationships between vectors and hosts are fundamental for successful transmission of the pathogen, from generation to generation.

In recent years, molecular detection of pathogenic microorganisms in ixodid ticks has been based on DNA amplification of the target pathogen. Utilizing a variety of molecular primers, probes and performing the polymerase chain reaction, the presence of multiple pathogens in ticks has been more evident (Sparagano, Allsopp, Mank, Rijpkema, Figueroa & Jongejan, 1999). More than a hundred tick-borne haemoprotozoan species of the genus *Babesia* infect a wide variety of animals, worldwide (Levine 1985; Piesman, 1987) and of the growing list of *Babesia* species, only a few prefer felids (Bosman, Venter & Penzhorn, 2007).

There is a sharp rise in tick populations across the globe due to either human-caused landscape alterations, poorly performed pesticide management (Jongejan & Uilenberg, 2004) as well as acaricide resistance (Nolan, 1990). Identification and knowledge of the life history of parasites of wildlife is imperative for the implementation of satisfactory control measures. Furthermore, establishing baseline data and pathogenicity of parasites is necessary for recovery and re-introduction programmes. Ixodid tick species can increase the potential for co-infections in vertebrate hosts by harbouring multiple disease agents (Jongejan & Uilenberg, 2004). As a result, knowledge of geographic range as well as seasonal activity of vector ticks is important for determining the possible risks of acquiring tick-borne infections. The majority of tick species adapt to a highly specific habitat and dynamically seek preferred hosts, preferably during that period of the year which they have the capability of transmitting the pathogen (Sonenshine, 1994), resulting in a well-established association between spatial and temporal distribution of vector ticks and tick-borne diseases (Fritz & Kjemtrup, 2003).

Generally babesiosis is a natural consequence of a protozoal infection transmitted through the bite of an ixodid tick in the tropical and subtropical regions of the world, therefore its occurrence is linked to the size of the tick population and the seasonality of the vectors (Hostis & Seegers, 2002). Many factors, including parasites, hosts and vectors can contribute to emergence of babesiosis, however, the re-emergence of babesiosis is often related to changes in vector control strategies, drug-resistant parasites or migration of hosts (Molyneux, 1998; Harrus & Baneth, 2005). The risk of being infected with *Babesia* species depends on the presence of the potential vector. Specific measures are therefore required to reduce the risk of tick bites and infection. Disease risk can often be related to the population fluctuation of a single host (Ostfeld & Keesing, 2000). In general, various variables may be associated with an increased risk of disease or infection in a defined population (Aktas, Altay & Dumanli, 2007; Raghavan, Glickman, Moore, Caldanaro, Lewis & Glickman, 2007). Risk factors are correlated to the occurrence of the disease but it does not mean that they are necessarily causal.

The prevalence of *Babesia ovis* in relation to the parameters describing the characteristics of the animals (species, age and tick burden) was monitored in sheep and goats (Aktas *et al.*, 2007). Results showed that the prevalence of *B. ovis* infection in relation to age of sheep were not different, as the statistical significance was defined at  $P > 0.05$ . However, there was a positive association between the prevalence of *Babesia* infection and tick burden ( $P < 0.05$ ). Comparable results were also obtained in a similar study on sheep and goats (Theodoropoulos, Gazouli, Ikononopoulos, Kantzoura & Kominakis, 2006). The statistical analysis of the data was indicative of the association between the presence of ticks and an animal testing positive for *Babesia*.

Studies on the frequency of *Babesia* species in cheetahs in South Africa are very limited. Taking into account the limitations of the conventional diagnostic methods, we determined the prevalence of the infection with *Babesia* species in South Africa by polymerase chain reaction (PCR) and Reverse Line Blot (RLB) hybridization assay. The aim of this study was to identify the possible associations between the likelihood of infection with *Babesia* species in captive cheetahs and various factors such as geographical location, gender, age and tick burdens of the hosts.

## Materials and methods

### 1. *Study localities and period*

The study was conducted during 2008 at two locations, namely the Ann van Dyk Cheetah Breeding Center-De Wildt/Brits and the Ann van Dyk-De Wildt Cheetah Breeding Center-De Wildt/Shingwedzi. The De Wildt/Brits inhabits 81 cheetahs (38 males and 43 females) with ages ranging from 3 to 13 years. Whereas, the De Wildt/Shingwedzi had a population of 22 cheetahs (10 males and 12 females). The youngest is 2 and the oldest is 6 years old. The cheetahs are kept individually in separate camps. They are regularly sprayed against ticks. For the purpose of the study, the treatment against tick infestation was halted.

### 2. *Tick sampling*

Two cheetah populations were considered for this study. A once-off tick-sampling from all individual cheetahs was performed ([Chapter 3](#)). For this purpose, the skin around the neck, shoulders, the perineal region and tail where the ticks mostly attach were assessed for the presence of tick infestation visually and also by palpation ([Bryson, Horak, Höhn & Louw, 2000](#)). To minimize stress to the cheetahs and prevent possible injuries to the researcher, wooden sticks were used to restrain the animals individually in a cage. The ticks were then manually removed and placed in labelled glass vials containing 70% ethanol. The collected ticks were identified to the species level according to various morphological features using a stereoscopic microscope ([Walker, Keirans & Horak, 2000](#); [Walker, Bouattour, Camicas, Estrada-Peña, Horak, Latif, Pegram & Preston, 2003](#)). They were subsequently counted as described in [Chapter 4](#).

### 3. *Blood sampling and molecular analysis*

A total of 103 whole blood samples in tubes with EDTA ([BD Vacutainer™](#) tubes, [Franklin Lake, USA](#)) were collected from individual cheetahs in both populations two weeks post tick-sampling. The tubes were transported in a cooler box to the molecular biology section in the Department of Veterinary Tropical Diseases at the Faculty of Veterinary Science, where they were processed by molecular techniques to detect *Babesia* species. Extraction of DNA was performed from 200 µl of whole blood using the [QIAamp® DNA Mini Extraction Kit](#) (Qiagen, Hilden, Germany), according to the method previously described in [Chapter 4](#).

The PCR was performed in a 9600 Perkin-Elmer touchdown thermocycler (Applied Biosystems, South Africa) in a total reaction volume of 25 µl containing enzyme platinum<sup>®</sup> Quantitative PCR Supermix-UDG and 20 pmol of each universal forward and reverse RLB primer and 2.5 µl of template DNA under the conditions described in chapter 4. A blood sample from a *Babesia*-positive cheetah (previously diagnosed via molecular tests) and distilled water were used as the positive and negative control, respectively, to validate the PCR. The PCR products were evaluated by agarose gel electrophoresis. Presence of *Babesia* species infection was consequently indicated by RLB assay.

#### 4. Data analysis

The main objective of the statistical analysis was to investigate the possible association between tick burden and presence of *Babesia* species infection. Other potential risk factors recorded were the locality, cheetah's age and gender. These data were obtained during sample collection. The association between age and tick burden was assessed using Spearman rank correlation. Tick burden was then categorized into terciles (low = 0-4; medium = 5-18; high = >18), and the prevalence of infection with *Babesia* species was initially compared between categories using a two-tailed Fisher's exact test (Thrusfield, 2005). Age was dichotomized ( $\leq 6$  years;  $> 6$  years) and the associations of age, gender and locality with the frequency of *Babesia* species isolation, as well as the associations of age, gender and locality with tick burden, were assessed by cross-tabulation and using Fisher's exact test. In order to account for possible confounding, the association between tick burden and *Babesia* species isolation was then estimated using a multiple logistic regression model, adjusting for age, gender and locality. Age was modelled as a continuous variable, because the prevalence of *Babesia* species isolation increased monotonically with increasing terciles of age. The fit of the logistic regression model was assessed using the Hosmer-Lemeshow goodness-of-fit test. A significance level of  $\alpha = 0.05$  was used. All statistical analyses were done using Stata 10.1 (Stata Corporation, College Station, TX, U.S.A.).

## Results

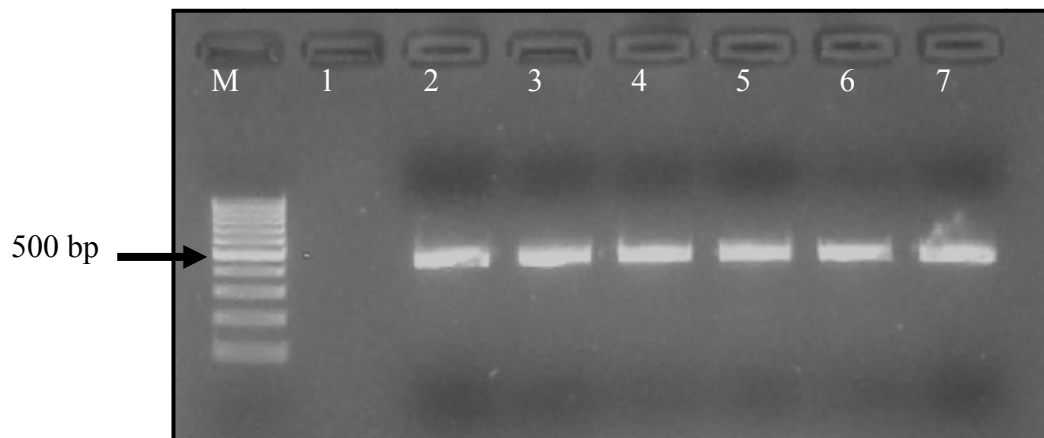
The prevalence of *Babesia* species in two captive cheetah populations in South Africa was determined using various molecular techniques. RLB primers successfully amplified the V4 hypervariable region of the target gene, the small subunit of the 18S rRNA, as a fragment of ~500 bp was produced on gel electrophoresis (Fig. 1). No DNA contamination was observed in the negative control. The PCR products obtained from 60 animals (58%) hybridised with the *Babesia* genus and/or species-specific RLB probes, whereas 43 animals (42%) were negative since they failed to amplify and to hybridise to any of the probes (Fig. 2). Forty-eight cheetahs (59%) and 12 cheetahs (54.5%) were positive at De Wildt/Brits and De Wildt/Shingwedzi, respectively (Table 1).

Tick-sampling showed that amongst the cheetahs in both populations, only 26 cheetahs harboured no ticks whereas the other 77 cheetahs harboured one or more ticks. A total of 1,137 ixodid ticks (924 at De Wildt/Brits and 213 at De Wildt/Shingwedzi) were recovered from cheetahs. All tick specimens were adults. The distribution of terciles of tick burden of cheetahs at the two localities (Brits and Shingwedzi) is shown in Table 4.

Comparison between the presence of *Babesia* species in blood samples and ticks on the skin surface of cheetahs indicated that the frequency of *Babesia* species infection in cheetahs was higher in animals with ticks present (66%) than in those without ticks (35%) ( $P = 0.006$ ) (Table 5). Moreover, the prevalence of infection increased with increasing tick burden (Table 6). The prevalence of infection in cheetahs did not differ significantly between locations or genders (Tables 2 & 3), but older cheetahs (> 6 years) had a higher prevalence of infection than younger ones ( $P = 0.02$ ) (Fig. 4). There was no significant association between age category and tick burden ( $P = 0.42$ ). A scatter plot of tick burden vs. age is shown in Fig. 3. Despite the slight increase in number of ticks as the cheetahs aged, there was a wide range of tick burdens at all ages and no significant linear relationship between the two variables was evident (Spearman's  $r = 0.133$ ;  $P = 0.182$ ).

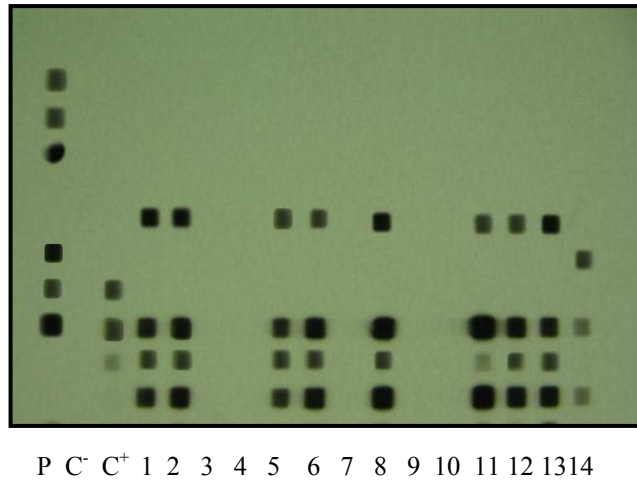
The results of the multiple logistic regression model are shown in Table 7. Adjusted for age, gender and locality, risk of *Babesia* species infection increased with increasing tick burden ( $P < 0.001$ ). There was a tendency for animals with medium tick burdens to be at greater risk of

harbouring *Babesia* species than those with low tick burdens (odds ratio = 2.65;  $P = 0.058$ ). Animals with high tick burdens were at far greater risk than both those with low tick burdens (odds ratio = 32.24;  $P < 0.001$ ) and those with medium tick burdens (odds ratio = 12.2;  $P = 0.002$ ). Adjusted for tick burden, gender and locality, the risk of harbouring *Babesia* species also increased significantly with increasing age of the cheetahs ( $P = 0.039$ ). There were no significant effects of gender or locality on risk of harbouring *Babesia* species (Table 7). The Hosmer-Lemeshow goodness-of-fit test statistic indicated adequate fit of the model.



**Fig. 1:** Detection of *Babesia* sp. in infected blood samples by PCR. Lane M: 100 bp DNA Ladder; Lane 1: water (negative control); Lane 2: piroplasm DNA obtained from infected cheetah blood (positive control); Lane 3 – 7: individual infected blood samples with *Babesia* species

*Babesia vogeli*  
*Babesia microti*  
*Babesia canis*  
*Cytauxzoon felis*  
*Babesia lengau*  
*Babesia felis*  
*Babesia rossi*  
*Theileria* and *Babesia* – genus specific  
*Babesia* II – genus specific  
*Babesia* I – genus specific



**Fig. 2:** Reverse line blotting (RLB) products positive for the specific oligonucleotides for *Babesia* species. Lanes represent as P: RLB plasmid control, C<sup>-</sup>: negative control, C<sup>+</sup>: positive control (*Babesia rossi*); 1-14: cheetah blood samples

**Table 1:** Association between the prevalence of *Babesia* species and the locations at the Ann van Dyk Cheetah Breeding Centers-De Wildt (Brits and Shingwedzi)

<i>Babesia</i> species	Locations	
	Brits	Shingwedzi
Positive	48 (59%)	12 (54.5%)
Negative	33 (41%)	10 (45.5%)
Number of cheetahs	81	22

Fisher's exact  $P = 0.81$

**Table 2:** Association between the prevalence of *Babesia* species and the gender of cheetahs at the Ann van Dyk Cheetah Breeding Centers-De Wildt

<i>Babesia</i> species	Gender	
	Male	Female
Positive	27 (56.2%)	33 (60%)
Negative	21 (43.8%)	22 (40%)
Number of cheetahs	48	55

Fisher's exact  $P = 0.84$

**Table 3:** Association between the prevalence of *Babesia* species and the age of cheetahs

<i>Babesia</i> species	Age	
	$\leq 6$ years	$> 6$ years
Positive	25 (46.3%)	35 (71.4%)
Negative	29 (53.7%)	14 (28.6%)
Number of cheetahs	54	49

Fisher's exact  $P = 0.02$

**Table 4:** Frequency of tick infestation on cheetahs at Ann van Dyk Cheetah Breeding Centers-De Wildt

<b>Classification of tick burdens</b>			
	<b>De Wildt/Brits</b>		
	Low (0 – 4)	Medium (5 – 18)	High (>18)
Number of cheetahs	28	27	26
<b>De Wildt/Shingwedzi</b>			
Number of cheetahs	9	9	4

**Table 5:** Association between the prevalence of *Babesia* species and the tick burden of cheetahs

<b><i>Babesia</i> species</b>	<b>Tick burden</b>	
	<b>No tick</b>	<b>One or more ticks</b>
Positive	9 (35%)	51 (66%)
Negative	17 (63%)	26 (34%)
Number of cheetahs	26	77

Fisher's exact  $P = 0.006$

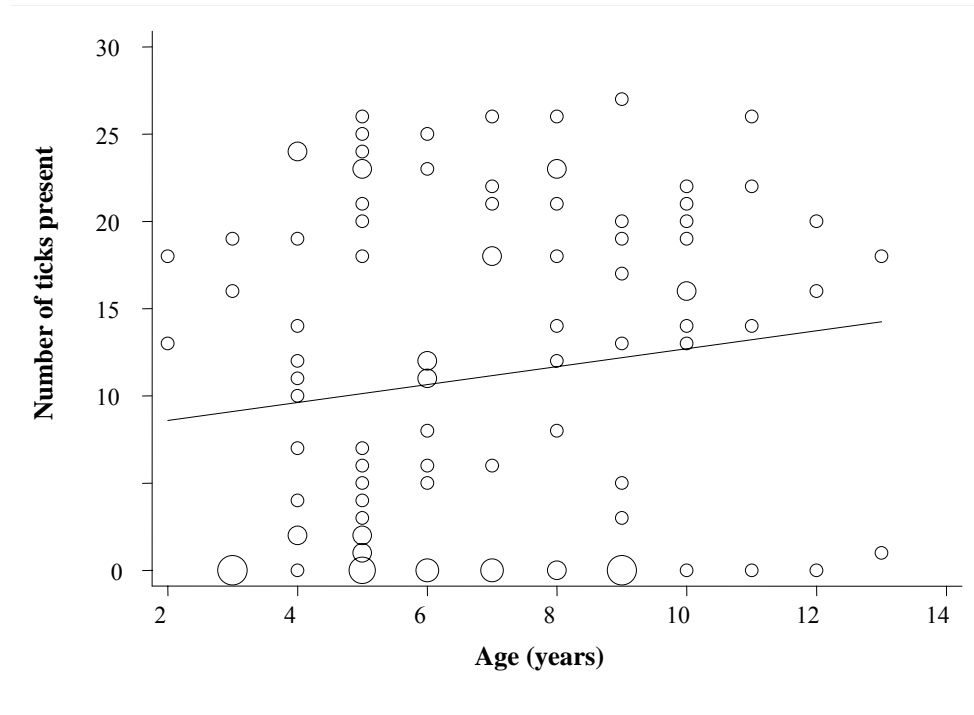


Fig. 3: Scatter plot of number of ticks present vs. age of cheetah. Area of circle is proportional to the number of data points. Spearman's  $r = 0.133$  ( $P = 0.182$ )

Table 6: Frequency of isolation of *Babesia* species from cheetahs grouped by terciles of tick burdens

Tick burden	<i>n</i>	Prevalence of <i>Babesia</i> species (%)
Low (0 - 4)	37	32.4 <sup>a</sup>
Medium (5 - 18)	36	55.6 <sup>a</sup>
High (>18)	30	93.3 <sup>b</sup>
Total	103	58.3

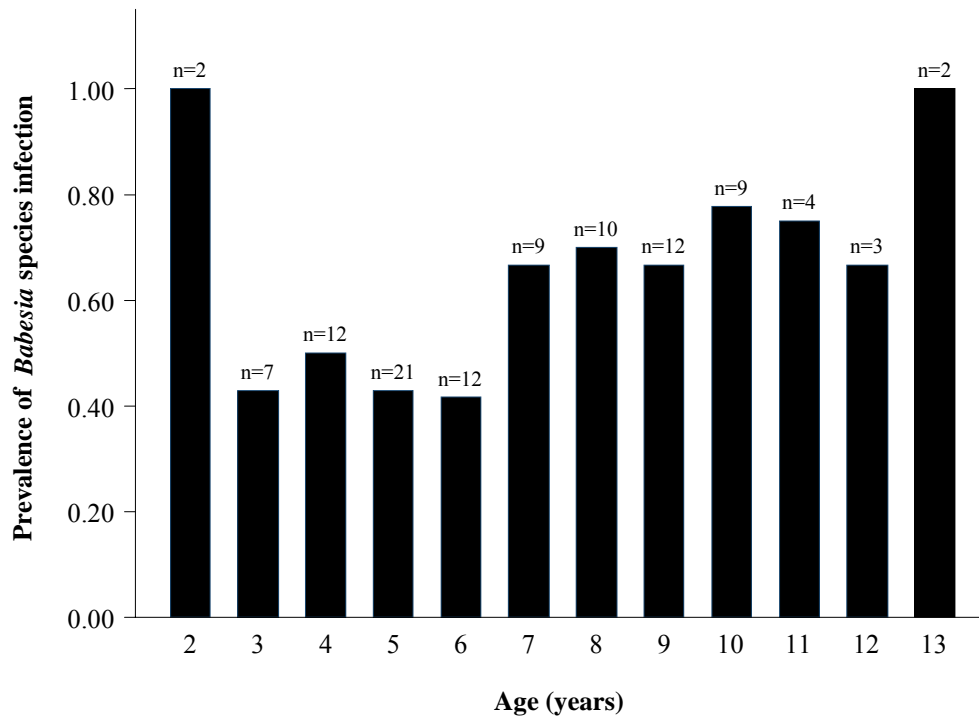
<sup>a,b</sup> Values with differing superscripts differ significantly ( $P < 0.05$ , Fisher's exact test)

**Table 7:** Risk factors for isolation of *Babesia* species from cheetahs: results of a multiple logistic regression model

Variables	Odds ratio	95% confidence interval	P-value
Tick burden			
Low (0 – 4)	1*	-	-
Medium (5 - 18)	2.65	0.97, 7.25	0.058
High (>18)	32.24	6.26, 166	<0.001
Age	1.26	1.01, 1.57	0.039
Gender (male vs. female)	0.78	0.30, 2.04	0.611
Locality (Brits vs. Shingwedzi)	0.42	0.12, 1.55	0.195

Hosmer-Lemeshow goodness-of-fit  $\chi^2 = 4.67$  (8 d.f.),  $P = 0.793$

\* Reference level



**Fig. 4:** Bar graph indicating the rate of babesial infection vs. age of cheetahs

## Discussion

The present study reports the association of potential risk factors with prevalence of *Babesia* species in captive cheetahs. Cheetah-associated *Babesia* species as a tick-borne pathogen remains a potential problem in the management of free-ranging and captive cheetah populations (Bosman, Oosthuizen, Peirce, Venter & Penzhorn, 2010). Despite the lack of information on the epidemiology of the cheetah-associated *Babesia* species in South Africa, it is believed that the frequency of infection is closely associated with the bioecology of the putative tick vector (Bosman *et al.*, 2010). Each cheetah in the present study served as a natural “trap animal” to collect questing (host-seeking) ticks at different locations. Two principles, suitable environment for the survival of the free-living stages and the presence of appropriate hosts in sufficient numbers, are required for tick populations to establish successfully in a region (Norval & Lightfoot, 1982).

A rise in the prevalence of a tick-borne pathogen is often expected in tick vector habitat areas (endemic areas) during the activity period of the tick. Ticks generally quest for hosts from a fixed vantage point on the vegetation (grass) at a preferred and desired height above the ground which places the tick in an advantageous position to make contact with the passing host (Fourie & Kok, 1992). By this definition, the rate of tick infestation is associated with the distribution of ticks as well as area inhabited by the host.

Tick numbers harboured by individual animals may vary considerably in a population, compared to the average number for the group, as our results showed that some individual cheetah may tend to have either higher or lower numbers of ticks. Although a number of factors at temporal and spatial scales influence this complex system, temporal overdispersion in tick infestations of a host may arise from diversity in the host’s ability to limit its tick burdens through various possible means such as immune response (Petney, Van Ark & Spickett, 1990; Hlatshwayo, Mbatlali & Dipeolu, 2002). Since captivity may be stressful for cheetahs and stress is suspected as a contributing factor to poor health and reproduction (Terio, Marker & Munson, 2004), the hormonal radioimmunoassay test may detect the status of immunosuppression. Given the cross-sectional nature of the study design, it also is possible that cheetahs that were already positive with *Babesia* species may have experienced altered immune function resulting in increased susceptibility to ixodid ticks. This is suggested by the presence of adult *A. hebraeum* ticks on

cheetahs, which is indicative of the environmental stress (Horak, Braack, Fourie & Walker, 2000).

The method of tick recovery from cheetahs was a modification of previously published methods (Horak, 1982; Bryson, Horak, Höhn & Louw, 2000). The count, therefore, reflected an estimated number of ticks harboured by each cheetah which would justify our categorizing the tick burdens into terciles rather than using actual counts. Factors such as host's body size, tick resistance (Johnston & Bancroft, 1918), host behavior (grooming) and habitat use (within and between host species) account for the differential susceptibility to tick infestation (Gallivan & Horak, 1997). Since tick burden is proportional to the host's body size, it is expected that larger animals harbour more ticks.

Among the variable risk factors examined in this study, the prevalence of ticks on cheetahs was associated with PCR-positive results, which indicates a high risk of infection with *Babesia* species in these animals. The prevalence of infection was 66% in the tick-infested animals. Irrespective of age, gender or locality (i.e. if these variables are held constant), tick burden was positively associated with prevalence of infection. The odds of infection in cheetahs with medium and high tick burdens were 2.65 and 32.24 times higher than that in those with low tick burdens, respectively. Since the prevalence of infection in our sample was high, these odds ratios are likely to overestimate the relative risk, but nonetheless they indicate a strong positive association between tick burden and risk of infection with *Babesia* species.

The logistic regression model showed the association of each individual predictor (ticks, age, gender and locality) with the outcome (*Babesia* species infection), adjusted for the effect of each of the other predictors in the model. The prevalence of infection in cheetahs did not differ significantly between locations or genders (Tables 2 & 3), but older cheetahs (> 6 years) had a higher prevalence of infection than younger cheetahs ( $P = 0.02$ ) (Fig. 3). There was no significant association between age category and tick burden (Fig. 4;  $P = 0.42$ ). Despite the slight increase in number of ticks as the cheetahs aged, there was a wide range of tick burdens at each age and no significant linear relationship between the two variables was evident (Spearman's  $r = 0.133$ ;  $P = 0.182$ ). Regardless of tick burden, age could be related to prevalence of infection, meaning that older cheetahs can have a higher prevalence of infection with *Babesia* species. This

consequently explains the fact that factors such as the senile-associated immunosuppression, environmental stress and various tick exposure may probably have contributed.

The present study was the first of its type to identify the association of certain potential risk factors with babesial infection in captive cheetah populations in South Africa. The finding that the prevalence of *Babesia* species infection was related to tick burden indicates a strong positive correlation between the prevalence of infection and presence of suspected vector ticks. These findings were of considerable interest, especially since at the time of study the cheetahs in both populations did not manifest clinical signs of infection with *Babesia* species. The results in this survey on cheetahs are consistent with those on ruminants ([Theodoropoulos \*et al.\*, 2005](#); [Aktas \*et al.\*, 2007](#)).

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## Chapter 8:

### General discussion

The results of the series of studies undertaken in this thesis demonstrated that the variety of tick species was very similar in the three cheetah populations, despite their geographic isolation. Similarities among *Babesia* species in the three cheetah populations were also evident. In general, local environmental conditions and vegetation types influenced the diversity of tick species at the various cheetah-breeding centres in South Africa. The similarity of the tick species diversity among different cheetah populations indicates that the number of tick species that can be supported, is a true attribute of the host (Hoogstraal & Aeschlimann, 1982). This implies the existence of a threshold of defence against parasites in a host species that limits the host's ability to cope with multiple parasite species. It is difficult to draw conclusions concerning ticks that have been collected from only a few individuals. As Cumming (1998) stated, however, there is a relationship between the number ticks occurring on a host and the number of times it is collected. This justifies the strict tick-control regimen followed at the breeding centres.

Ticks have been implicated as a source of disease for more than 100 years (Smith & Kilbourne, 1893). *Babesia* species go through a series of complex developmental stages to complete their

life cycles in the tick for transmission to take place. This development depends on an equilibrium and stability between the capability of the tick to launch a defence response against the parasite and the ability of the parasite to protect itself from the tick's immune response (Gray, 1982).

From the information about tick species collected through drag-sampling, patterns in the distribution of tick diversity amongst cheetah populations and geographical areas were clearly demonstrated. With the exception of *Rhipicephalus (Boophilus) decoloratus*, all the ticks collected from the study sites were three-host tick species, meaning that they require three different hosts to complete their life cycles. Three species, namely *Amblyomma hebraeum*, *R. (B.) decoloratus* and *R. zambeziensis*, primarily parasitise members of the family Bovidae. Among the collected species, instars of some prefer rodents and lagomorphs as hosts while adults quest for large mammals (Hoogstraal, 1956; Braack, Horak, Jordaan, Segerman & Louw, 1996; Mehlhorn, 2008). Ticks commence exploratory behaviour and, if on a suitable host, they commence attachment behaviour and insert their mouthparts into the host skin to begin feeding. Under favourable conditions, the life cycle of three-host ticks, from the time of hatching of the larvae to the hatching of the next generation, is completed in less than 1 year, in the natural environment (Sonenshine, 1991).

The results presented in this thesis showed the significance of polymerase chain reaction (PCR) and reverse line blot (RLB) hybridisation assay in surveying the presence of *Babesia* species in captive cheetah populations that potentially confuses the epidemiology of babesiosis in wild felids in South Africa. The results highlighted the potential problem of misdetection of the parasite, which was emphasized in a recent study (Bosman, Venter & Penzhorn, 2007), comparing PCR detection with the examination of both blood smears and serodiagnosis. The amplification of nuclear ribosomal RNA genes (ssu 18S rRNA) spanning the V4 hypervariable region (Ellis, Hefford, Baverstock, Dalrymple & Johnson, 1992) along with RLB hybridisation assay contributed greatly towards our understanding of *Babesia* species occurring in cheetahs in South Africa. The comparison between the full sequences of the small-subunit ribosomal RNA gene obtained and representatives of different *Babesia* species in Genbank along with phylogenetic analysis, confirmed the RLB results (Schnittger, Yin, Qi, Gubbels, Beyer, Niemann, Jongejan, & Ahmed, 2004; Tait, & Oura, 2004). It is interesting to note that most of the samples that were tested for *Babesia* by RLB hybridisation assay corresponded to infection

with *Babesia* species even though a number of them were negative on microscopic examination. However, no quantitative evaluation was made in the study. This random comparison highlighted the importance of there being carriers of low levels of haemoparasite after recovery from the disease (M'ghirbi, Hurtado, Barandika, Khlif, Ketata & Bouattour, 2008).

The *Babesia* genus-specific probes I and II, along with the *Theileria/Babesia* genus-specific probe hybridised on the RLB membrane, were used as extra confirmatory markers to display the possible positive reaction with the target sample DNA. The BLASTn homology search showed that each probe represents a variety of DNA fragment of *Babesia* and *Theileria* species of variable length. Moderate to high sequence similarity to the genus-specific probes may result in hybridisation reaction. No reaction of the target DNA with either of those genus-specific probes may be indicative of dissimilarity of sequence of the target DNA with *Babesia* and *Theileria* species.

The maintenance of *Babesia* species is dependent on two classes of hosts, one is a mammal and the other is a specific tick vector that must feed on the mammal host. The level of aggregation is as vital as tick burden in terms of the *Babesia* species transmission (Brunner & Ostfeld, 2008). Maintenance of a close association between larvae and nymphs in the life cycle of ixodid ticks, nymphs infect mice hosts through transmission of *Babesia* parasites, which are subsequently transmitted to the larvae through blood meals (Brunner & Ostfeld, 2008). This epidemiological pattern potentiates the distribution of babesial infection within a mouse population providing that tick instars share the same host population. The infected larvae ensure the transstadial transmission of *Babesia* species from one generation to another (Brunner & Ostfeld, 2008).

Since *Babesia* trophozoites can not undergo further development in a non-vector tick, sporozoites that are infective for vertebrate host animals are not formed. The results of this study that detected *Babesia* parasites in only *H. elliptica* ticks is indicative of the midgut acting as a major barrier for prevention of further development of *Babesia* parasites in non-vector ticks (Battsetseg, Matsuo, Xuan, Boldbaatar, Chee, Umemiya, Sakaguchi, Hatta, Zhou, Verdidia, Taylor & Fujisaki, 2006). The differences between *Babesia* transmission in potential vector and non-vector ticks may further help to explain the mechanisms by which *Babesia* species invade the tick vector and are transmitted to vertebrate hosts. In addition, *Babesia* species must

overcome the immune responses of the vector tick for successful transmission. However, understanding natural immunity of ticks against parasitic pathogens, especially *Babesia* species, is still in its infancy.

Contamination with the DNA non-specific organisms remains a major dilemma in molecular diagnostics. The risk of contamination that can result in the reporting of false positive results increases since most of molecular methods require post-amplification handling of PCR products for confirmation of a positive result. This creates a challenge to differentiate between true positive and false positive results.

Detecting *B. lengau* in mouse blood was a novel finding. The tendency of mice to live in small clans in permanent burrow systems could facilitate tick infestation resulting in transmission of *Babesia* species. It is not clear whether mice are preferred hosts for this *Babesia* species remains unclear. Our results may merely indicate an association of mice with *B. lengau* and further study is required to establish the role of mice in the maintenance of infection at the cheetah-breeding centres. However, our results were consistent with findings that *Aethomys* species are preferred hosts for *H. elliptica* and *R. simus* in South Africa (Braack *et al.*, 1996). Deleterious effects of tick infestation and *Babesia* parasites on the rodents are unknown. No *Babesia* trophozoites were observed in the red blood cells on the prepared smears.

## Conclusions

Understanding the epidemiology of feline babesiosis was hampered by the poor availability of diagnostic tests. The combination of PCR and RLB hybridisation assay used in this study enabled detection of prevalence of infection in various captive cheetah populations. The probability of detecting the relevant parasite DNA template in RLB hybridisation assay may be increased by either increasing input DNA or extracting total DNA from a larger volume of infected blood. The specificity of the RLB hybridisation assay could not be further improved using the suu 18S rRNA gene and was beyond the scope of this study. Perhaps in future, more specific and sensitive tests will be developed.

This study represents the first report on the molecular epidemiology of *Babesia* species in cheetahs in South Africa. Under natural conditions, *Babesia* species are thought to be host

specific (Young & Morzaria, 1986). Consideration of ectoparasites and their natural hosts throughout the country offers valuable insights into their distributions and the *Babesia*-associated problems, which they can cause particularly if they transfer to domestic and wild animals. Results from this study open up a new understanding of *Babesia* parasites infecting cheetahs and have shown that *Babesia* species such as *B. canis rossi*, *B. felis* and *B. lengau* were present at cheetah-breeding centres in South Africa, indicating that these species may share the same potential tick vector. In South Africa, the coastal provinces comprise the endemic regions for *B. felis* and its tick vector (Jacobson, Schoeman & Lobetti, 2000). Infection with *B. canis rossi* and *B. felis* may not be clinically significant in cheetahs (the identity of the *Babesia* species involved has clearly been confirmed) but it indicates that the cheetahs' close proximity to feral domestic cats and wild dogs at the centres, can lead to infection.

The molecular tools detected the babesial infection in cheetahs (a subclinical form), in mice that live in endemic areas and also in *H. elliptica* tick (as potential vector). Although we managed to detect the parasite in the instars and adults of *H. elliptica* ticks, more research is required to distinguish the developmental stages of the parasite in the tick. However, the fact that a mouse can be a preferred host for *Babesia lengau* remains speculation and a further comprehensive study is indicated to establish the role of this rodent in the emergence, re-emergence and epidemiology of the diseases.

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