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Molecular confirmation of high prevalence of species of *Hepatozoon* infection in free-ranging African wild dogs (*Lycaon pictus*) in the Kruger National Park, South Africa

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ABSTRACT

Reports in the literature indicate that species of *Hepatozoon* commonly occur in African wild dog (AWD) or painted wolf (*Lycaon pictus*) populations. These findings were based on examination of blood smears by microscopy, and specific identity of the *Hepatozoon* sp. gamonts seen could not be confirmed. We present the first in-depth molecular data on the prevalence of species of *Hepatozoon* in a free-ranging AWD population. In a general health survey of AWDs in the Kruger National Park, blood specimens (n = 74) collected from 54 individuals were examined for the presence of *Hepatozoon* spp. At first sampling, specimens from 42 of 54 individuals (77.7%) were positive, based on the primer set HepF300 and HepR900. Twenty individuals were resampled between 51 and 69 days after first sampling; one of these was resampled twice. Samples from six individuals that had tested negative previously now reacted positive. Assuming that all 54 individuals were still alive, the prevalence had therefore increased to 48 individuals infected, or 88.8%. Resultant 18S rDNA sequences isolated from these specimens share high similarity to other *Hepatozoon canis* genotypes. Phylogenetic analysis recovered the *Hepatozoon* sp. isolated from AWDs within the *H. canis* cluster, which includes species of *Hepatozoon* from other canid and tick hosts.

1. Introduction

African wild dogs (AWD) or painted wolves (*Lycaon pictus*), which originally inhabited most of the non-forested areas of sub-Saharan Africa, currently occur as scattered remnant populations (Woodroffe and Sillero-Zubiri, 2020). With overall numbers declining, primarily related to human activities, the species is classified as Endangered (Woodroffe, 2011; Woodroffe and Sillero-Zubiri, 2020). Pack sizes can fluctuate considerably over relatively short periods and only the alpha male and female in a pack reproduce (Mills, 1993). The number of mature animals, defined as those possibly reproducing during the current breeding season, rather than overall population numbers are therefore a better indication of the conservation status of AWDs (Woodroffe and Sillero-Zubiri, 2020). About one half of the estimated 1,400 surviving mature AWDs occur in southern Africa. The *ca.* 42 mature animals in the Kruger National Park (KNP) and adjoining areas constitute the largest population in southern Africa. AWDs are not distributed evenly throughout the KNP, but occur primarily south of the Olifants River, i.e. the southern half of the park, which falls in Mpumalanga Province (Higgitt et al., 2019).

Diseases can pose a serious threat to AWD populations. Rabies caused extinction of various populations, e.g. in Serengeti National Park, Tanzania (Burrows et al., 1994), Maasai-Mara National Reserve, Kenya (Kat et al., 1995) and the re-established population in Madikwe Nature

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Reserve, South Africa (Hofmeyr et al., 2000). AWDs are also susceptible to canine distemper (Goller et al., 2010; van Heerden et al., 1989). This disease was incriminated in the extinction of a relatively small AWD population in Chobe National Park, Botswana (Alexander et al., 1996). Furthermore, an unconfirmed disease caused the demise of five AWD dog packs over 4 weeks in a 2600 km^2 area in the Okavango Delta, Botswana (Alexander et al., 2010).

Vectors play a significant role in the transmission of diseases and parasites, and may be responsible for cross-species transmission of various diseases between wild and domestic animals (Miller et al., 2017). A general health survey of the AWD population in the KNP, primarily in the southern half of the park, offered the opportunity of examining a large set of blood samples for the presence of haemoprotozoa, specifically species of *Hepatozoon*. The genus *Hepatozoon* is one eight genera of blood parasites collectively referred to as haemogregarines. Species of *Hepatozoon* follow a heteroxenous life cycle involving an intermediate vertebrate host, as well as a haematophagous definitive invertebrate host (Smith, 1996). Within the definitive host syzygy (paring) occurs in the digestive tract and sporogony in the haemocoel of the tick host, resulting in the formation of oocysts enclosing sporocysts with sporozoites, the infective stages of the parasite (Baneth et al., 2007). In the case of wild and domestic canids infection occurs via ingestion of an infected tick host containing sporulated oocysts (Baneth et al., 2003). Merogonic development and the formation of meronts containing merozoites occurs within the bone marrow of the infected canid host. Rupturing of meronts release merozoites that enter neutrophils where they develop into gamonts circulating in the peripheral blood and awaiting ingestion by a hematophagous tick host (Baneth et al., 2007; Léveillé et al., 2019).

In a previous survey, *Hepatozoon* gamonts resembling those of *H. canis* were reported from 26 (89.7%) of 29 AWD blood smears from KNP (Van Heerden et al., 1995). High prevalence of species of *Hepatozoon* in free-ranging AWDs have also been reported elsewhere: in Serengeti, Tanzania, 81.5% (13/16) individuals were infected (Peirce et al., 1995) and in Zambia, 55% (6/11) individuals were infected (Williams et al., 2014). Species of *Hepatozoon* have also been reported from captive AWDs in South Africa (Matjila et al., 2008).

Canine hepatozoonosis is recognised as a clinical disease ranging from relatively asymptomatic in low level cases, to severe in high level cases (Baneth et al., 2003). Typical characteristics include fever, lethargy, myalgia, lameness and mucopurulent ocular discharge in severe cases (Elias and Homans, 1988; Little et al., 2009). In domestic dogs, *H. canis* appears to be well adapted to their canid hosts, mostly producing a subclinical to mild disease, whereas *Hepatozoon americanum*, whose infections are frequently fatal to their hosts, apparently only recently crossed the species barrier from wild hosts to domestic dogs (Baneth et al., 2003; Vincent-Johnson et al., 1997). Reasons for the difference in the clinical signs between the two species, apart from infection to novel hosts, may be due to the fact that merogonic development takes place in different target organs (Baneth et al., 2003). *Hepatozoon americanum* mainly infects the skeletal and cardiac muscles causing pyogranulomatous myositis, whereas *H. canis* primarily infects the spleen, bone marrow and lymph nodes (Baneth et al., 2003).

With only limited data available on species of *Hepatozoon* from AWDs, baseline data is needed to determine to what extent species of *Hepatozoon* occur in these hosts. Confirming the identity of the *Hepatozoon* sp. infecting AWDs in the KNP was important, as well as to which species are these haemogregarines closely related to and if the occurrence of these species of *Hepatozoon* hold possible threats to these hosts.

2. Material and methods

2.1. Sample collection

During a general health survey, AWDs in the KNP were immobilised in the field for sample collection. Due to the difficulty of individual recognition under field conditions, some individuals were immobilised more than once. Blood samples (n = 74) were collected from 54 free-ranging AWDs, none of which showing overt clinical signs of disease. Twenty individuals were resampled (one of these being resampled twice), between 51 and 69 days after the first sampling. Blood was drawn from the cephalic vein and collected into EDTA tubes. A microchip was inserted subcutaneously into each animal to enable future identification. With the exception of one collection locality (3 samples collected) between the Olifants and Letaba Rivers, samples were collected at 27 localities (Supplementary Table S1) throughout the southern half of the KNP, South Africa (Fig. 1).

2.2. DNA extraction, amplification and sequencing

Total genomic DNA was extracted from the blood samples using the KAPA Express Extract Kit (Kapa Biosystems, Cape Town, South Africa) according to the manufacturers' instructions. Following Penzhorn et al. (2018) identification of species of *Hepatozoon* was initially completed using the 18S ribosomal ribonucleic acid (rRNA) gene primer sets HepF300 and HepR900 (Ujvari et al., 2004). The PCR reactions were run targeting a fragment of approximately 600 base pairs (bp) of the 18S rRNA gene. An additional PCR was performed using the primer set EF and ER, targeting a longer fragment of approximately 1400 bp of the 18S rRNA gene on four (n = 4) samples (Kvičerová et al., 2008). Thermocycling conditions for the first primer set (HepF300 and HepR900) were as follows: initial denaturation at 94 °C for 3 min, followed by 35 cycles, entailing a 95 °C denaturation for 30 seconds, annealing at 60 °C for 30 seconds with an end extension at 72 °C for 1 minute, and following the cycles a final extension of 72 °C for 10 min. For the second primer set (EF and ER) thermocycling conditions included initial denaturation at 95 °C for 5 minutes, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 50 °C for 30 seconds and elongation at 72 °C for 2 min, terminated by one cycle of elongation at 72 °C for 10 min.

Resultant amplicons were viewed under UV-light on a 1% agarose gel that was stained with gel red. The PCR products of each sample were then classified as "positive" or "negative" based on gel electrophoresis visualisation. All positive PCR products of the HepF300 and HepR900 primer sets (n = 60) as well as the additional four PCR products using the EF and ER primer sets (n = 4) were submitted to a commercial company (InqabaBiotec™, Pretoria, South Africa) for purification and sequencing. Resultant sequences were then assembled using Geneious R9.1 (http://www.geneious.com). The Basic Local Alignment Search Tool (BLAST) was used to verify the sequence identity. Sequences obtained were deposited in the NCBI GenBank database under the following accession numbers (GenBank: MW676058 – MW676121).

Comparative sequences of *Hepatozoon*, and *Karyolysus* spp. were downloaded from GenBank and aligned using the MUSCLE alignment tool implemented from within Geneious R11 under the default settings. *Adelina dimidiata* (GenBank: DQ096835) and *Adelina grylli* (GenBank: DQ096836) were selected as outgroup taxa. The alignment (1469 base pairs) consisted of 35 sequences. A model test was performed to determine the most suitable nucleotide substitution model, according to the Bayesian information criterion (BIC) using jModelTest 2.1.7 (Darriba et al., 2012). The model with the best BIC score for the 18S rDNA sequence alignment was the Hasegawa-Kishino-Yano (Hasegawa et al., 1985) model, with estimates of invariable sites, and a discrete gamma distribution (HKY + I + Γ). Bayesian inference (BI) was used to infer phylogenetic relationships and was preformed using MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001) implemented from within Geneious R11. For the BI analysis, the Markov Chain Monte Carlo (MCMC) algorithm was run for 10 million generations, sampling every 100 generations. The first 25% of the trees were discarded as 'burn-in' with no 'burn-in' samples being retained. Results were visualized using the Tracer tool (implemented from within Geneious R11), to assess convergence and the burn-in period (Rambaut et al., 2018).

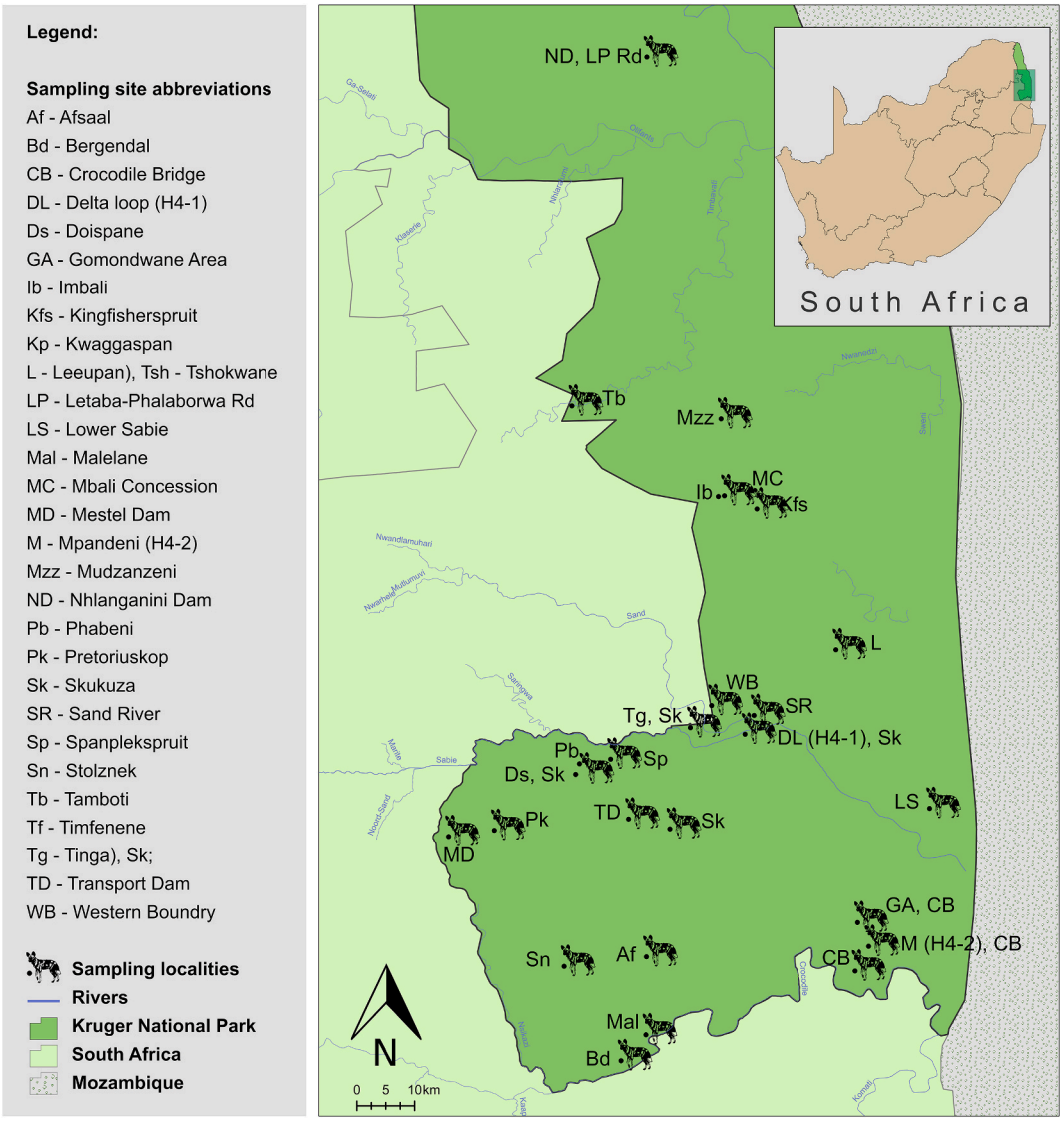

Fig. 1. Map of the sampling localities of African wild dogs (*Lycaon pictus*) in the Kruger National Park, South Africa.

3. Results and discussion

Our study confirmed a high prevalence of *H. canis* genotypes in free-ranging AWDs in the KNP. Seventy-four blood samples from 54 AWDs from the KNP were screened through PCR amplification to determine the presence of species of *Hepatozoon*. Agarose gel electrophoresis results indicated that 60 (81.1%) samples reacted positively using the primer sets HepF300 and HepR900. At first sampling, specimens from 42 of 54 individuals (77.7%) were positive (Supplementary Table S1). Twenty individuals were resampled (one individual was resampled twice) between 51 and 69 days after first sampling (Supplementary data S1). Samples from six individuals that had tested negatively previously now reacted positively. Assuming that all 54 individuals were still alive, the prevalence had therefore increased to 48 individuals infected, or 89%. According to Baneth et al. (2007), parasitaemia of circulating gamonts of *H. canis* occurs anything from 28 to 43 days post infection. These findings are consistent with the results from the present study. The six resampled individuals negative at first sampling became infected after first sampling, or they were already infected, but the parasites were still limited to the host's tissues and had not entered the circulating blood. Furthermore, no individuals positive at first sampling were negative at subsequent collections. Thus, the *Hepatozoon* sp. gamonts remain present in the host's peripheral blood for extended periods of time.

A total of 64 samples were sent to InqabaBiotec for purification and sequencing.

Of the resultant sequences (n = 57) were identical and BLAST results of 18S rDNA sequences isolated from *L. pictus* (GenBank: MW676058) revealed a species of *Hepatozoon* genotype (designated herein as *Hepatozoon* sp. A) with a 99.8% identity to *H. canis* (GenBank: KU893123) over a query length of 1432 bp. Two specimens (GenBank: MW676060 and MW676061) revealed a *Hepatozoon* sp. genotype (designated herein as *Hepatozoon* sp. B) with a 100% identity to *Hepatozoon* sp. (GenBank: MG919980) isolated from a black-backed jackal (*Canis mesomelas*) from South Africa, and a 99.9% identity to *H. canis* (GenBank: LC331052) isolated from a domestic dog (*C. familiaris*) from Zambia over a query length of 1390 bp. Additionally, two specimens (GenBank: MW676096 and MW676116) contained mixed infections with both genotypes (*Hepatozoon* sp. A and B).

Phylogenetic analysis based on 18S rDNA sequences recovered species of *Karyolysus* isolated from lizard hosts (clade 7), sister to a large clade comprising species of *Hepatozoon* isolated from large mammals, carnivores and ticks (clade 1–6) (Fig. 2). In the present study the *Hepatozoon* sp. isolated from AWDs was recovered within the *H. canis* cluster, comprising species of *Hepatozoon* from other canid and tick hosts (clade 1). Sequences obtained from AWDs with mixed *Hepatozoon* sp. infections were not included in the phylogenetic analysis, as sequences

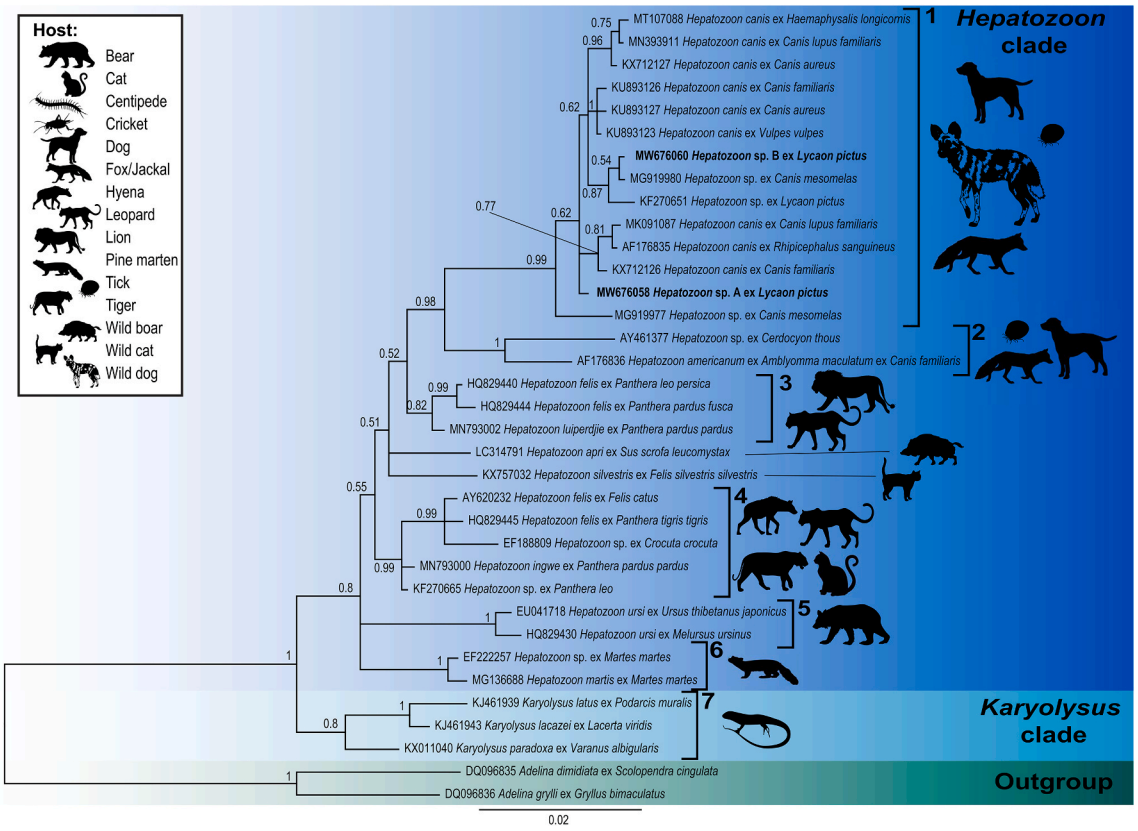

Fig. 2. Bayesian inference (BI) phylogram based on 18S rDNA sequences. Phylogram illustrating the phylogenetic relationships between *Hepatozoon* sp. isolated from African wild dogs (shown in bold) with 33 representative sequences of other species of *Hepatozoon* and *Karyolysus* retrieved from GenBank. *Adelina dimidiate* and *Adelina grylli* were selected as the outgroup. Posterior probability values lower than 0.50 were omitted. The scale-bar represents 0.02 nucleotide substitutions per site. Distinct clades are presented in alternating colours and numbers 1 to 6 highlight distinct clades. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

contained a double chromatogram peak or two separate bases at the same positions (heterozygous positions). The *H. canis* group fell sister to a clade comprising *H. americanum* and a *Hepatozoon* sp. isolated from the crab-eating fox (*Cerdocyon thous*) (clade 2). Within the *H. canis* group (clade 1) the *Hepatozoon* sp. (GenBank: KF270651) isolated from AWDs from Zambia was recovered sister to the *Hepatozoon* sp. (GenBank: MG919980) genotype isolated from black-backed jackals from South Africa and the *Hepatozoon* sp. B genotype isolated from AWDs from the present study (GenBank: MW676060). The AWD *Hepatozoon* sp. A genotype (GenBank: MW676058) from the present study fell separate from the AWD isolate (GenBank: KF270651) from Zambia, showing slight genetic diversity (<1%) among the different populations. However, the *Hepatozoon* sp. sequence (GenBank: KF270651) from the Zambian AWD is only 548 bp long. Thus, more data and longer 18S rDNA sequences for this conservative marker may provide better insight for comparison of the genetic diversity of species of *Hepatozoon* among these populations. Furthermore, the *H. canis* genotype from the present study was distinct from *H. felis*, *H. luiperdjie*, *H. ingwe* *H. apri*, *H. silvestris*, *H. ursi*, and *H. martis*, respectively (clade 3–6). Since members of this group have been reported from domestic dogs and indigenous canid species from widely scattered localities globally, further investigation into delineation of new taxa from this group could be fruitful.

Hepatozoon canis is classified as one of the most widespread tick-borne protozoal parasites and even though its primary suspected vector is *Rhipicephalus sanguineus* sensu lato, host specificity across different invertebrate hosts or vectors has not yet been fully explored (Baneth et al., 2001, 2007). Therefore, the possible transmission of *H. canis* and closely related species via other tick species cannot be eliminated (Farkas et al., 2014). Furthermore, transmission of *H. canis* is transstadial and not transovarial (Baneth et al., 2001). Other possible vectors transmitting *H. canis* to domestic dogs have been identified, e.g., *Amblyomma ovale* and *Rhipicephalus microplus* in Brazil (de Miranda et al., 2011; Forlano et al., 2005; Rubini et al., 2009), *Haemaphysalis longicornis* and *Haemaphysalis flava* in Japan (Murata et al., 1995) and *Haemaphysalis concinna* in Hungary (Hornok et al., 2013). Since *R. sanguineus* s.l. has not been reported from AWDs in South Africa (Horak et al., 2018), at least one vector other than *R. sanguineus* must be involved. The most prevalent tick infesting AWDs in South Africa is *Rhipicephalus simus*, followed by *Haemaphysalis elliptica* (Horak et al., 2018). The latter was the most prevalent tick species infesting a black-backed jackal population in South Africa, followed by *R. simus* (Penzhorn et al., 2020). The prevalence (15.4%) of *Hepatozoon* cf. *canis* infection in this jackal population was substantially lower than the prevalence in AWDs in the KNP (Penzhorn et al., 2018). This suggests that *R. simus* rather than *H. elliptica* may be involved in the transmission of *H. canis* in non-domestic canids.

Disease manifestations related to *H. canis* have apparently not been reported from other canid species. However, a *Hepatozoon* sp. (GenBank: EF188809) distinct from *H. canis* has been attributed to the mortality in young spotted hyaenas (*Crocuta crocuta*) (East et al., 2008). In experimental infections of domestic dogs immunosuppressed with corticosteroids, *H. canis* gamonts appeared rapidly in the blood, suggesting that immune mechanisms play an important role in preventing development of severe clinical signs (Baneth et al., 2001). The potential role of *H. canis* as a comorbidity should therefore be borne in mind. Although *H. canis* infections have not been incriminated in causing disease in AWDs, possible detrimental effects cannot be ruled out, especially in severe infections and where there are comorbidities. Significant tissue

lesions attributed to species of *Hepatozoon* were found in three black-backed jackals in the KNP with schizonts occurring in the lungs, bone marrow and especially skeletal muscles (McCully et al., 1975). The resulting myositis was severe, with individual cells becoming necrotic.

In conclusion, the present study provides data on the high prevalence of *H. canis* genotype infections in free-ranging AWDs from the KNP. Although in most cases in domestic dogs, *H. canis* infection is subclinical or results in mild disease, in severe cases or where comorbidities occur, infection can be fatal. Thus, distinguishing between closely related lineages, and gathering data on the host condition, may provide insights into the differences in pathogenicity and virulence of *H. canis*-like genotypes (Penzhorn et al., 2018). This knowledge is important, especially for conservation areas such as the KNP which protects the largest AWD population in southern Africa (Higgitt et al., 2019). Through constantly screening more taxa and building up a more comprehensive molecular database, knowledge can be gained on how to optimally protect these host species. Future work should include research on potential vectors and life cycle elucidation, which would provide valuable insights on how these parasites are transmitted, the rate of infection, and vulnerability of the endangered AWD to infections by species of *Hepatozoon*.

Ethics declarations

This study was approved by the Animal Ethics Committee of the University of Pretoria (ref. V031-17), the Animal Use and Care Committee of South African National Parks (ref. 013/16), and the Department of Agriculture, Land Reform and Rural Development (then Department of Agriculture, Forestry and Fisheries) (ref. 12/11/1/1/6) in terms of Section 20 of the Animal Diseases Act (Act No. 35 of 1984).

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijppaw.2021.03.002.

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