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Prevalence and diversity of *Babesia, Hepatozoon, Ehrlichia*, and *Bartonella* in wild and domestic carnivores from Zambia, Africa

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ORIGINAL PAPER

Prevalence and diversity of *Babesia*, *Hepatozoon*, *Ehrlichia*, and *Bartonella* in wild and domestic carnivores from Zambia, Africa

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Abstract A molecular survey was conducted for several hemoparasites of domestic dogs and three species of wild carnivores from two sites in Zambia. Three *Babesia* spp. were detected including *Babesia felis* and *Babesia leo* in lions (*Panthera leo*) and a *Babesia* sp. (similar to *Babesia lengau*) in spotted hyenas (*Crocuta crocuta*) and a single lion. All wild dogs (*Lycaon pictus*) and domestic dogs were negative for *Babesia*. High prevalences for *Hepatozoon* were noted in all three wild carnivores (38–61 %) and in domestic dogs (13 %). Significantly higher prevalences were noted in hyenas and wild dogs compared with domestic dogs and lions. All carnivores were PCR negative for *Ehrlichia canis*, *Ehrlichia ewingii*, and *Bartonella* spp. Overall, high prevalences and diversity of *Babesia* and *Hepatozoon* were noted in wild carnivores from Zambia. This study is the first molecular characterization of *Babesia* from any hyena species and is the first report of a *Babesia* sp. closely related to *B. lengau*, a parasite previously only reported from cheetahs (*Acinonyx jubatus*), in lions and hyenas. Although usually benign in wild carnivores, these hemoparasites can be pathogenic under certain circumstances. Importantly, data on vectors for these parasites are lacking, so studies are needed to identify vectors as well as determine transmission routes, infection dynamics, and host specificity of these hemoparasites in wildlife in Africa and also the risk of transmission between domestic animals and wildlife.

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Introduction

In many parts of Africa, large predator populations have undergone severe declines, primarily due to anthropogenic factors; however, disease issues have also caused local declines and concern for species conservation (Ray et al. 2005; Munson et al. 2008). Three species included in this study, the African wild dog (*Lycaon pictus*), the African lion (*Panthera leo*), and the spotted hyena (*Crocuta crocuta*), are listed as endangered or threatened in all or parts of their ranges. An understanding of the natural history of pathogens in these carnivores is complicated by their threatened/endangered status, changes in their ecosystems, and interactions with domesticated animals (Woodroffe et al. 2012).

Infections with hemoparasites in the genera *Babesia* and *Hepatozoon* and vector-borne bacteria in the genera *Ehrlichia* and *Bartonella* are common in many wildlife species (Breitschwerdt and Kordick 2000; Penzhorn 2006; Rar and Golovijova 2011). Although these infections are typically asymptomatic, they can be pathogenic under certain circumstances (e.g., unnatural hosts, stress due to captivity, habitat degradation or climate fluctuation or immunosuppression) (Penzhorn 2006; Munson et al. 2008; East et al. 2008). For

example, *Babesia* and canine distemper virus coinfections have caused severe mortality in African lions (Munson et al. 2008) and *Hepatozoon*, which normally causes asymptomatic infections in carnivores, is suspected to have caused mortality in juvenile spotted hyenas in Tanzania (East et al. 2008).

Hemoparasite research in carnivores in Zambia is limited, and data on the natural history of wild carnivores is also lacking. In Lusaka, the capital city, a low percentage of domestic dogs (*Canis familiaris*) were positive for *Babesia* (Nalubamba et al. 2011) and a dog recently exported from Zambia was infected with *Ehrlichia canis* (Baba et al. 2012). Related work on wild carnivores in Zambia indicates that infection with gastrointestinal parasites is common (Berentsen et al. 2012). To better understand the prevalence and diversity of vector-borne hemoparasites in Zambian carnivores, we conducted a molecular survey for *Babesia* spp., *Hepatozoon* spp., *Ehrlichia canis*, *Ehrlichia ewingii*, and *Bartonella* spp. in domestic dogs, lions, spotted hyenas, and African wild dogs from two regions in Zambia, Africa.

Materials and methods

Study Area

Samples were collected in a 3,000-km^2 area of east-central South Luangwa National Park (SL) and in Liuwa Plain National Park (LP) which are located in Zambia's Eastern and Western Provinces, respectively (Fig. 1). South Luangwa National Park is a mosaic of deciduous forest, grasslands, and woodlands (Astle et al. 1969; White 1983; Astle 1988). The Luangwa River forms the eastern border for most of the park. Liuwa Plain National Park is approximately 3,500 km^2 of grasslands, floodplains, and woodlands and is home to the second largest wildebeest (*Connochaetes taurinus*) migration on the continent. The park is recovering from decades of poaching which resulted in a reduction of prey species and, consequently predators. New management of LP has resulted in increased game populations in recent years, and a high population of spotted hyenas. Other

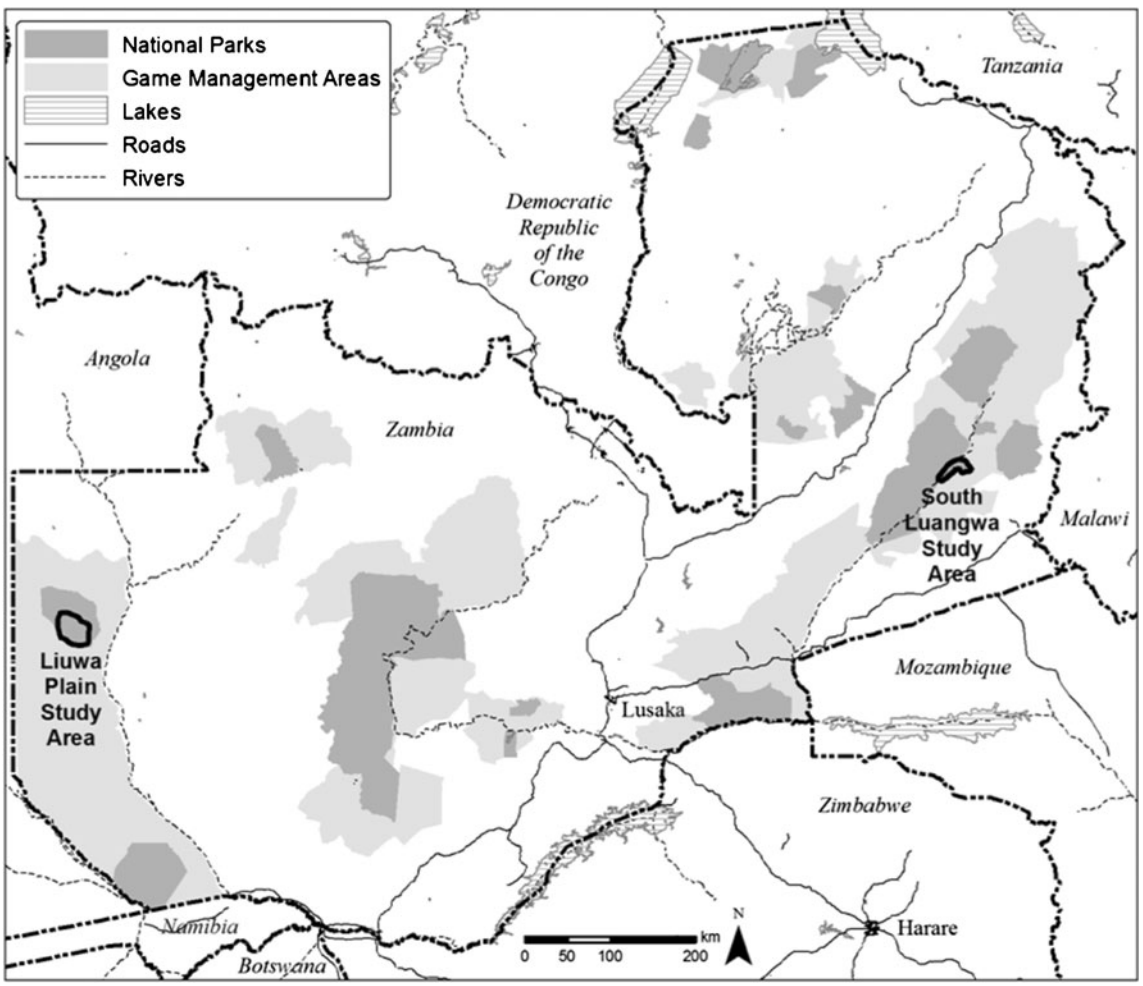

Fig. 1 Location of Liuwa Plains National Park and South Luangwa National Park, Zambia (adapted from Berentsen et al. 2013)

predators are returning, including lions, African wild dogs, and cheetahs (*Acinonyx jubatus*).

Sample collection

Samples were collected from May 2009–August 2011. Domestic dogs were physically restrained and blood samples obtained by venipuncture of the cephalic vein. Samples from wild carnivores were collected during chemical immobilization during concurrent studies of carnivore ecology or during removal of poaching snares. Wild carnivores were immobilized with a mixture of zoletil and medetomidine, reversed with atipamezole (Kock et al. 2006) and blood samples were obtained via venipuncture of the jugular, cephalic, or saphenous vein. Thick blood smears were immediately prepared in the field, fixed in ethanol, and maintained at room temperature until testing. All immobilization procedures followed animal welfare standards and protocols required by the Zambia Department of Veterinary and Livestock Development and the Zambia Wildlife Authority. All procedures were approved by the National Wildlife Research Center's Institutional Animal Care and Use Committee under Protocol QA-1725.

Molecular Analysis

Genomic DNA was extracted from thick blood smears using the Qiagen DNA Purification Kit (Germantown, MD) following the manufacturer's protocol except that the dried blood was scraped from the slide after a 10-min incubation with proteinase K and ATL buffer. For detection of *Babesia* and *Hepatozoon* infections, a PCR protocol targeting a ~600-bp region of the 18S rRNA gene was conducted using primers KIM18SF (5′-GAAATTAGAGTGTTTC) and KIMR2 (5′-ACCCTATTTAGCAGGTTAAG). For each PCR reaction, 5 μl of DNA was added to 20 μl of a master mix containing 11 μL of water, 2.5 μL of 25 μM $MgCl_2$, 5 μL of 5X GoTaq® Flexi Buffer, 0.25 μL of 0.2 mM each dNTP (Promega), 0.5 μL of each primer (50 μM), and 0.25 μL GoTaq® Flexi DNA Polymerase (Promega). The cycling parameters were 94 °C for 2 min followed by 40 cycles of 94 °C for 45 s, 45 °C for 1 min, and 72 °C for 1 min. For additional characterization and to confirm results of the 18S rRNA gene sequencing for *Babesia* spp., a PCR protocol that amplifies the entire internal transcribed spacer (ITS)-2 rRNA region was conducted on selected samples (Shock et al. 2012). For two samples that were positive for a *Babesia* species similar to *Babesia lengau*, the near full-length 18S rRNA was amplified as described (Yabsley et al. 2009).

Nested PCRs specific for the 16S rNA gene of *E. canis* and *E. ewingii* were conducted as described using primers ECC and ECB in a primary PCR (Dawson et al. 1994) followed by two separate nested PCRs using either primers ECA and HE3 for *E. canis* (Wen et al. 1997) or primers EE72 and HE3 for *E. ewingii* (Anderson et al. 1992a; b). To detect *Bartonella* spp., a PCR protocol that amplifies the ITS-1 rRNA region was conducted (Roux et al. 2000). Amplified products were separated in 2 % agarose gels, stained with ethidium bromide, and visualized with UV light. A negative water control was included in each set of DNA extraction, and a different water control was included in each set of primary and secondary PCR reactions. Appropriate positive controls (i.e., DNA from a culture of *E. chaffeensis*, dog blood positive for *E. ewingii*, feline blood positive for *Bartonella*, and a puma (*Puma concolor*) blood sample positive for a *Babesia* sp. distinct from any detected in this study) were included in each batch of PCR reactions.

To confirm identity, all 18S rRNA gene amplicons and representative ITS-2 amplicons were purified with a Qiagen gel extraction kit (Germantown, MD) and bi-directionally sequenced at the Georgia Genomics Facility (Athens, GA). Chromatograms were analyzed with Sequencher 5.0 (Gene Codes, MI). Sequences of 18S rRNA were aligned with those from related organisms obtained from GenBank using a basic local alignment search tool (BLAST) search (National Center for Biotechnology Information, Bethesda, MD) (Altschul et al. 1990). Phylogenetic analyses were conducted using Molecular Evolutionary Genetics Analysis (MEGA) using the neighbor-joining and minimum evolution analyses with the Kimura 2-parameter model and maximum parsimony using a close-neighbor-interchange search. Bootstrap analyses (1,000 replicates) were used to assess branch reliability. Sequences from this study have been deposited in the GenBank database (KF270642–KF270678, KF510019–KF510025).

Data Analysis

Fischer's exact test was performed to detect differences in prevalence between species and location ($p < 0.05$ was considered significant).

Results

Babesia infections were noted in two wild carnivore species, lions and hyenas, with similar prevalences noted for each (Table 1). Similarly, no differences in *Babesia* prevalence were noted between the two sites for either species. Based on sequence analysis of one or both gene targets, at least three *Babesia* spp. were detected including *Babesia felis* and *Babesia leo* in lions and a *Babesia* sp. (similar to *Babesia lengau*) in the six positive spotted hyenas and a single lion. The near full-length sequence (1,361 bp) of the *Babesia* from the hyenas was 99 % similar to *B. lengau* from cheetahs (Fig. 2, Bosman et al. 2010). Other closely related species

Table 1 PCR results for *Babesia* and *Hepatozoon* of lions (*Panthera leo*), spotted hyenas (*Crocuta crocuta*), wild dog (*Lycaon pictus*), and domestic dogs collected from Liuwana Plains National Park (LP) and South Luangwa National Park (SL), Zambia

Site	Species	Number	No. positive (%)	
			Babesia	*Hepatazoon*
Liuwa Plain National Park (LP)	Domestic dog	2	0	0
	Lion	1	0	1 (100)
	Spotted hyena	9	3 (33)	5 (56)
	Wild dog	4	0	2 (50)
South Luangwa National Park (SL)	Domestic dog	6	0	1 (20)
	Lion	23	6 (26)	13 (57)
	Spotted hyena	10	3 (30)	2 (20)
	Wild dog	7	0	4 (57)
Both sites	Domestic dog	8	0	1 (13)[a]
	Lion	24	6 (25)	9 (38)[a]
	Spotted hyena	19	6 (32)	11 (61)[b]
	Wild dog	11	0	6 (55)[b]

Different letters indicate a significant difference ($p<0.05$) in prevalence between species

included *Babesia conradae* (AF158702; 98 %) from dogs in CA, USA and *Babesia duncani* (HQ289870; 96.8 %) from humans in the western USA. Based on sequence analysis of ITS-2 from four hyenas and a lion infected with the *Babesia* sp. related to *B. lengau*, two genetic groups were detected. For one group, the ITS-2 sequences from two hyenas were identical and 305 bp (KF510023 and KF510019). These two sequences were most similar to *B. lengau* (GQ411430, 87 %) followed by *B. duncani* (AY998763, 79 %) and *B. conradae* (AY965739, 74 %) which is similar to the 18S rRNA data for these two hyenas. The other ITS-2 sequences of *Babesia* sp. from two hyenas and the single lion sample (KF510025, KF510020, and KF510024) were identical but only 243 bp in length. These two sequences were most similar to *B. duncani* (AY998763, 55 %).

High prevalences for *Hepatozoon* were noted in all three wild carnivores (38–61 %) and in domestic dogs (13 %) (Table 1). Significantly higher prevalence rates were noted in hyenas and wild dogs compared with domestic dogs and lions (all $p<0.05$). Although the *Hepatozoon* sequences were highly conserved among the different hosts, some differences were noted and some sequences were highly similar to *Hepatozoon canis* or *Hepatozoon felis* (KF270642–4, KF270646, KF270651, KF270654, KF270658–60, KF270663–5, KF270667–9, and KF270673). *Hepatozoon canis*-like sequences were detected in hyena, lions, and wild dogs whereas *H. felis*-like sequences were detected in lions and hyenas. Coinfections with *Babesia* and *Hepatozoon* were noted in one lion at the SL site. All carnivores were PCR negative for *E. canis*, *E. ewingii*, and *Bartonella* spp.

Discussion

In the current study, at least three species of *Babesia* in two species of wild carnivores from Zambia were detected. Previous studies have reported *Babesia* in several species of African carnivores including domestic dogs, domestic cats, lions, cheetahs, wild cats (*Felis silvestris*), caracal (*Felis caracal*), and hyenas (Van den Berge 1937; Barnett and Brocklesby 1968; Collett 2000; Penzhorn et al. 2001; Bosman et al. 2007; 2010; Munson et al. 2008; Bosman

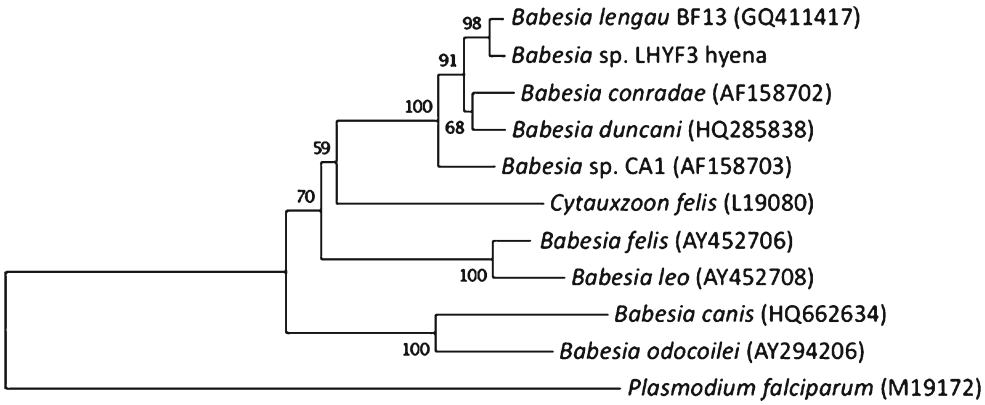

Fig. 2 Phylogenetic analysis of near full-length 18S rRNA gene sequence of a *Babesia lengau*-like sp. from spotted hyenas from Zambia

et al. 2013). Although none of the domestic dogs in our study were positive for *Babesia*, a low prevalence has been reported in the capital of Zambia and infection is common in domestic dogs (~10 % prevalence) in South Africa (Collett 2000; Nalubamba et al. 2011). We found no evidence of *Babesia* in wild dogs; however, *B. canis rossi* has been reported from free-ranging wild dogs from South Africa and uncharacterized *Babesia* have been detected from wild dogs from South Africa and Tanzania (Matija et al. 2008; Peirce et al. 1995; Van Heerden et al. 1995). In addition, wild dogs are experimentally susceptible (Matija et al. 2008; Peirce et al. 1995; Van Heerden et al. 1995) and a fatal case in a captive wild dog has been reported from South Africa (Colly and Nesbit 1992). Our failure to detect *Babesia* in wild dogs could be because sampled animals may have little contact with areas that contain domestic dogs or vectors or because the prevalence is too low to detect with our sample sizes. There may also be geographic differences as Prager et al. (2012) sampled 264 wild dogs from five populations in southern Africa from 1998 to 2009 and did not detect any *Babesia* infections.

To our knowledge, this is the first report of *Babesia* in lions from Zambia although lions are commonly infected with *Babesia* spp. in other southern African counties (e.g., South Africa, Swaziland) (Lopez-Rebollar et al. 1999; Penzhorn et al. 2001; Bosman et al. 2007; Munson et al. 2008; Githaka et al. 2012). In the current study, lions were infected with three *Babesia* species; *B. leo* and *B. felis* were the two most common, and both of these *Babesia* spp. have been reported from a wide range of African felids, including lions (Bosman et al. 2007). Domestic cats and leopards (*Panthera pardus*) are experimentally susceptible to both of these *Babesia* spp. (Penzhorn et al. 2001; Lopez-Rebollar et al. 1999). Previous work on free-ranging lions indicated that coinfections with *B. leo* and *B. felis* were common (Bosman et al. 2007). The failure to detect *Babesia* spp. coinfections in the current study could be related to the testing method (direct sequencing of PCR products) since previous studies have used reverse line blot hybridization (RLBH), a more sensitive assay for detecting mixed infections.

Sequence analysis of our products also detected a single lion infected with a *Babesia* sp. related to *B. lengau* which is the first report of this parasite species in lions. Interestingly, use of RLBH with genus and species-specific probes in a previous study showed that an unknown species of *Babesia* was detected in lions from southern Africa (Bosman et al. 2007). Because this species was not characterized by sequence analysis, its relationship to the *Babesia* sp. identified in this study is not clear. Additional sequence analysis of *Babesia* samples from lions in other countries is necessary to determine if the *Babesia* species detected in the current study is widely distributed. Recently, *B. lengau* was reported in two cases of severe cerebral and hemolytic babesiosis in two domestic cats in South Africa (Bosman et al. 2013).

The only previous reports of *Babesia* from the Family Hyaenidae include *Babesia alberti* described from the spotted hyena (Van den Berge 1937) and an unnamed *Babesia* sp. from the aardwolf (*Proteles cristata*; Peirce et al. 2001). This study is the first molecular characterization of *Babesia* from any species of hyena, and is the first report of a *Babesia* sp. related to *B. lengau*. Although *B. lengau* has only been detected in cheetahs, a *Babesia* recently detected from clinically ill domestic sheep in Greece was reported to be 99 % similar to *B. lengau* (Giadinis et al. 2012); however the sequence of the Greece sheep *Babesia* was not available in Genbank for comparison with our sequences (as of 6 November 2013). The host range of *Babesia* sp. related to *B. lengau* now includes three species of African carnivores and possibly domestic sheep. In the current study, ITS-2 sequences from two hyenas confirmed the close relationship of the hyena *Babesia* with *B. lengau*. However, the significance of two distinct ITS-2 sequences among the *B. lengau*-like species detected in this study is unknown. It is unknown if the *Babesia* sp. detected in the current study is the same as *B. alberti* because thin blood smears were not available for morphological analysis and genetic characterization of *B. alberti* has not been conducted.

A high prevalence of *Hepatozoon* detected in all species tested was not unexpected because *Hepatazoon* infections have been reported from numerous free-ranging African carnivores including lions, spotted hyenas, hyenas, wild dogs, and domestic dogs (Brocklesby 1971; McCully et al. 1975; Averbeck et al. 1990; Dubey and Bwangamoi 1994; Peirce et al. 1995; Van Heerden et al. 1995; East et al. 2008). Most infections have been identified by examination of blood smears and were classified as *H. canis*, a cosmopolitan parasite of canids; however, recent genetic characterization has revealed that numerous variants, subspecies, or cryptic species of *H. canis* exist worldwide (East et al. 2008; Gabrielli et al. 2010; Starkey et al. 2013). In wild canids, *Hepatozoon* infections are typically subclinical (McCully et al. 1975), but a non-*H. canis* species of *Hepatozoon* was reported to cause clinical disease in spotted hyenas in Tanzania (East et al. 2008). Based on the gene target used in the current study, several *Hepatozoon* sequences were detected, but some samples were very similar to *H. canis* and *H. felis*. A recent PCR study on wild felids and canids in India detected *H. felis* only in felids (lions, tigers, and leopards) and *H. canis* only in canids (domestic dogs and Indian wild dogs) (Pawar et al. 2012); however, these parasites are not considered to be specific to the suborders of the Carnivora and *H. canis* and *H. felis* have been reported in both canids and felids (Rubini et al. 2006; Jittapalapong et al. 2006; Baneth et al. 2013). Similarly, in the current study, *H. canis*-like sequences were detected in felids (lions) and hyena and *H. felis*-like sequences were detected in hyenas.

No evidence of *E. canis*, *E. ewingii*, or *Bartonella* DNA was detected in any wild carnivores or in domestic dogs.

Among domestic dogs, *E. canis* has been reported throughout sub-Saharan African including Sudan, Cameroon, Gabon, Kenya, and South Africa and in a dog that had recently been imported to the Netherlands from Zambia (Kaminjolo et al. 1976; Ndip et al. 2005; Matjila et al. 2008; Baba et al. 2012). Using PCR, *E. ewingii* has been detected in domestic dogs from Cameroon (Ndip et al. 2005). Based on serologic testing, antibodies reactive to *E. canis* have been reported in domestic dogs from Maasai Mara, Kenya, but not in sympatric African wild dogs (Alexander et al. 1993). Three other studies detected antibodies in domestic cats, domestic dogs, jackals, and Africa wild dogs in Kenya and South Africa (Alexander et al. 1994; Matthewman et al. 1996; Flacke et al. 2010; Woodroffe et al. 2012). Although not confirmed, a decline in wild dog numbers in South Africa was suspected to have been caused by ehrlichiosis (Neitz and Thomas 1938). A single study on 21 lions in Botswana failed to detect antibodies to *E. canis* (Ramsauer et al. 2007). Despite reports of antibodies in some wildlife species, no *Ehrlichia* species has been detected in wild carnivores in Africa by PCR, similar to our negative PCR data (Matjila et al. 2008). Unfortunately, serum samples were not available for serologic testing; however, future studies should incorporate both molecular and serologic testing to identify the species of *Ehrlichia* responsible for antibody responses previously detected in domestic and wild canids. For example, an *Ehrlichia ruminantium*-like sp. has been detected by PCR in domestic dogs in South Africa (Allsopp and Allsopp 2001) and the serologic cross-reactivity of *E. canis* and *E. ruminantium* has been demonstrated (Kelly et al. 1994). Previous studies have identified *Bartonella henselae* in lions, but both serologic and molecular-based prevalence rates are low (Molia et al. 2004; Pretorius et al. 2004). The lack of *Bartonella* PCR positives in the current study is not unexpected because culture enrichment followed by PCR is the most sensitive method for detection (Duncan et al. 2007).

The findings of the current study indicate that there is considerable variation in the prevalence of *Babesia* and *Hepatozoon* among the tested carnivore species. The reason for differences in *Babesia* and *Hepatozoon* prevalence between hosts is unknown, primarily because very little is known about the life cycles, transmission routes, host specificity, or pathogenic potential of these carnivore parasites. Asymptomatic infections with *Babesia* and *Hepatozoon* wild African carnivores are common (McCully et al. 1975; Averbeck et al. 1990; Van Heerden et al. 1995; Lopez-Rebollar et al. 1999; Penzhorn et al. 2001; Penzhorn 2006; Bosman et al. 2007; Bosman et al. 2010; Githaka et al. 2012); however, there are reports of disease in hyenas with *Hepatozoon* (East et al. 2008) and *Babesia* can cause disease in lions if parasites are present in high numbers or if hosts are immunosuppressed or stressed (Penzhorn 2006; Munson et al. 2008). Finally, a combination of morphological and molecular characterization of these parasites would greatly enhance our ability to compare contemporary studies with historical studies. Future studies should focus on understanding vectors, transmission routes, infection dynamics, parasite diversity, and host specificity of these hemoparasites in African wildlife and the risk of transmission between domestic animals and wildlife.

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Conflict of interest The authors have no knowledge of a conflict of interest.

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