UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Campus de Botucatu

DETECÇÃO E CARACTERIZAÇÃO DE HEMOPROTOZOÁRIOS E ECTOPARASITAS EM *Nasua nasua* E *Didelphis* spp.

MARIA REGINA LUCAS DA SILVA

Tese apresentada ao Instituto de Biociências, Campus de Botucatu, UNESP, como parte dos requisitos para obtenção do título de Doutora no Programa de Pós-Graduação em Biologia Geral e Aplicada, Área de concentração Biologia de Parasitas e Microorganismos.

Profa. Adjunta Lucia Helena O'Dwyer de Oliveira

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SUMÁRIO

RESUMO

Animais silvestres são considerados reservatórios para uma infinidade de patógenos transmitidos por ectoparasitas, dentre os quais *Hepatozoon* spp. e piroplasmas; e os ectoparasitas atuam como vetores desses micro-organismos. O presente estudo teve por objetivo investigar a ocorrência de *Hepatozoon* spp., piroplasmas e ectoparasitas em *Nasua nasua* e *Didelphis* spp. da região peri-urbana e urbana dos municípios de Botucatu, Palmital e São Paulo, além de identificar os ectoparasitas e caracterizar morfologicamente e molecularmente os hemoparasitas encontrados. Foram coletados ectoparasitas e amostras de sangue de 69 *Didelphis albiventris*, 11 *Didelphis aurita* e 83 *N. nasua*. Também, foram coletadas amostras de baço e fígado de dois *N. nasua* e de 25 *D. albiventris*, para análise histológica. Os carrapatos foram identificados e dissecados para pesquisa de oocistos de *Hepatozoon* spp. e alguns exemplares tiveram seu DNA extraído para verificar a presença de hemoparasitas. Para identificação dos hemoparasitas foram realizados esfregaços sanguíneos, a PCR, clonagem, e posterior sequenciamento das amostras. Primeiramente, *Hepatozoon procyonis* foi identificado em *N. nasua*. Dois carrapatos *Amblyomma ovale*, coletados em *N. nasua*, também foram positivos para *Hepatozoon* spp. Um espécimen foi positivo para *H. procyonis* e o outro para *Hepatozoon* sp. próximo de um haplótipo de *Hepatozoon americanum.* Um piroplasma próximo de *Babesia* sp. capybara foi detectado em *Amblyomma sculptum*, também coletado em *N. nasua*. Foi detectado, pela primeira vez, infecção por *Hepatozoon canis* em espécimes de *D. albiventris*, que também foram positivos para quatro haplótipos diferentes de piroplasmas. Para finalizar, *D. aurita* mostrou-se infectado por *Hepatozoon* sp. próximo de *Hepatozoon* sp. detectado em um marsupial chileno (*Dromiciops gliroides*). Como conclusão, nós fornecemos dados sobre a epidemiologia de hemoparasitas em *N. nasua* e, redescrevemos *H. procyonis* baseado em características morfológicas, morfométricas e moleculares. Um haplótipo de *H. americanum* foi detectado, pela

primeira vez, em *A. ovale* e pode representar risco de infecção por *H. americanum* em cães da região de Botucatu. Provavelmente, *D. albiventris* é um hospedeiro acidental para *H. canis,* mas, se infecta com piroplasmas que devem ser mais bem estudados para serem corretamente caracterizados.

Palavras-chave: Animais silvestres, *Hepatozoon americanum*, *Hepatozoon canis*, *Hepatozoon procyonis*, piroplasmas.

ABSTRACT

Wild animals are considered reservoirs for several pathogens transmitted by ectoparasites, among which *Hepatozoon* spp., and piroplasms, and the ectoparasites are the vectors of these microorganisms. The present study aim to investigate the occurrence of *Hepatozoon* spp., piroplasms and ectoparasites in *Nasua nasua* and *Didelphis* spp., from peri-urban and urban regions of Botucatu, Palmital and São Paulo municipalities, besides to identify ectoparasites and characterize morphologically and molecularly the hemoparasites found. For this purpose, were collected ectoparasites and blood samples from 69 *Didelphis albiventris*, 11 *Didelphis aurita* e 83 *N. nasua*. In addition, tissue samples were collected from two *N. nasua* and 25 *D. albiventris* for histological analysis. Ticks were identified and dissected to recover oocysts of *Hepatozoon* spp. Some specimens of ticks had their DNA extracted for identification of hemoparasites. For parasitological diagnosis, blood smears were performed, for subsequent identification and morphometry of the parasites. The molecular diagnosis was performed by PCR or cloning and sequencing. First, we detected *Hepatozoon procyonis* infecting *N. nasua*. *Amblyomma ovale* collected on *N. nasua* was also infected by *H. procyonis* and by *Hepatozoon* sp. closely related to a *Hepatozoon americanum* haplotype. A piroplasm closely related to de *Babesia* sp. capybara was detected in *Amblyomma sculptum* collected also on *N. nasua*. We detected, for the first time, *H. canis* infecting specimens of *D. albiventris*, which was also infected with four different haplotypes of piroplasms. To finalize, *Hepatozoon* sp. closely related to *Hepatozoon* sp. detected from a Chilean marsupial infected *D. aurita*. In conclusion, we provided epidemiological data on hemoparasites infection in *N. nasua,* and redescribed *H. procyonis* based on morphological, morphometrical and molecular characteristics. One *H. americanum* haplotype was detected, for the first time, in *A. ovale* representing a risk of infection for dogs from Botucatu region.

Probably, *D. albiventris* is an accidental host for *H. canis*, but is infected with piroplasms that should be better studied and characterized.

Keywords: *Hepatozoon americanum*, *Hepatozoon canis*, *Hepatozoon procyonis*, piroplasms, wild animals.

1. INTRODUÇÃO

Inúmeras espécies de animais silvestres atuam como reservatórios de protozoários e, devido às mudanças climáticas, aquecimento global e urbanização, muitos desses animais adquiriram hábito sinantrópico (LLEDÓ et al., 2010). Consequentemente, os mamíferos silvestres infestados por ectoparasitas podem entrar em contato com animais domésticos, com quem compartilham os mesmos ectoparasitas, oferecendo risco de transmissão de doenças aos animais domésticos e ao homem (FIGUEIREDO et al., 1999).

Dentre os micro-organismos transmitidos por artrópodes, e que tem animais silvestres como reservatórios, estão o *Hepatozoon* spp. e os piroplasmas. Hepatozoonose é a doença ocasionada pelos protozoários do gênero *Hepatozoon* e acomete cães de áreas urbanas e rurais (O'DWYER et al., 2004; RUBINI et al., 2008). Piroplasmas são hemoparasitas de importância médica e veterinária, que incluem espécies de *Babesia* e *Theileria* (BARBOSA et al., 2017). *Babesia* spp. causam no homem, animais domésticos, animais de produção e animais silvestres, uma doença denominada de babesiose (LLEDÓ et al., 2010; YABSLEY e SHOCK, 2013).

Artrópodes como os carrapatos e as pulgas são ectoparasitas de ação irritativa e espoliadora e além do mais, assumem grande importância médica e veterinária por serem vetores de patógenos que causam doenças graves aos seres humanos e aos animais domésticos e silvestres (MULLER et al., 2005). A coleta dos ectoparasitas, identificação e exames com a finalidade de detectar patógenos, podem conduzir à descoberta de novas infecções parasitárias emergentes em animais e humanos (NELDER et al., 2005).

2. REVISÃO DE LITERATURA

2.1. *Nasua nasua*

Mamíferos da espécie *N. nasua,* conhecidos como quatis, são animais de médio porte da Classe Mammalia, Ordem Carnivora e Família Procyonidae. Esses animais são onívoros e

possuem uma alimentação generalista, composta principalmente de pequenos insetos, frutos e pequenos vertebrados (ALVES-COSTA et al., 2004).

Os quatis são amplamente distribuídos na América do Sul e são capazes de se adaptarem a diferentes ambientes e se alimentarem de diversificados itens como os mencionado anteriormente. Devido a esse comportamento, os indivíduos desta espécie são capazes de fazer intercâmbio entre áreas silvestres e domésticas (RODRIGUES e MASSARD, 2006).

2.2. *Didelphis* spp.

Popularmente conhecidos como gambás, marsupiais do gênero *Didelphis* são distribuídos amplamente nas Américas e possuem considerável importância na relação ecológica parasito-hospedeiro (SALVADOR et al., 2007). Esses animais pertencem à Classe Mammalia, Subclasse Theria, Infraclasse Metaheria, Ordem Marsupiala e Família Didelphidae (MIZIARA et al., 2008).

Didelphis spp. possuem hábitos noturnos, e por serem onívoros e oportunistas adaptam-se a diversos ambientes, exibem elevada sinantropia e cada vez é mais frequente sua convivência com seres humanos nas cidades (MULLER et al., 2005). Devido a essas características, são considerados disseminadores de agentes causadores de doenças entre os animais silvestres, domésticos e o homem. Esses animais podem atuar como hospedeiros definitivos ou reservatórios de protozoários, helmintos e artrópodes (MULLER et al., 2005).

2.3. Ectoparasitas

Algumas espécies de pulgas e carrapatos apresentam pouca especificidade e podem se infectar e transmitir patógenos aos seres humanos e aos animais domésticos e silvestres. O conhecimento de sua associação aos hospedeiros e a distribuição dos ectoparasitas de animais é

fundamental para à compreensão da ecologia dos artrópodes transmissores de micro-organismos (NELDER et al., 2005).

2.3.1. Carrapatos

Carrapatos são ectoparasitas hematófagos e possuem distribuição cosmopolita. Devido à espoliação, inoculação de toxinas e transmissão de micro-organismos ocasionam doenças em animais e no homem. Protozoários, fungos, helmintos, bactérias e vírus são alguns dos patógenos transmitidos por carrapatos (FIGUEIREDO et al., 1999).

No Brasil, *Rhipicephalus sanguineus* senso lato foi detectado parasitando *N. nasua* (DANTAS-TORRES et al., 2010). Esse carrapato tem como hospedeiro principal o cão, mas também pode ser encontrado no homem e em pássaros (DANTAS-TORRES et al., 2010). É importante frisar que este ectoparasita é o principal transmissor de babesiose canina no Brasil (PASSOS et al., 2005). Outro carrapato identificado em *N. nasua* é *Amblyomma sculptum* publicado como *A. cajennense*, vetor de *Rickettsia rickettsi*, bactéria causadora da febre maculosa brasileira (FIGUEIREDO et al., 1999). Sousa et al. (2017a) relataram 70,9% dos quatis infestados por carrapatos no Pantanal brasileiro. As espécies de carrapatos detectadas em *N. nasua* foram: *A. sculptum*, *Amblyomma parvum*, *Amblyomma ovale*, *Rhipicephalus micropulus* and *Amblyomma* spp.

De acordo com Muller et al. (2005), no Rio Grande do Sul, 43% dos 30 *Didelphis albiventris* analisados apresentaram carrapatos das espécies *Ixodes loricatus* e *Amblyomma aureolatum*. Estes autores sugeriram que os gambás se infestaram com *A. aureolatum* por frequentarem as mesmas áreas que canídeos e felídeos silvestres. Além disso, destacaram que a presença de *I. loricatus* e *A. aureolatum* representa alerta para disseminação de patógenos causadores de severas doenças aos humanos, como *R. rickettsi*, que pode ser transmitida por *A. aureolatum*.

2.3.2. Pulgas

As pulgas são insetos hematófagos na fase adulta e tem como hospedeiros animais endotérmicos, principalmente mamíferos. Estes insetos atuam como parasitas ou como vetores de patógenos. Quando atuam como agentes infestantes provocam irritação, espoliação e reação inflamatória. Como vetores ou hospedeiros intermediários são responsáveis pela transmissão de viroses, doenças bacterianas, protozooses e helmintoses (LINARDI, 2011).

A ordem Siphonaptera é composta por aproximadamente 3000 espécies e subespécies, que parasitam animais silvestres e domésticos. Merece destaque *Ctenocephalides felis* por ser o mais importante ectoparasita de cães e gatos no mundo inteiro, devido à sua distribuição geográfica, perdas econômicas, resistência aos inseticidas e por atuarem como vetores e como parasitas hematófagos (LINARDI e SANTOS, 2012). As pulgas do gênero *Polygenis* também são importantes por manterem *Yersinia pestis*, o agente etiológico da peste bubônica, circulando entre roedores silvestres ou entre roedores silvestres e domiciliares (LINARDI, 2011).

O número de relatos de pulgas parasitando *N. nasua*, no Brasil é reduzido e são descritas poucas espécies infestando esses animais, sendo elas: *C. felis*, encontrada em área urbana, e *Rhopalopsyllus lutzi lutzi*, encontrada em área rural (RODRIGUES e MASSARD, 2006; FIGUEIREDO et al., 2010). Portanto, *N. nasua* mantém espécies de ectoparasitas e podem intercambiá-los entre os ambientes silvestre e urbano.

Por outro lado, são inúmeros os sifonápteros relatados em *Didelphis* spp. As pulgas presentes nestes animais são as seguintes: *Adorotopsylla intermedia, Craneopsylla minerva minerva*, *C. felis, Leptopsylla segnis, Polygenis atopus, Polygenis klagesi samuelis, Polygenis rimatus rimatus, Pulex irritans, R. lutzi lutzi, Xenopsylla cheopis* (RAFAEL, 1982; BARROS-BATESTI e ARZUA, 1997; MULLER et al., 2002; BOTÊLHO et al., 2003; MORAES et al., 2003; SALVADOR et al., 2007).

2.4. *Hepatozoon* spp.

O gênero *Hepatozoon* é classificado no Filo Apicomplexa, Subordem Adeleorina e Família Hepatozoidae (WENYON, 1926). São descritas mais de 300 espécies, que infectam leucócitos de mamíferos e eritrócitos de anfíbios, aves e répteis. O ciclo de vida desses parasitas é heteroxeno e envolve um hospedeiro intermediário vertebrado e um hospedeiro invertebrado definitivo como mosquitos, pulgas, e ácaros (Mesostigmata e Metastigmata). O oocisto do parasita se desenvolve no hospedeiro definitivo e os hospedeiros vertebrados se infectam quando ingerem o invertebrado contendo oocistos maduros (SMITH, 1996).

Segundo Baneth et al. (2000), a hepatozoonose canina é causada por *Hepatozoon americanum* ou *Hepatozoon canis*, e tem como vetores carrapatos ixodídeos. Na região sudeste do Brasil, a caracterização molecular de *Hepatozoon* demonstrou que a espécie que infecta cães é *H. canis* (RUBINI et al., 2005). Análises filogenéticas revelam a presença de *H. canis* em animais silvestres, como por exemplo, em raposas (*Vulpes vulpes*) (CRIADO-FORNELIO et al., 2003). Dessa forma, os animais selvagem podem atuar como reservatórios de *H. canis*.

Em procionídeos, *Hepatozoon* spp. foram detectados em *Procyon lotor* e *Procyon cancrivorus panamensis,* conhecidos como mão pelada, e em *N. nasua* (RICHARDS, 1961; SCHNEIDER, 1968; CLARK et al., 1973; RODRIGUES et al., 2007; SOUSA et al., 2017a). No Brasil, *Hepatozoon procyonis* foi detectado em esfregaço de sangue de *N. nasua* e de *P. cancrivorus*. O gametócito desse protozoário foi caracterizado morfologicamente e biometricamente aonde demonstrou similaridade com *H. procyonis* descrito anteriormente nos Estados Unidos (RODRIGUES et al., 2007). No Pantanal, por análise molecular, 41,9% dos *N. nasua* avaliados foram positivos para *Hepatozoon* sp. (SOUSA et al., 2017a).

Ayala et al. (1973) relataram duas hemogregarinas distintas infectando *Didelphis marsupialis* na Colômbia, uma delas identificada como *Hepatozoon didelphydis*. Na Guiana Francesa, 25% e 17% dos *D. marsupialis* e *D. albiventris*, respectivamente, estavam parasitados

por *Hepatozoon* spp. *Hepatozoon didelphydis* foi identificado em *D. marsupialis* e *Hepatozoon* sp. presente em *D. albiventris*, provavelmente, é uma nova espécie (THOISY et al., 2000). No Pantanal do Brasil, Sousa et al. (2017a) detectaram o marsupial *Thylamys macrurus* infectado com *Hepatozoon* sp. Em contraste, na região sul do país Criado-Fornelio et al. (2006), ao analisarem 15 *D. albiventris*, não detectaram a presença de *Hepatozoon* spp. No Pantanal, também não foi identificado nenhum *D. albiventris* infectado por *Hepatozoon* spp., porém, apenas três animais foram examinados (WOLF et al., 2016).

2.5. Piroplasmas

Piroplasmas são parasitas que acometem animais silvestres, domésticos e o homem, podendo causar doenças graves. Entre os agentes infecciosos transmitidos por carrapatos para animais, os piroplasmas são os mais prevalentes (ALVARADO-RYBAK et al., 2016). Esses hemoparasitas pertencem ao Filo Apicomplexa e incluem espécies de *Babesia*, *Theileria* e *Cytauxzoon*, sendo este último parasita praticamente exclusivo de felídeos.

O ciclo de vida de piroplasmas é heteroxeno com a presença de um vetor invertebrado, que são carrapatos ixodídeos, e um hospedeiro vertebrado. Os carrapatos transmitem os piroplasmas para os mamíferos através da picada, durante o repasto sanguíneo. Quando o hospedeiro vertebrado é infectado, esporozoítos de *Babesia* spp. vão diretamente para corrente sanguínea e infectam eritrócitos. Enquanto nas espécies de *Theileria* os esporozoítos penetram, primeiramente, nos linfócitos ou outros leucócitos e depois, migram para os eritrócitos (ALVARADO-RYBAK et al., 2016).

2.5.1 *Babesia* spp.

Hemoprotozoários do gênero *Babesia* são classificados taxonomicamente dentro do Filo Apicomplexa, Classe Aconoidasida, Ordem Piroplasmida e Família Babesidae (HOMER et al.,

2000). Estes protozoários infectam eritrócitos e ocasionam uma doença de caráter zoonótico denominada babesiose.

A babesiose é uma enfermidade emergente, transmitida por carrapatos ixodídeos e os animais silvestres atuam como hospedeiros reservatórios para as espécies de *Babesia* zoonóticas. Nos Estados Unidos, *Babesia microti* é a principal espécie que causa babesiose em humanos. Na Europa, a babesiose humana geralmente é provocada por *Babesia divergens* (YABSLEY e SHOCK, 2013).

Na América do Sul, a infecção por *Babesia* spp. em humanos foi relatada na Colômbia e no Brasil. Em exames sorológicos realizados na Colômbia, com utilização de antígenos preparados a partir de cepas de *Babesia bovis* e *Babesia bigemina*, sete indivíduos, dos 194 analisados, foram positivos para *Babesia* spp. e um foi positivo em exame parasitológico (RÍOS et al., 2003). No Pernambuco, Brasil, a primeira descrição de infecção por *Babesia* sp. em humanos foi identificada por exame direto em lâminas de sangue, em um paciente adulto (ALECRIN et al., 1983). Posteriormente, foi diagnosticada uma criança assintomática com uma possível infecção por *B. microti* (RECH et al., 2004). Em ambos os relatos não ocorreu a identificação molecular dos protozoários.

Babesia spp. muitas vezes são associadas a animais domésticos, porém, inúmeros animais silvestres são reservatórios para este parasita (YABSLEY e SHOCK, 2013). Em carnívoros da família Procyonidae esse hemoprotozoário foi detectado na América do Norte, América Central e na Ásia (TELFORD e FORRESTER, 1991, KAWABUCHI et al., 2005, BIRKENHEUER et al., 2006, JINAI et al., 2007; BIRKENHEUER et al., 2008; CLARK et al., 2012, MEHRKENS et al., 2013). A infecção por *B. microti* ocorreu em 82% dos *P. lotor* analisados na Flórida. Vale ressaltar que *B. microti* ocasiona babesiose em humanos, portanto, nesta região os ectoparasitas destes animais apresentam risco considerável ao homem (CLARK et al., 2012). Na Costa Rica, 100% dos *Nasua narica* estudados estavam parasitados por

Babesia sp., filogeneticamente próxima de *Babesia* sp. detectada em guaxinins nos EUA, entretanto, os sinais clínicos e a importância epidemiológica desse parasita em quatis são desconhecidos (MEHRKENS et al., 2013).

Paparini et al. (2012) identificaram uma nova espécie de *Babesia* em *Bettongia penicillata ogilbyi* (marsupiais) na Austrália. Neste mesmo país, outra espécie de *Babesia* foi descrita parasitando *Macropus gigantes* (canguru). Estes animais apresentavam sintomas de babesiose (anemia, apatia e sinais neurológicos) e foram positivos tanto no esfregaço de sangue quanto na PCR. Por meio do sequenciamento foi possível caracterizar uma nova espécie (DAWOOD et al., 2013). Barbosa et al. (2017), ao investigarem a prevalência de hemoparasitas e parasitas entéricos em mamíferos Australianos, também detectaram *Babesia* sp. em um marsupial (*Isoodon macrourus*). No Brasil, *Monodelphis domestica* capturada no Pantanal estava infectada com *Babesia* sp. filogeneticamente próxima de *Theileria bicornis* (WOLF et al., 2016).

Em relação aos marsupiais do gênero *Didelphis*, na Colômbia foram encontrados *D. marsupialis* infectados por *Babesia brasiliensis* (AYALA et al., 1973). Thoisy et al. (2000) identificaram *Babesia* sp. em 4% de *D. albiventris* estudados, na Guiana Francesa. No Brasil, uma possível infecção de *D. marsupialis* com piroplasma foi relatada em animais provenientes do Pará por Deane e Deane (1961). A presença de *Babesia* sp. também foi observada em *D. albiventris* e *D. marsupialis* procedentes do Pará, Rio de Janeiro e de São Paulo (SERRA-FREIRE, 1979). Recentemente, *Babesia* sp. foi detectada, por meio de análise molecular, em *D. marsupialis*, no Pará (SOARES et al., 2017). Entretanto, dados morfológicos desse parasita não foram analisados.

Yabsley e Shock (2013) salientaram a importância da caracterização molecular para distinção das espécies de *Babesia*, bem como a investigação dos dados morfológicos e

morfométricos, hospedeiros reservatórios e vetores, que se fazem necessários para uma melhor compreensão da epidemiologia destes patógenos.

2.5.2. *Theileria* spp.

Theileria são protozoários obrigatórios pertencentes à Família Theileridae, infectam principalmente ruminantes e são importantes pelas perdas econômicas que causam na agropecuária (BISHOP et al., 2004).

Apenas duas espécies de *Theileria* foram descritas em carnívoros silvestres e são nomeadas como *Theileria parva* e *Theileria sinensis* (ALVARADO-RYBAK et al., 2016). Recentemente, no Pantanal Brasileiro, Estado do Mato Grosso do Sul, Sousa et al. (2017b) detectaram *N. nasua* infectado por *Theileria* sp. filogeneticamente associada a *Theileria equi.*

Em marsupiais, *Theileria* spp. são hemoparasitas frequentemente detectados. Na Austrália, sete espécies de *Theileria* foram encontradas infectando estes animais (PAPARINI et al., 2012). A alta prevalência de *Theileria penicillata* foi detectada em *Bettongia penicillata* (RONG et al., 2012). Além disso, inúmeros outros marsupiais são infectados por *Theileria* spp. como por exemplo: *Bettongia lesueur* e *Potorous gilbertii*, (LEE et al., 2009; PAPARINI et al., 2012). No Brasil, há uma ampla diversidade de marsupiais, com 55 espécies descritas (PAGLIA et al., 2012) porém, nenhuma espécie de *Theileria* foi relatada infectando esses animais. A ausência de detecção de *Theileria* spp. em marsupiais no país, pode ser devido à escassez de estudos que avaliaram a presença destes parasitas em marsupiais.

3. Objetivos

No Brasil, os poucos estudos sobre a infecção por *Hepatozoon* spp. e piroplasmas em *Nasua nasua* e *Didelphis* spp., bem como em seus ectoparasitas, e a frequente constatação desses

animais em regiões peri-urbanas e urbanas nas cidades de Botucatu, Palmital e São Paulo incentivou-nos a realizar esta pesquisa.

Perante o exposto, investigamos a ocorrência de *Hepatozoon* spp., piroplasmas e ectoparasitas em *N. nasua* e *Didelphis* spp., provenientes das regiões urbana e peri-urbana dos municípios de Botucatu, Palmital e São Paulo. Essa pesquisa resultou em quatro artigos científicos intitulados:

1. "**A survey of haemoparasites e ectoparasitas in *Nasua nasua* with a redescription of *Hepatozoon procyonis* based on morphological and molecular data**".
2. "**Molecular detection of hemoprotozoan in ticks collected on *Nasua nasua* from São Paulo, Brazil**
3. **"*Didelphis albiventris* naturally infected with *Hepatozoon canis* in southeastern Brazil"**
4. **"Morphological and molecular characterization of hemoparasites in *Didelphis* spp. from Brazil".**

Esses artigos integram o capítulo 1, 2, 3 e 4 dessa tese, respectivamente

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- Referências citadas de acordo com as normas da ABNT

5. CAPÍTULO 1

ARTIGO CIENTÍFICO:

A survey of haemoparasites and ectoparasites in *Nasua nasua* with a redescription of *Hepatozoon procyonis* based on morphological and molecular data

❖ **A versão final deste artigo foi publicada na revista Parasitology Research https://doi.org/10.1007/s00436-018-5903-x**

A survey of haemoparasites and ectoparasites in *Nasua nasua* Linnaeus, 1766 with a redescription of *Hepatozoon procyonis* Richards, 1961 based on morphological and molecular data

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Abstract

Haemoparasites are vector-borne parasites that infect wild carnivores worldwide. Since data about haemoparasite infections in *Nasua nasua* from Brazil are lacking, the aim of this study was to investigate the occurrence of haemoparasites and ectoparasites in *N. nasua* from different areas of Brazil. Blood samples and ectoparasites from 83 *N. nasua* were collected in Botucatu, Palmital, and São Paulo municipalities. Samples were screened via microscopy and molecular methods to detect haemoparasites. Tissues from two *N. nasua* were obtained for histopathological and molecular analyses. All 83 samples were negative for piroplasms on morphological and molecular examination. Thin blood smears of nine animals were positive for *Hepatozoon* gamonts. The gamonts shared morphological characteristic of *Hepatozoon procyonis.* Meronts were detected in the liver and spleen tissue of one animal. Twenty-one blood samples and four tissue samples were PCR-positive for *Hepatozoon* sp. The sequences obtained were 97% identical to those of *Hepatozoon felis*, *Hepatozoon ursi*, and *Hepatozoon* sp. Based on searches for similarity and morphology, we identified the sequences to be from *H. procyonis.* In conclusion, we provide epidemiological data on haemoparasite infections and redescribe *H. procyonis* based on morphological, morphometrical, and molecular analyses.

Keywords: coatis, *Hepatozoon procyonis*, histology, molecular characterisation, tick-borne pathogens

Introduction

The ring-tailed coati (*Nasua nasua*) are medium-sized animals that belong to the order Carnivora and family Procyonidae. *Nasua nasua* are omnivores that have a generalist diet, comprised mainly of insects, fruits, and small vertebrates (Alves-Costa et al. 2004). They are broadly distributed in South America and adapt to different environments and food, making it possible for them to live in urban areas and allowing them to interchange between wild and domestic environments (Rodrigues et al. 2006). Vector-borne haemoparasites are important worldwide, as they can cause diseases in animals and human. Many of these pathogens are maintained in wildlife reservoirs (Herrera et al. 2008; Shock et al. 2011; Yabsley and Shock 2013; Alvarado-Rybak et al. 2016). For example, the coatis are considered reservoirs for *Trypanosoma cruzi* and *Trypanosoma evansi* (Phylum: Sarcomastigophora), and can become infected with several other haemoparasites, including members of phylum Apicomplexa, as piroplasms and *Hepatozoon* spp. (Herrera et al. 2004; Rodrigues et al. 2007; Herrera et al. 2008; Sousa et al. 2017a; Sousa et al. 2017b).

Piroplasms are intraerythrocytic tick-borne parasites that include species within the genera *Theileria*, *Babesia*, and *Cytauxzoon* (Barbosa et al. 2017). These protozoa have a zoonotic and/or veterinary importance. *Cytauxzoon* spp. infection is mainly reported in felids (Alvarado-Rybak et al. 2016). Some species of *Theileria* are highly pathogenic to cattle and small ruminants, causing significant economic loss (Bishop et al. 2004). *Babesia* spp. are considered the causative agents of a zoonosis named babesiosis and wildlife animals act as possible reservoirs for zoonotic *Babesia* (Yabsley and Shock 2013). Piroplasm infections have been reported in several carnivore species, including members of the Procyonidae family (Alvarado-Rybak et al. 2016). In the Brazilian Pantanal, *Theileria* sp. closely related to *Theileria equi* has been detected infecting *N. nasua* (Sousa et al. 2017b). However, information is lacking regarding piroplasm infection in *N. nasua* from other regions of Brazil.

Hepatozoon species (Adeleorina: Hepatozoidae) are blood parasites that infect domestic and wild animals worldwide. They are mainly reported to infect carnivores and rodents, among the mammals (Modrý et al. 2017). Within the Procyonidae family, *Hepatozoon* sp. were first reported in *Procyon lotor* from the United States, and consequently named *H. procyonis* (Richards 1961). *Procyon cancrivorus* were also demonstrated to be infected with *H. procyonis* (Schneider 1968; Rodrigues et al. 2007). In Brazil, *H. procyonis* were detected in *N. nasua* for the first time by morphological analysis (Rodrigues et al. 2007). Sousa et al. (2017b) performed the first molecular detection of the *Hepatozoon* sp. in *N. nasua*.

However, the studies that previously reported the *Hepatozoon* spp. infection in coatis were based solely on either morphological (Rodrigues et al. 2007) or molecular examination (Sousa et al. 2017a). Studies involving both morphological and molecular analyses are needed to improve characterisation of haemoparasite species in *N. nasua*, allowing for a better understanding of their epidemiology.

Ticks and fleas are hematophagous ectoparasites that can cause irritation and spoliation. Moreover, they are of great medical and veterinary importance, because these ectoparasites play a role as vectors of pathogens for human and animals (Muller et al. 2005). Therefore, the study of ectoparasites associated with wildlife are necessary to elucidate whether these arthropods may represent a risk to the health of humans and animals (Dantas-Torres et al. 2010). In Brazil, few recent studies have investigated the presence of ectoparasites infesting coatis (Dantas-Torres et al. 2010; Martins et al. 2017; Sousa et al. 2017a).

In this context, the aim of the present study was to investigate the occurrence of haemoparasites and ectoparasites in *N. nasua* from different regions in Brazil, using both morphological and molecular analyses. In addition, we redescribe *H. procyonis* based on its morphological and molecular characteristics.

Material and methods

Study area and sample collection

Between September 2013 and June 2017, blood samples and ectoparasites were collected from *N. nasua* in three municipalities of the state of São Paulo (Botucatu, Palmital, and São Paulo), Brazil (Fig. 1). In Botucatu (22° 53′ 25″ S, 48° 27′ 19″ W), 30 animals were sampled from two forest fragments within the "Universidade Estadual Paulista (UNESP)". Forty *N. nasua* were captured in a park located in the central region of the Palmital municipality (22° 47′ 1.7″ S, 50° 12′ 24.8″ W). The animals sampled in Palmital were part of a population control program developed by veterinarians from the "Centro de Medicina e Pesquisa em Animais Selvagens (CEMPAS)", in which the males and females were subjected to vasectomy and tubal ligation, respectively. Moreover, food (fruits and a mixture of cornmeal, bananas, and dog food) was offered daily to these animals. Thirteen samples were collected from *N. nasua* in the "Centro de Triagem de Animais Silvestres (CETAS) of the Parque Ecológico do Tietê", from the urban area of the São Paulo municipality (23° 29′ 29.0″ S, 46° 31′ 15.3″ W). In Botucatu and São Paulo regions,

111 the coatis were free to move between urban and wild environments, whereas in the Palmital municipality,
112 the animals remained restricted to the park (urban region) owing to the absence of nearby floret
113 fragments.

114 The animals were anaesthetised with a combination of ketamine and midazolam. The sex and
115 age of the *N. nasua* were recorded. Age was estimated based on tooth wear, body size, and weight, and
116 the animals were classified as either young or adult (Herrera et al. 2008). Blood samples were collected
117 from the jugular vein and stored in EDTA tubes at -20 °C until DNA isolation. For microscopic
118 examination, thin blood smears were performed, fixed in absolute methanol, stained with 10% Giemsa,
119 and screened for the presence of piroplasms and *Hepatozoon* spp. The parasitemia of the positive coatis
120 was estimated by counting the number of gamonts in 500 leukocytes (Aktas et al. 2015).

121 *Nasua nasua* were also inspected for the presence of ectoparasites. Tick and flea specimens were
122 stored in 70% ethanol. Morphological identification of arthropods was performed following taxonomic
123 keys (Barros-Battesti et al. 2006; Martins et al. 2010; Linardi and Santos 2012). To detect the presence of
124 *Hepatozoon* oocysts in the tick haemocoel, 35 specimens (nymphs and adults) were randomly selected
125 and dissected as previously described by Demoner et al. (2013).

126

127 **Histopathological analysis**

128 All animals were apparently healthy except for two *N. nasua* that died in the CETAS of the
129 "Parque Ecológico do Tietê". One animal was bitten by a dog and died owing to respiratory insufficiency,
130 and the other was euthanized because it was severely injured with myiasis, muscular atrophy, and bone
131 exposure of the lumbar spine. They were necropsied and tissues samples (liver and spleen) were collected
132 for histopathological and molecular analyses. For histopathology, tissues were formalin-fixed, paraffin-
133 embedded, sliced at a thickness of 5 µm, and stained with haematoxylin and eosin (H&E).

134

135 **Morphometric analysis**

136 The parasites detected in the thin blood smears and in the histopathological analyses were
137 measured via a computerized image analysis system, using the software QWin Lite 2.5 (Leica). All the
138 detected *Hepatozoon* gamonts had their length, width, and cytoplasm projection measured. The number of
139 gamonts measured by animal ranged from 1 to 23 gamonts. The nuclei, when visible, were also measured.
140 We further measured the length and width of the *Hepatozoon* stages detected in the tissues.

DNA extraction, amplification, and sequencing

Genomic DNA of each sample was isolated from 200 µL of blood or 20–50 mg of tissues (liver and spleen), using the illustra™ blood genomicPrep Mini Spin Kit and the illustra™ tissue and cells genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, UK), respectively, following the manufacturer's instructions. DNA concentrations (ng/µL) and quality (A_{260nm}/A_{280nm}) were measured using spectrophotometry (NanoDrop® ND-2000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA).

Piroplasm detection was performed by conventional PCR using the primers BAB2 143-167 (5'-CCG TGC TAA TTG TAG GGC TAA TAC A-3') and BAB2 694-667 (5'-GCT TGA AAC ACT CTA RTT TTC TCA AAG-3'), targeting a ≈ 500-bp fragment of the 18S rRNA gene of *Babesia* spp., *Theileria* spp., and *Cytauxzoon* spp. (Almeida et al. 2012). PCR reactions were performed using the following amplification conditions: 95 °C for 5 min, 58 °C for 1 min, and 72 °C for 2 min with 35 repetitive cycles of 94 °C for 30 sec, 58 °C for 20 sec, and 72 °C for 30 sec followed by a final extension at 72 °C for 7 min.

DNA samples were screened for the presence of *Hepatozoon* spp., using the HepF300 (5'-GTT TCT GAC CTA TCA GCT TTC GAC G-3') and Hep900 (5'-CAA ATC TAA GAA TTT CAC CTC TGA C-3') primer pair, which amplifies 600 bp of the 18S rRNA gene (Ujvari et al. 2004). The PCR conditions were 94 °C for 3 min, 35 cycles at 94 °C for 45 sec, 56 °C for 1 min, 72 °C for 1 min, and a final extension step at 72 °C for 7 min. All positive samples in the first PCR were also amplified by nested PCR with the primers HAM-1 (5'-GCC AGT AGT CAT ATG CTT GTC-3') and HPF-2 (5'-GAC TTC TCC TTC GTC TAA G-3') (Criado-Fornelio et al. 2006), followed by 4558 (5'-GCT AAT ACA TGA GCA AAA TCT CAA-3') and 2733 (5'-CGG AAT TAA CCA GAC AAA T-3') (Mathew et al. 2000), which targeted a larger fragment (1,120 bp) of the 18S rRNA gene for sequencing and phylogenetic analysis. The PCR conditions for the primary PCR (primers HAM-1 and HPF-2) consisted of a pre-PCR step at 95 °C for 3 min, followed by 40 cycles of 95 °C for 1 min, 56 °C for 1 min, an extension at 72 °C for 1 min and 30 sec, and a final extension at 72 °C for 7 min. The PCR conditions of the secondary PCR (primers 4558 and 2733) consisted of a pre-PCR step at 94 °C for 3 min, followed by 40 cycles of 94 °C for 1 min, 55 °C for 2 min, an extension at 72 °C for 2 min, and a final extension at 72 °C for 10 min.

To avoid misdiagnosis of *Hepatozoon* meronts with *Toxoplasma gondii* cysts, a PCR was performed on the *N. nasua* DNA tissues with the primers TOX4 (5'-CGC TGC AGG GAG GAA GAC

GAA AGT TG-3') and TOX5 (5'- CGC TGC AGA CAC AGT GCA TCT GGA TT-3'), which targeted a 529-bp fragment of the *T. gondii* repeated sequence (Homan et al. 2000).

All the PCRs were carried out in a total volume of 25 µL: 12.5 µL of GoTaq® Colorless Master Mix (Promega Corporation, WI, USA), 1.25 µL (10 pmol/µL) of each primer, 5 µL of DNA, and 5 µL of nuclease free water (Thermo Scientific). A negative (distilled sterile water) and a positive control (*Hepatozoon canis* or *Babesia vogeli* DNA isolated from naturally infected dogs, or *T. gondii* DNA extracted from experimentally infected mice) were used in each reaction.

Amplified products were visualized by electrophoresis on 1% agarose gels stained with GelRed™ (Biotium, Hayward, USA). The amplicons were purified using illustra ExoProStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) in agreement with the manufacturer's instructions. PCR products were sequenced using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Foster City, CA, USA) and ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Phylogenetic analysis

The obtained sequences were edited using the BioEdit software v7.2.5 (Hall 1999) and compared for similarity with sequences available in GenBank® by BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). Multiple alignment, with 628 bp, for four sequences obtained in this study and 32 sequences retrieved from GenBank®, was performed using the MUSCLE algorithm and the GENEIOUS v.7.1.3 software (Biomatters; http://www.geneious.com). The best evolutionary model for maximum likelihood analysis was identified using the jModelTest v.2.1.10 program (Darriba et al. 2012). The best fitting model based on the Akaike Information Criterion (AIC) was TPM2uf + G. The phylogenetic tree was implemented by the maximum likelihood methods using PhyML v.3.0 (Guindon et al. 2010). To estimate internal nodes of the tree, a bootstrap with 1000 replicates was used. An alignment with 1,085 bp from one *Hepatozoon* sp. sequence obtained in this study and five *Hepatozoon* sp. sequences isolated from carnivores available on GenBank®, was used to estimate the percentage of nucleotide divergence. This analysis was performed on the MEGA6 software, using the Kimura 2 Parameter substitution model (Tamura et al. 2013).

Statistical analysis

The prevalence and confidence intervals (95%) for *Hepatozoon* spp. were calculated using Sourceforge.net® (http://sampsize.sourceforge.net/iface/index.html). Association between *Hepatozoon* sp. DNA positivity with origin (Botucatu, Palmital or São Paulo), age (young or adult), sex (female or male), tick (presence or absence) and fleas (presence or absence) was assessed using Pearson Chi Square test (all categories sample sizes>5) or Fisher's exact test (any category sample size<5). Association between infection with age, sex and ectoparasites was evaluated by region studied. Palmital region was excluded from the analysis because all samples were negative for *Hepatozoon* spp. An unpaired Student's t-test was used to determine statistically significant differences in the parasitemia between the age and sex. Analyses of the obtained data were carried out on the BioEstat 5.0 software (Ayres et al. 2007). *P*-values ≤ 0.05 were considered to indicate statistically significant differences.

Ethical statement

All procedures used in this study were approved by the local ethics committee on animal use (protocol no: 561-CEUA) and authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA; SISBIO authorization no: 42263-2).

Results

The 83 samples examined were negative for piroplasms in the thin blood smear and also in the PCR. Nine animals (10.8%; CI: 5–19.5; n = 83) were positive for *Hepatozoon* gamonts (Table 1). All positive samples in the thin blood smear were from the Botucatu municipality. The gamonts were elongated, with an eccentric nucleus and a curved cytoplasmic projection located opposite to the nucleus (Fig. 2), which is characteristic of *H. procyonis* (Richards 1961; Rodrigues et al. 2007). The average *Hepatozoon* parasitemia of the nine animals was 0.40%, ranging from 0.2% to 1%. No statistical difference was observed in the parasitemia of the groups: young (n=2) and adult (n=7) ($p = 0.57$); female (n=4) and males (n=5) ($p = 0.13$). However, the number of animals that had the parasitemia calculated was very small. In the histopathological analysis, several stages of *Hepatozoon* meronts were detected in the liver and spleen of one out of the two sampled animals (Figs. 3 and 4). The blood from positive animal in the histopathology was negative, for *Hepatozoon* spp., in the microscopic examination and

positive in the molecular analysis. All samples that presented as positive for infection in the thin blood smear and histopathology were also positive in the PCR.

Hepatozoon DNA was detected in 21 animals (25.3%; CI: 16.4–36; n = 83), using PCR (Table 1). A higher prevalence of *Hepatozoon* infection was registered in *N. nasua* from Botucatu, with 17 positive animals (56.7%; CI: 37.4–74.5; n = 30). In São Paulo, four animals (30.8%; CI: 9–61.4; n = 13) were infected with *Hepatozoon* sp. However, *Hepatozoon* infection was not found in *N. nasua* from the Palmital municipality. The prevalence of *Hepatozoon* infection between Botucatu and São Paulo municipalities was not statistically different (p = 0.18). However, statistic differences were observed between Botucatu and Palmital (p = 0.00), and between São Paulo and Palmital (p = 0.00), as in Palmital no infected animal was detected. Although the prevalence of *Hepatozoon* sp. was higher in female that in male and in adults that in young coatis, no significant association ($p > 0.05$) was observed between sex or age and the presence of *Hepatozoon* sp. in the evaluated regions (Table 2).

The liver and spleen of the two necropsied coatis were also PCR-positive for *Hepatozoon* sp. and PCR-negative for piroplasm and *T. gondii*. Twenty-five 18S rRNA sequences were obtained from the blood, liver, and spleen samples of the 21 positive animals, and were identified to be identical to each other. A BLAST search showed that the *Hepatozoon* sp. sequences isolated in this study were distinct from the *Hepatozoon* spp. sequences available in GenBank®. The obtained sequences demonstrated a 97% similarity to those of *Hepatozoon felis* (KX017290, AY628681, and AY620232), *Hepatozoon* sp. (EF222257, KU198330, and FJ719813), and *Hepatozoon ursi* (EU041718), and a 96% similarity to those of *Hepatozoon canis* (KX712126, DQ439540, and AY150067). Based on the similarity search and morphological characteristics of the *Hepatozoon* gamonts, we identified the sequences to belong to *H. procyonis*. All 25 *H. procyonis* sequences obtained in this study were deposited in GenBank® under the following accession numbers: MF685386–MF685410. The percentage of nucleotide divergence between *H. procyonis* and *Hepatozoon* spp. in carnivores ranged from 3.3–4.2% (Table 3).

The maximum likelihood tree (Fig. 5), inferred from a 640-bp fragment of the 18S rRNA gene from 32 *Hepatozoon* sequences, showed three principal clades: one comprised of *Hepatozoon* spp. sequences from carnivores, and the other two clades grouped *Hepatozoon* spp. sequences from amphibians, reptiles, rodents, and marsupials. Moreover, *H. procyonis* detected in this study clustered with *Hepatozoon* sp. sequences previously isolated from *N. nasua* in Brazil (KX779359 and KX779361) and formed a unique clade strongly supported with 100% bootstrap.

A total of 129 tick specimens were collected from 31 *N. nasua* (37.3%; CI: 26.9–48.6; n = 83). Tick specimens were morphologically identified as *Amblyomma dubitatum*, *Amblyomma ovale*, *Amblyomma sculptum*, *Amblyomma* sp., and *Rhipicephalus sanguineus* sensu lato (Table 1). Of the 21 coatis that were positive for *H. procyonis*, 18 were also positive for ticks (85.7%; CI: 63.6– 96.9). However, the presence of ticks was not significantly correlated with the presence of *H. procyonis* ($p > 0.05$). *Amblyomma ovale* and *A. sculptum* were the most prevalent tick species infesting *N. nasua*. *Amblyomma ovale* was detected in 15 of the 31 animals infested with ticks (43.9%; CI: 30.1–66.9; n = 31) and *A. sculptum* was present on 16 coatis (51.6%; CI: 33–69.8; n = 31). To detect the presence of *Hepatozoon* oocysts, 35 tick specimens (30 *A. ovale* adults and five *A. sculptum* nymphs) were dissected. All the evaluated tick specimens were negative for *Hepatozoon* oocysts. Additionally, 12 specimens of fleas were also found infesting seven *N. nasua* (8.4%; CI: 3.4–16.6; n = 83). Ten fleas were identified as *Rhopalopsyllus lutzi lutzi* and two as *Ctenocephalides felis* (Table 1). No significant association was observed between the flea infestation and the *H. procyonis* infection ($p > 0.05$).

Redescription of *Hepatozoon procyonis* Richards, 1961

Gamonts: Gamonts were located in the cytoplasm of neutrophils and monocytes. They were elongated, surrounded by a capsule, and presented a mean size of 10.35 ± 0.53 µm × 3.21 ± 0.29 µm (9.06–11.34 µm × 2.53–4.01 µm; n = 62). The *H. procyonis* gamonts showed a curved cytoplasmic projection that was located opposite to the nucleus, which is characteristic to this species (Fig. 2). The cytoplasmic projection measured 4.33 ± 0.35 µm (3.66–5.15 µm; n = 62). It was not possible to visualize the nucleus in most of the gamonts. When visible, the compact nucleus was located eccentrically and showed a mean size of 4.07 ± 0.44 µm × 2.22 ± 0.22 µm (2.97–4.36 µm × 1.81–2.53 µm; n = 13).

Meronts: Several developmental stages of meronts were observed in the liver and spleen of *N. nasua* (Figs. 3 and 4). The *H. procyonis* meronts were round to oval and separated from the surrounding tissues by a thick membrane, which enveloped them. Immature meronts contained amorphous material (Fig. 3) and measured 15.39 ± 2.4 µm × 12.99 ± 2.29 µm (10.07–20.39 µm × 8.58–18.84 µm; n = 53). Almost-mature meronts presented visible nuclei without distinct merozoites (Fig. 4). The mean size was observed to be 21.63 ± 0.61 µm × 18.94 ± 2.31 µm (21.1–22.45 µm × 16.69–21.34 µm; n = 4). No mature meronts were observed.

Taxonomic summary

Type host: *Procyon lotor* Linnaeus, 1758 (Carnivora: Procyonidae) (Richards 1961; Clark et al. 1973)

Other host: *Procyon cancrivorus* Cuvier, 1798 (Carnivora: Procyonidae) (Schneider 1968; Rodrigues et al. 2007); *Nasua nasua* Linnaeus, 1766 (Carnivora: Procyonidae) (Rodrigues et al. 2007; present study)

Vector: Unknown

Site infection: Gamonts were observed in peripheral and total blood (Richards 1961; Schneider 1968; Clark et al. 1973; Rodrigues et al. 2007; present study). Meront stages were detected in the heart (Richards 1961; Schneider 1968; Clark et al. 1973), skeletal muscle (Clark et al. 1973), and liver and spleen tissue (present study).

Type locality: Southwestern Georgia (Richards 1961)

Other localities: Panama (Schneider 1968); Texas, United States (Clark et al. 1973); Rio de Janeiro State, Brazil (Rodrigues et al. 2007); Botucatu (22° 53′ 25″ S, 48° 27′ 19″ W) and São Paulo (23° 29′ 29.0″ S, 46° 31′ 15.3″ W) municipalities, São Paulo State, Brazil (present study)

Prevalence (present study): Botucatu: 17 out of 30 (56.57%); São Paulo: four out of 13 (30.77%)

DNA sequences: Twenty-five 18S rRNA sequences (964–1064 bp) were deposited in GenBank® (Accession numbers: MF685386–MF685410).

Material deposited: Blood smear and histology slides will be deposited at the "Coleção Helmintológica do Instituto de Biociências de Botucatu (CHIBB)'', at the Parasitology Department, IBB, UNESP, São Paulo, Brazil.

Discussion

Piroplasms have been reported to infect a wide range of wild carnivores worldwide, including members of the Procyonidae family such as the raccoon (*P. lotor*) in the USA and Japan, and the white-nosed coatis (*Nasua narica*) in Costa Rica (Kawabuchi et al. 2005; Birkenheuer et al. 2006; Birkenheuer et al. 2008; Jinnai et al. 2009; Clark et al. 2012; Mehrkens et al. 2013; Alvarado-Rybak et al. 2016). In Brazil, these protozoa have been detected via molecular methods in carnivores such as the pampas cat (*Oncifelis colocolo*), common genet (*Genetta genetta*), crab-eating fox (*Cerdocyon thous*), pampas fox (*Lycalopex gymnocercus*), jaguar (*Panthera onca*), little spotted cat (*Leopardus tigrinus*), margay (*Leopardus wiedii*), ocelot (*Leopardus pardalis*), pallas's cat (*Otocolobus manul*), puma (*Puma concolor*), jaguarundi (*Puma yagouaroundi*), maned wolf (*Chrysocyon brachyurus*) and ring-tailed coati (*N. nasua*)

(André et al. 2009; André et al. 2011; Soares et al. 2014; Silveira et al. 2016; Furtado et al. 2017; Soares et al. 2017; Sousa et al. 2017b). However, piroplasms were not detected in *N. nasua* from the Botucatu, Palmital, and São Paulo municipalities. The absence of the piroplasm infection in coatis from these regions may be related to the geographical distribution of these protozoa and unavailability of competent vectors, considering that ring-tailed coatis from Pantanal region of Brazil have been infected with *Theileria* sp., closely related to *T. equi* (Sousa et al. 2017b).

Hepatozoon procyonis was the only haemoparasite found infecting *N. nasua* from the regions of our study. This parasite was first reported infecting *P. lotor* specimens from Georgia, United States (Richards 1961). *Hepatozoon procyonis* was also detected in *P. cancrivorus* from Panama and Brazil (Schneider 1968; Rodrigues et al. 2007). By morphological analysis, Rodrigues et al. (2007) identified *H. procyonis* in *N. nasua* from Brazil and morphometrically characterized the gamonts of this species. Recently, *Hepatozoon* sp. was detected infecting *N. nasua* from the Brazilian Pantanal via molecular methods, but the *Hepatozoon* species that affected these animals was not identified (Sousa et al. 2017a). To our knowledge, this is the first report that characterizes *H. procyonis* from *N. nasua* morphologically, morphometrically, and molecularly. Based on these characteristics, we also redescribed this parasite.

We detected *Hepatozoon* gamonts in thin blood smears of the *N. nasua*. The gamonts were elongated with eccentric nuclei and presented a cytoplasmic projection. The morphological data obtained in this study agreed with previously described gamont characteristics for *H. procyonis* (Richards 1961; Schneider 1968; Rodrigues et al. 2007). The study by Rodrigues et al. (2007) reported that the mean size of the *H. procyonis* gamonts from *N. nasua* was 10.45 × 4.03 µm. In our study, similar data was obtained, with gamonts measuring 10.35 × 3.21 µm on mean. The parasites observed in *P. lotor* had their nuclei located in the central region of the gamont (Richards 1961). However, the nuclei of the *H. procyonis* gamonts from *N. nasua* were reported to be eccentric and measured 4.20 × 2.03 µm on mean (Rodrigues et al. 2007). The gamonts identified in this study also exhibited eccentric nuclei with a mean size of 4.07 × 2.22 µm. However, the nuclei of most of the gamonts were not visible. The morphometric data obtained in this study with respect to cytoplasmic projection, a characteristic of *H. procyonis*, were similar to that described by Rodrigues et al. (2007). Cytoplasmic projection measured 4.33 µm in this study, whereas Rodrigues et al. (2007) reported measurements of 4.08 µm.

Hepatozoon procyonis meronts have been found in the heart and skeletal muscle tissues of *P. lotor* and in the heart tissue of *P. cancrivorus* (Richards 1961; Schneider 1968; Clark et al. 1973). We

detected, for the first time, *H. procyonis* meronts in the liver and spleen tissues of *N. nasua*. The almost-mature meronts found in this study measured a mean of 21.63 × 18.94 µm and were smaller than the meronts detected in *P. lotor* and *P. cancrivorus* (Schneider 1968; Clark et al. 1973). *Hepatozoon procyonis* meronts observed in *P. lotor* measured 31.2 × 22.7 µm on mean (Clark et al. 1973), and those detected in *P. cancrivorus* presented a mean size of 40.3 × 27.1 µm (Schneider 1968). The differences observed in the size of the meronts may be due to the developmental stages of the measured meronts. Other explanations could be associated with the techniques of fixation and preparation of the samples, errors in morphometric analysis, or affiliation with other *Hepatozoon* species (Baneth and Shkap 2003; Kubo et al. 2010; Hodžić et al. 2017).

Phylogenetic analysis demonstrated that the *H. procyonis* sequence obtained in this study grouped in the same clade as the *Hepatozoon* sp. sequences isolated from *N. nasua* in the Brazilian Pantanal (Sousa et al. 2017a). Therefore, the *Hepatozoon* sp. detected in the Pantanal is probably *H. procyonis*, which suggests that this parasite appears to be common among coatis from several regions of Brazil.

Hepatozoon procyonis was detected in two (Botucatu and São Paulo) out of the three studied regions. No *H. procyonis* infection was observed in coatis from Palmital. This was probably because the animals lived restricted to the park and did not move between the urban and wild environments, consequently being less exposed to vectors. Moreover, the coatis from Palmital were fed daily and exposed to good nutritional conditions, suggesting robust immune systems for the animals. Our results are in agreement with Calegaro-Marques and Amato (2014) that stated the influence of the degree of urbanization on helminthic richness, abundance and community structure of *Turdus rufiventris.* These authors reported that urbanization disrupts host-parasite interactions and a higher environmental diversity allows the survival of intermediate hosts, vectors and parasites (Calegaro-Marques and Amato, 2014). Additionally, the prevalence of vectors as fleas and ticks infesting dogs is frequently higher in rural areas than in urban areas (Abarca et al. 2016).

Usually, the *Hepatozoon* infected animals showed low parasitemia (Mundim et al. 2008). In this study the parasitemia mean of *H. procyonis* was relatively low (0.4%), which indicates that the coatis evaluated were probably in the chronic stage of infection. In addition, no statistical difference was observed in the parasitemia with age or sex of animals. New studies are needed for evaluated this association in a higher number of animals.

The prevalence of haemoparasites was suggested to be affected by several factors, such as the host's sex and age (Aktas and Ozubek 2017). In our study, no significant difference was observed between a positive *H. procyonis* infection and the sex or age of the host. This information corroborates those in previous studies (Baneth et al. 2013; Cardoso et al. 2014; Aktas and Ozubek 2017) and suggests that coatis were infected when young. No association was observed between *H. canis* infection in red foxes (*Vulpes vulpes*) and the sex or age of the animals, and the authors suggested that the foxes were infected by a vector or by transplacental transmission when young (Cardoso et al. 2014). However, the possibility of transplacental transmission for *H. procyonis* infection have not yet been studied.

In the present study, five tick species were detected on *N. nasua* (*A. dubitatum*, *A. ovale*, *A. sculptum*, *Amblyomma* sp., and *R. sanguineus* s. l.). We further identified, for the first time, nymphs of *A. dubitatum* infesting *N. nasua*. The other stages and species of ticks were previously detected on coatis in studies performed in Brazil (Dantas-Torres et al. 2010; Martins et al. 2017; Sousa et al. 2017a). However, the *H. procyonis* vector remains unknown. We failed to detect *Hepatozoon* oocysts in ticks. One explanation for not detecting the oocysts in the ticks might be the fact that the development of *Hepatozoon* oocysts needs about 30 days in *Rhipicephalus turanicus*, and ~ 14 days in *A. ovale* (Giannelli et al. 2017). So, probably in the fresh taken ticks from the positive hosts there was not enough time for the *Hepatozoon* to develop further and mature oocysts. *Amblyomma ovale* was among the most frequent tick species collected from *N. nasua* in this study, and most of the *H. procyonis* positive animals were also found to be infested by *A. ovale*. Interestingly, *A. ovale* has been considered a potential vector for other *Hepatozoon* species (Forlano et al. 2005; Rubini et al. 2009; Demoner et al. 2013). These observations suggest that *A. ovale* could act as vector for *H. procyonis*, but further studies are required to confirm this hypothesis.

Conclusion

This study provides epidemiological data on haemoparasite infection in *N. nasua* from 3 distinct regions of Brazil. Furthermore, we characterized *H. procyonis* morphologically, morphometrically, and molecularly, and resdescribed this species. Further studies are required to elucidate the life cycle of *H. procyonis* and its vectors.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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568 Table 1. Prevalence of *Hepatozoon procyonis* infection and ticks species collected from *Nasua nasua* in the municipalities of Botucatu, Palmital and São Paulo, São Paulo
569 State, Brazil.

Origin	**Nº of animals sampled**	**Nº of animals Infected (n/%)**		**Nº of animals infested (n/%)**		**Nº de ticks specimens collected**							**Nº de fleas specimens collected**		
		Blood smear	PCR	Ticks	Fleas	*A. dubitatum*	*A. ovale*		*A. sculptum*		*Amblyomma* sp.	*R. sanguineus* s. l.	*R. lutzi lutzi*		*C. felis*
						N	F	M	F	N	L	F	F	M	F
Botucatu	30	9 (30.0)	17 (56.7)	26 (86.7)	6 (20.0)	5	27	27		39	20		8	2	
Palmital	40	0	0	3 (7.5)	0	1	1					1			
São Paulo	13	0	4 (30.8)	2 (15.4)	1 (7.7)				1	2	5				2
Total	83	9 (10.8)	21 (25.3)	31 (37.3)	7 (8.4)	6	28	27	1	41	25	1	8	2	2

570 Nº - number; *A. dubitatum - Amblyomma dubitatum; A. ovale – Amblyomma ovale; A. sculptum – Amblyomma sculptum; R. sanguineus* s. l. – *Rhipicephalus sanguineus* sensu
571 lato*; R. lutzi lutzi - Rhopalopsyllus lutzi lutzi; C. felis - Ctenocephalides felis;* N – Nymph; F – Female; M – Male; L – Lavae.

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575 Table 2. Comparison of *Hepatozoon procyonis* prevalence by PCR, in *Nasua nasua* from Botucatu, Palmital and São Paulo, in relation to ectoparasites, gender and age.

Origin	**Nº**	**Ticks**			**Fleas**			**Gender**						**Age**		
		Presence of ticks (n/%)	Positive (n/%)	*P* - *value*	Presence of fleas (n/%)	Positive (n/%)	*P* - value	Female		Male		*P* - value	Young		Adult		*P* - value
								Nº	Positive (n/%)	Nº	Positive (n/%)		Nº	Positive (n/%)	Nº	Positive (n/%)	
Botucatu	30	26 (86.7)	16 (61.5)	0.29	6 (20.0)	4 (66.7)	0.67	14	8 (57.1)	16	9 (56.2)	0.96	13	6 (46.1)	17	11 (64.7)	0.31
Palmital	40	3 (7.5)	0	*	0	0	*	24	0	16	0	*	15	0	25	0	*
São Paulo	13	2 (15.4)	2 (100)	0.08	1 (7.7)	1(100)	0.30	6	3 (50.0)	7	1 (14.3)	0.26	8	1 (12.5)	5	3 (60.0)	0.21
Total	83	31 (37.3)	18 (58.1)		7 (8.4)	5 (71.4)		44	11 (25.0)	39	10 (25.6)		36	7 (19.4)	47	14 (29.8)	

576 Nº - number of animals sampled

577 **P*- value not measured because in Palmital municipality all samples were negative for *Hepatozoon procyonis*

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581 Table 3. Distance matrix among *Hepatozoon* spp. sequences isolated from carnivorous. Upper triangle shows the percentage of nucleotide difference, while the lower triangle
582 shows the number of nucleotide difference.

Sequences	**1 (AF176836)**	**2 (KU569168)**	**3 (EU041718)**	**4 (MF685403)***	**5 (AY620232)**	**6 (KX757032)**
1. *Hepatozoon americanum*	-	3.2%	3.4%	3.6%	3.3%	2.7%
2. *Hepatozoon canis*	32	-	4.4%	4.2%	3.6%	3.6%
3. *Hepatozoon ursi*	34	44	-	3.8%	3.4%	3.5%
4. *Hepatozoon procyonis* *	36	42	38	-	3.3%	3.6%
5. *Hepatozoon felis*	33	37	35	34	-	2.2%
6. *Hepatozoon silvestris*	28	37	36	37	23	-

583 *Sequence isolated in this study.

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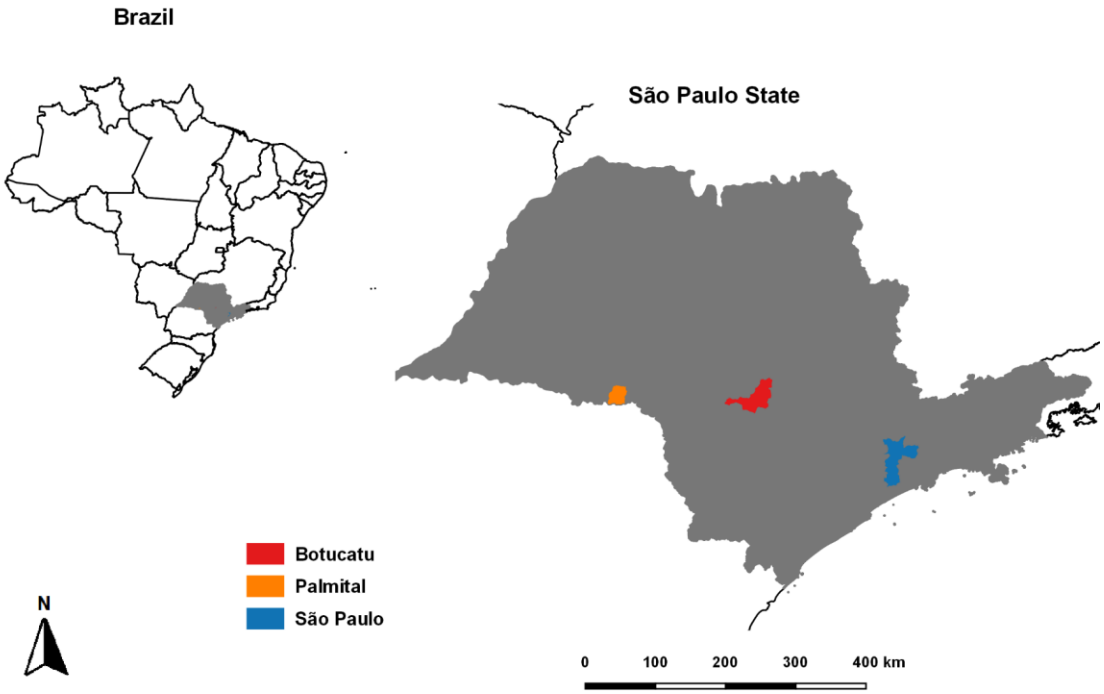

Fig. 1. Map of São Paulo State, indicating the three municipalities where samples were collected.

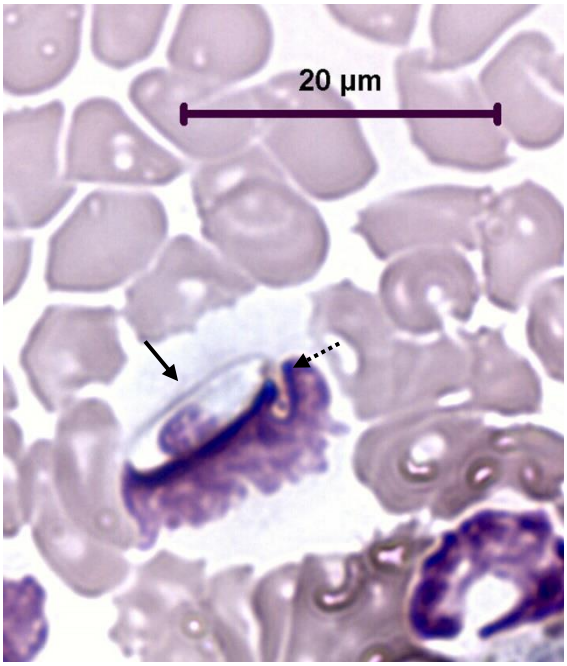

Figure 2. Gamont of *Hepatozoon procyonis* in the total blood of a *Nasua nasua*. (⇢) Gamont. (→) cytoplasmic projection. Giemsa stain.

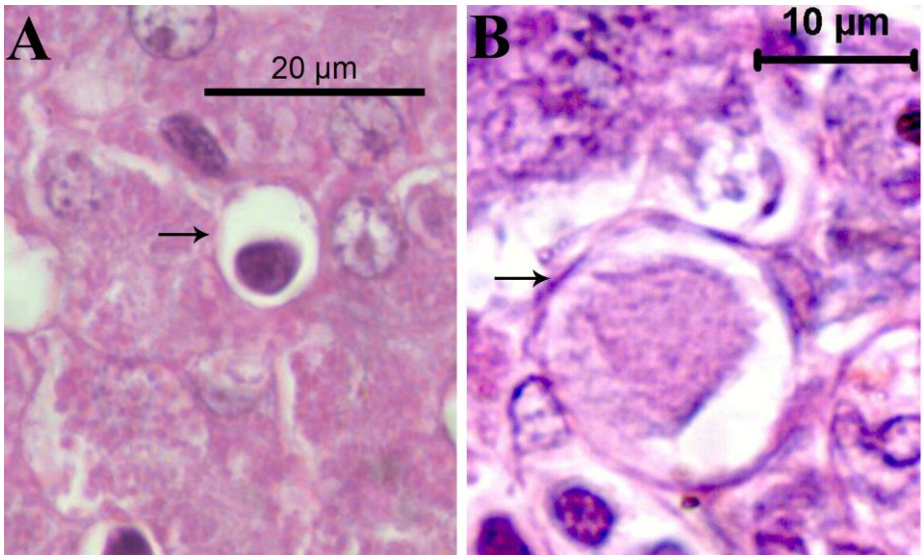

Figure 3. Imature meronts of *Hepatozoon procyonis* in the liver and spleen tissues from *Nasua nasua*. (A) An early *H. procyonis* meront in the liver. (B) An immature *H. procyonis* meront in the spleen. Hematoxylin & Eosin stain.

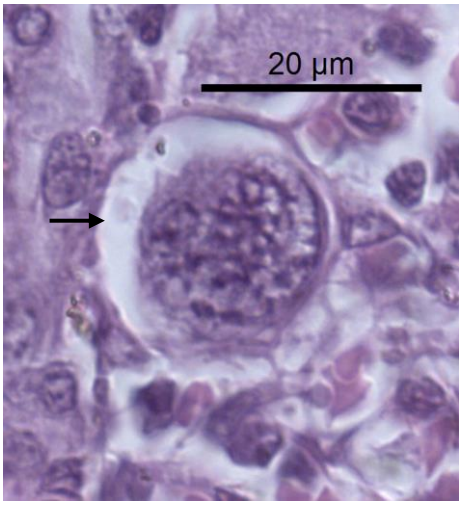

Figure 4. Almost mature meront of *Hepatozoon procyonis*, in the spleen of a *Nasua nasua*, with merozoites nuclei visible. Hematoxylin & Eosin stain.

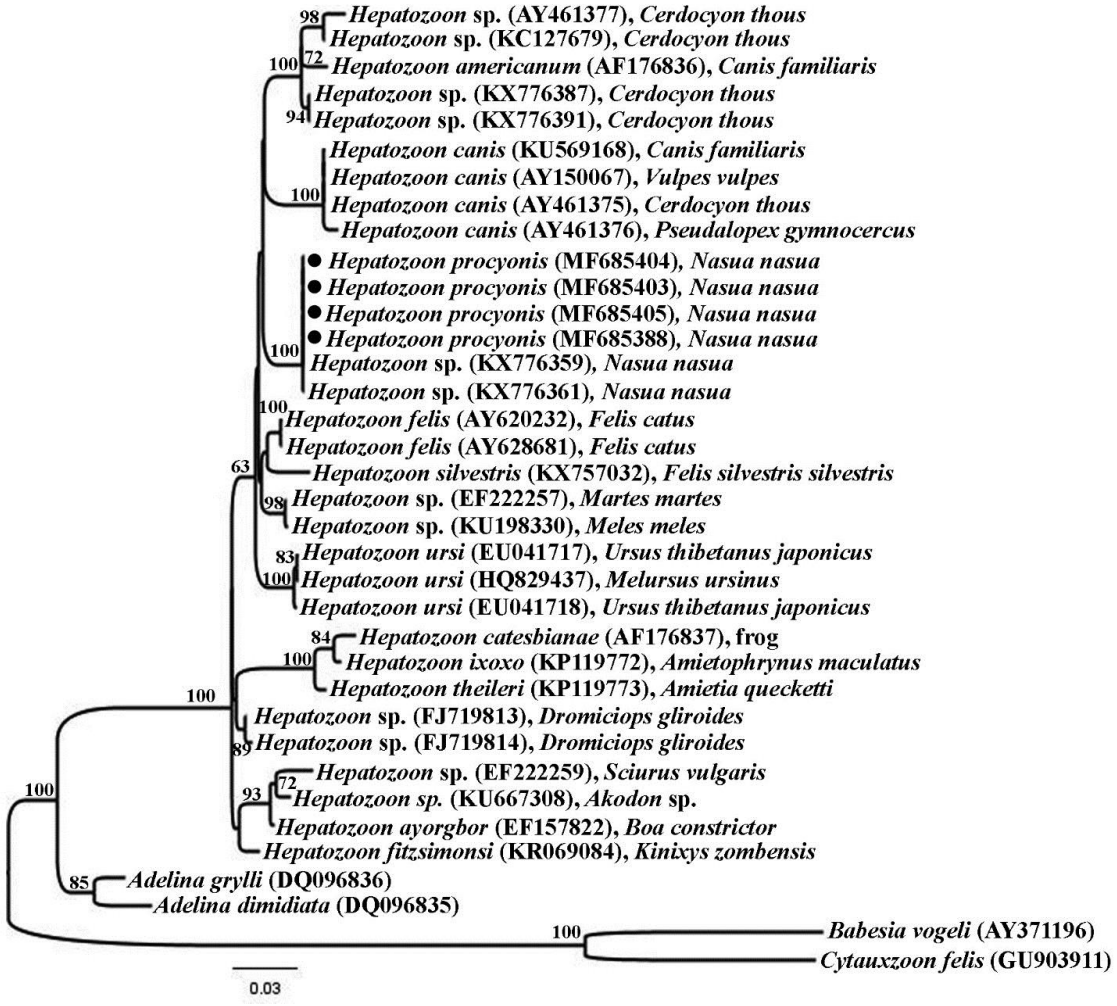

Fig. 5. Phylogenetic tree of maximum likelihood analysis based on the partial sequences (640 bp) of the 18S rRNA gene of *Hepatozoon* species from *Nasua nasua,* isolated in this study and sequences deposited in GenBank. The branch length scale represents 0.03 substitutions per site. *Cytauxzoon felis*, *Babesia vogeli*, *Adelina dimidiata* and *Adelina grylli* were used as outgroups. (●) sequences obtained in this study.

6. CAPÍTULO 2

ARTIGO CIENTÍFICO:

Molecular detection of hemoprotozoan in ticks collected on *Nasua nasua* from São Paulo, Brazil

Molecular detection of hemoprotozoan in ticks collected on *Nasua nasua* from São Paulo, Brazil

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ABSTRACT

Piroplasms and *Hepatozoon* spp. are tick-borne Apicomplexa protozoa that cause disease in both humans and animals. In Brazil is scarce the information about which protozoa are circulating in ticks from wild animals is scarce. Therefore, the aim of present study was to investigate the occurrence of hemoprotozoa in ticks from *Nasua nasua*, and molecularly characterize the parasites detected. Forty-eight tick samples were screened via molecular analysis to detect piroplasms and *Hepatozoon* species. Two *Amblyomma sculptum* samples were PCR positive for piroplasms. The Piroplasmida sequences obtained presented 99% similarity to *Babesia* sp. from capybara (EF222255) and 89% similarity to *Theileria orientalis*, *Theileria sergenti* and *Theileria annulata*, and in the phylogenetic analysis grouped with *Babesia* sp. (EF222255). In the PCR for *Hepatozoon* spp. detection, two *Amblyomma ovale* were positive. One of the detected sequences was 100% similar with *Hepatozoon procyonis*, while the other sequence showed 99% of identity for *Hepatozoon americanum* haplotype. In the phylogenetic analysis, *Hepatozoon* sp. from *A. ovale* clustered in the same clade with *H. americanum* haplotypes and *H. procyonis* sequence grouped with *H. procyonis* sequences from *N. nasua*. In conclusion, we detected *Hepatozoon* spp. and Piroplasmida in ticks from *N. nasua*. *Hepatozoon americanum* haplotype was detected in *A. ovale* for the first time and this parasite can represent a risk of infection for dogs.

Keywords: *Amblyomma ovale; Amblyomma sculptum; Hepatozoon americanum; Hepatozoon procyonis;* Piroplasmida.

1. Introduction

Ticks are arthropods with medical and/or veterinary relevance, distributed in the Argasidae, Ixodidae and Nuttalliellidae families (Guglielmone et al., 2010). They are haematophagous ectoparasites that infest vertebrates worldwide, and can cause irritation, dermatitis and anemia (Peter et al., 2005). However, the main relevance of ticks is that they are vectors of bacteria, viruses, and protozoa (Jongejan and Uilenberg, 2004). In addition, ticks and wildlife are the main reservoirs of tick-borne microorganisms, and certainly most tick-borne agents of humans and animals circulate between wild animals and ticks (Dantas-Torres et al., 2012; Baneth, 2014). Recently, the occurrence and diversity of tick-borne infections in humans and animals have increased due to factors, such as the existence of better diagnostic tools, global climate change and urbanization, that increased contact of humans and domestic animals with wildlife and vectors (Dantas-Torres, 2015; Alvarado-Rybak et al., 2016).

Wild carnivores belong to the same Order as dogs and cats, sharing several parasites (Alvarado-Rybak et al., 2016). *Nasua nasua* are medium-sized carnivores that belong to family Procyonidae. They are broadly distributed in South America and, in some regions of Brazil, the coatis live in urban areas and can make interchange between wild and domestic environments (Rodrigues et al., 2006; Silva et al., 2018). *Nasua nasua* are host of several tick species as *Amblyomma auricularium*, *Amblyomma brasiliense*, *Amblyomma dubitatum*, *Amblyomma parkeri*, *Amblyomma parvum*, *Amblyomma ovale*, *Amblyomma sculptum*, *Rhipicephalus microplus* and *Rhipicephalus sanguineus* sensu lato (Dantas-Torres et al., 2010; Martins et al., 2017; Sousa et al., 2017a; Silva et al., 2018).

The piroplasms, which include *Babesia* spp. and *Theileria* spp., and *Hepatozoon* spp. are tick-borne protozoa that belong to the Phylum Apicomplexa. *Babesia* spp. and

Theileria spp. are mainly transmitted by Ixodid ticks, via saliva, during the course of the blood meal (Homer et al., 2000; Baneth et al., 2014). These parasites infect erythrocytes of mammals and can cause a disease named piroplasmosis. Among arthropod-borne diseases of animals, piroplasmosis is one of the most prevalent (Alvarado-Rybak et al., 2016). *Hepatozoon* spp. are protozoa closely related to the piroplasms, and infect vertebrates through the ingestion of an arthropod containing mature oocysts (Smith, 1996). *Hepatozoon americanum* and *Hepatozoon canis* are the causative agents of canine hepatozoonosis. The vector of *H. americanum* is the tick *Amblyomma maculatum* and, *H. canis* is mainly transmitted by the tick *R. sanguineus* (Baneth et al., 2011), nevertheless, in Brazil can be transmitted by *A. ovale* (Forlano et al., 2005; Rubini et al., 2009; Demoner et al., 2013).

The molecular detection of DNA from parasites in ticks does not necessarily mean that they are biological vectors (Masatani et al., 2017). However, monitoring of pathogen occurrence in tick specimens can increase knowledge about the diversity and, improve the understanding of the epidemiology of tick-borne microorganisms (Anderson et al., 2017; Blanco et al., 2017). In Brazil, although there are some recent researches of detection of tick-borne pathogens in ticks from wild mammals, the majority of these studies evaluated the occurrence of bacteria (Almeida et al., 2013; Acosta et al., 2016; Witter et al., 2016; Dall'Agnol et al., 2017; Fontalvo et al., 2017; Labruna et al., 2017; Martins et al., 2017; Weck et al., 2017; Sousa et al., 2018c). Moreover, there were few molecular epidemiological studies on detection of protozoan parasites from ticks of wildlife (Almeida et al., 2013; Wolf et al., 2016; Blanco et al., 2017; Sousa et al., 2017a; Sousa et al., 2017b). In addition, most of these studies were performed in Brazilian Pantanal (Wolf et al., 2016; Sousa et al., 2017a; Sousa et al., 2017b). Therefore, currently are scarce the information about which protozoa are

circulating in ticks collected from wild animals in some regions of Brazil. Within that context, this study aimed to detect *Babesia* spp., *Theileria* spp., and *Hepatozoon* spp. in ticks from *N. nasua* and phylogenetically characterize the protozoa detected.

2. Materials and Methods

2.1. Collection of tick samples

From September 2013 to June 2017, 129 tick specimens were collected on 83 *N. nasua* from urban and peri-urban region of Botucatu (22° 53′ 25″ S, 48° 27′ 19″ W), Palmital (22° 47′ 1.7″ S, 50° 12′ 24.8″ W), and São Paulo (23° 29′ 29.0″ S, 46° 31′ 15.3″ W) municipalities, São Paulo State, Brazil (Silva et al., 2018). Details about the municipalities, capture and management of animals have been described by Silva et al., (2018).

Thirty-five of 129 ticks were dissected to detect the presence of *Hepatozoon* oocysts in the hemocoel (Silva et al., 2018). Twenty-five larvae were not identified to species level, and not used for molecular detection of hemoprotozoa. The other 69 tick specimens were identified and stored in 70% ethanol until DNA extraction (Silva et al., 2018).

2.2. DNA extraction, amplification and sequencing

DNA from adult ticks was isolated individually, while DNA from nymphs was extracted individually or in pools. Nymphs were pooled into groups consisting of 2-5 ticks, according to species and host. Each pool was considered as one tick sample. Prior to DNA isolation, ticks were washed with PBS and homogenized using a fine sterile scalpel blade. The Illustra Tissue Mini Spin Kit® (GE Healthcare, Buckinghamshire, UK) was used to DNA extraction from the ticks, following the manufacturer's

instructions. Spectrophotometry (NanoDrop® ND-2000 Spectrophotometer, Thermo Scientific, Wilmington, DE, USA) was used to measure DNA concentrations (ng/μL) and quality (A260nm/A280nm).

All samples (individual ticks and pools) were screened for the presence of piroplasms (*Babesia* spp., and *Theileria* spp.) and *Hepatozoon* spp. DNA. Piroplasms detection was first performed with the primers BAB2 143-167 and BAB2 694-667 (Almeida et al., 2012), following the conditions described by Silva et al. (2018). For additional sequencing and phylogenetic analysis two other protocols were performed (Table 1) (Jefferies et al., 2007; Paparini et al., 2012). The presence of *Hepatozoon* DNA was screened using the primers pair Hep300 and Hep900, which targets a 600-bp fragment of the 18S rRNA gene of *Hepatozoon* spp. (Ujvari et al., 2004). To amplify and sequence a larger segment of the 18S rRNA gene, the positive samples for *Hepatozoon* DNA were also submitted to a nested PCR protocol (Table 1) (Mathew et al., 2000; Criado-Fornelio et al., 2006).

All the PCRs were carried out in a total volume of 25 μL: 12.5 μL of GoTaq® Colorless Master Mix (Promega Corporation, WI, USA), 1.25 μL (10 pmol/μL) of each primer, 5 μL of DNA, and 5 μL of nuclease free water (Thermo Scientific). In the case of nested PCR, only 1 μl of DNA was used. In all PCRs, negative (distilled sterile water) and positive (*Hepatozoon canis* or *Babesia vogeli* DNA isolated from naturally infected dogs) controls were included.

The amplified DNA segments were visualized by electrophoresis on 1% agarose gels stained with GelRed™ (Biotium, Hayward, USA). The amplicons were purified using illustra ExoProStar™ 1-Step (GE Healthcare, Buckinghamshire, UK) in agreement with the manufacturer's recommendations. PCR products were sequenced using the BigDye™ Terminator v.3.1 Cycle Sequencing Ready Reaction Kit (Applied

Biosystems, Foster City, CA, USA) and ABI 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Phylogenetic analysis

The resultant sequences were edited with BioEdit, version 7.2.5 (Hall, 1999), and compared for homologous sequences in the GenBank databases, using BLASTn (http://www.ncbi.nlm.nih.gov/BLAST). The MUSCLE algorithm, in the software GENEIOUS v.7.1.3 (Biomatters, http://www.geneious.com) was used to performer the multiple alignments with sequences obtained from this study and sequences available in GenBank. The jModelTest v.2.1.10 program (Darriba et al. 2012) determined the best evolutionary models for maximum likelihood analysis. The best fitting models, based on the Akaike Information Criterion (AIC), for phylogenetic reconstruction of piroplasms and *Hepatozoon* spp. were TIM+I+G and HKY+G, respectively. Maximum likelihood trees were inferred using PhyML v.3.0 (Guindon et al. 2010), with 1000 bootstrap replicates.

2.4. Ethical approved

All procedures used in this study were approved by the local ethics committee on animal use of the Unesp-Botucatu (protocol nº: 561-CEUA) and in accordance with the licenses obtained from the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA; SISBIO authorization nº: 42263-2) (Silva et al., 2018).

3. Results

A total of 48 tick samples (individual ticks and pools) were screened for the presence of piroplasm and *Hepatozoon* spp. DNA. The samples consisted of 5 *A.*

dubitatum, 25 *A. ovale*, 17 *A. sculptum* and, 1 *R. sanguineus* sensu lato (Table 2). Of the 48 tick samples screened, only two samples (*A. sculptum*: one female and one pool of nymphs) were collected on *N. nasua* from São Paulo municipality. Three samples (*A. ovale*: one female; *A. sculptum*: one nymph; *R. samguineus* s.l: one female) were collected on animals from Palmital municipality. In Botucatu municipality, 43 tick samples were collected (*A. dubitatum:* four nymphs and one pool of nymphs; *A. ovale*: nine females and 15 males; *A. sculptum*: six nymphs and eight pools of nymphs).

In the PCR with the primers BAB2 143-167 and BAB2 694-667 (Almeida et al., 2012), two of the 48 samples were positive for piroplasms. Positive samples were one pool and one nymph of *A. sculptum* collected on two *N. nasua* from Botucatu municipality (Table 2). The two positive samples were successively tested with primers BT18SF1/R1 and BT18SF2/R2 (Paparini et al. 2012), however, no fragment was amplified. The samples were so amplified and sequenced using the primer pairs BTF1/R1 and BTF2/R2 (Jefferies et al., 2007). The sequences obtained were identical to each other, and BLAST search demonstrated 99% similarity to *Babesia* sp. from a Brazilian capybara (EF222255) and 89% similarity to *Theileria orientalis* (MF576178), *Theileria sergenti* (AF081137) and *Theileria annulata* (MF287950). The maximum likelihood phylogenetic tree based on a 709-bp fragment of the 18S rRNA gene of 47 piroplasms sequences, and *Plasmodim falciparum* and *Plasmodium ovale* as outgroups (Fig. 1), revealed that Piroplasmida sequences detected in *A. sculptum* in this study grouped with *Babesia* sp. obtained from a capybara with 100 bootstrap. In addition, our sequences and *Babesia* sp. capybara formed sister clades to the *Babesia* spp., with low bootstrap (31), and positioned distant of *Theileria* clade (Fig. 1).

Two *A. ovale* adults comprises the positive samples for *Hepatozoon* spp. DNA (Table 2). These positive ticks were collected on two distinct animals from urban and

peri-urban region of Botucatu municipality. The two sequences obtained were identified to be different to each other. BLAST analysis revealed that the sequence detected in an *A. ovale* female show 100% identity to *Hepatozoon procyonis* from *N. nasua* of Botucatu municipality (MF685388). While the sequence obtained from an *A. ovale* male presented 99% similarity to *Hepatozoon* sp. (AY461377) from a Brazilian crab-eating fox, and 97% of identity with *H. americanum* (AF176836). A phylogenetic tree (Fig. 2) was inferred from a 637-bp alignment of the 18S rRNA gene from 32 *Hepatozoon* sequences and, including sequences of *Babesia vogeli, Adelina grylli* and, *Adelina dimidiata* as outgroups. The phylogenetic analysis showed that *H. procyonis* sequence, detected in an *A. ovale*, grouped in a clade with *H. procyonis* sequence detected in *N. nasua* from Botucatu and, with *Hepatozoon* sp. sequence obtained from *N. nasua* in Brazilian Pantanal. *Hepatozoon* sp. sequence that was closely related to *H. americanum* grouped in the same clade that *H. americanum* sequence (AF176836) detected in a dog from United States and *H. americanum* genotypes (AY461377; KC127679; KX776387; KX776391) previously isolated from *Cerdocyon thous* in Brazil, with bootstrap strongly supported (100), based on maximum likelihood analysis (Fig. 2).

4. Discussion and Conclusion

Our study reports the molecular detection of *Hepatozoon* spp. and a Piroplasmida haplotype in ticks collected from *N. nasua* in urban and peri-urban region of Botucatu municipality. Previous studies had reported the occurrence of Hemoprotozoa in ticks collected from wild mammals in other regions of the Brazil (Blanco et al., 2017; Sousa et al., 2017b). In the Santa Catarina state, *Hepatozoon* sp. DNA was detected in *Amblyomma fuscum* collected on a specimen of *Akodon montensis*

(Blanco et al., 2017). In Brazilian Pantanal, *A. parvum* from *C. thous*, *Thrichomys fosteri* and *Clyomys laticeps*, *A. ovale* from *C. thous*, and *A. sculptum* from *C. thous* were PCR-positive for piroplasms and a high diversity of piroplasm species was reported (Sousa et al., 2017b).

For piroplasm diagnostic and sequencing, three PCR protocols were used here. Of the tested protocols, only the primers BTF1/R1 and BTF2/R2 (Jefferies et al., 2007) generated sequences with high quality. However, this primers pair amplifies approximately 850 bp (Jefferies et al., 2007), so we obtained short sequences. Paparini et al. (2015) observed similar results when performed molecular characterization of *Theileria ornithorhynchi*. Therefore, in the future, primers that amplify a larger fragment of 18S rRNA gene and presenting a highest sensivity should be designed.

Piroplasmida species detected from *A. sculptum*,in our study is a probable haplotype of *Babesia* sp. detected from a capybara in Brazil (Criado-Fornelio et al., 2009). Criado-Fornelio et al. (2009) provisionally identified this protozoan as *Babesia* sp. capybara 1, since in the phylogenetic analysis *Babesia* sp. grouped as a sister lineage of the babesids, but the bootstrap support was low (45). Posteriorly, in a phylogenetic analysis, *Theileria youngi* from a *Neotoma fuscipes* was grouped closed to the *Babesia* sp. isolated from the capybara, but also without statistical support (Lack et al., 2012). Similarly to previous studies, phylogenetic analysis for piroplasm performed here was not strongly supported. New studies involving morphological and molecular methods with more appropriated primers are necessary for the correct identification of this piroplasm. Considering that, *Babesia* sp. capybara 1 has been reported only in *Hydrochoerus hydrochaeris* and *A. sculptum* can infest these animals (Martins et al., 2017), we hypothesis that nymphs might become infected by feeding previously in the larval stage on Piroplasmida positive capybaras.

In the present study, *Hepatozoon procyonis* DNA was detected in *A. ovale* for the first time. Silva et al. (2018) did not detect *Hepatozoon* oocysts in ticks. However, *A. ovale* was the most prevalent tick infesting *N. nasua* infected by *H. procyonis*, suggesting this tick species as probable vector of *H. procyonis*. The presence of *Hepatozoon* DNA in *A. ovale* reinforce this hypothesis. Nevertheless, the detection of *Hepatozoon* DNA in ticks does not confirm the vector capacity. For ticks obtained from animals, it is unclear whether the ticks are vector or just got the pathogens from the infected host during feeding (Anderson et al., 2017). In addition, *A. ovale* has been considered a competent vector for other *Hepatozoon* species such as *H. canis* (Forlano et al., 2005; Rubini et al., 2009; Demoner et al., 2013). Therefore, the capacity of *A. ovale* to act as a *H. procyomis* vector should be better investigated.

To the best of our knowledge, this is the first report on detection of *Hepatozoon* sp. DNA closely related to *H. americanum* in *A. ovale. Hepatozoon americanum* is the causative agent of canine hepatozoonosis in the south-central and southeastern United States (Ewing and Panciera, 2003). American canine hepatozoonosis is a tick-borne disease highly debilitating and often fatal (Baneth et al., 2003; Ewing and Panciera, 2003). In Brazil, canine hepatozoonosis is caused by *H. canis* (Rubini et al., 2005; Demoner et al., 2016). However, *H. americanum* haplotypes have been detected in dogs from Northern region of Brazil (Gomes et al., 2016) and wild carnivores from South, Southern and Midwestern regions (Criado-Fornelio et al., 2006; André et al., 2010; Almeida et al., 2013; André et al., 2015; Sousa et al., 2017a). Crab-eating foxes from several Brazilian states have been identified as hosts for *H. americanum*-related haplotypes (Criado-Fornelio et al., 2006; André et al., 2010; Almeida et al., 2013; Sousa et al., 2017a). Sousa et al. (2017a) suggested that these haplotypes appears to be common in *C. thous* from Brazil. However, clinical cases of canine hepatozoonosis

caused by *H. americanum* have not been reported in Brazil. *Amblyomma ovale* nymphs preferentially infest small rodents and marsupials (Martins et al., 2016; Blanco et al., 2017), but can also parasitize wild and domestic carnivores (Guglielmone et al., 2003). Since *A. ovale* PCR-positive, closely related to *H. americanum*, was collected in a coati negative for *Hepatozoon* sp., this tick might had fed on infected carnivore hosts, such as *C. thous*, in a previous life stage.

In addition, *A. maculatum* is considered the vector of *H. americanum* (Baneth et al., 2011). However, the occurrence of this tick species has never been reported in Brazil. In a previous study, only *A. sculptum* nymphs were collected in *C. thous* positive for *H. americanum* haplotype and these ticks were PCR-negative for *Hepatozoon* (Almeida et al., 2013). As *Amblyomma* species presented in Brazil has parasitized crab-eating foxes, the authors suggested that the native species of *Amblyomma* should be considered as potential vectors of *H. americanum* haplotype, detected in *C. thous* (Almeida et al., 2013). *Amblyomma ovale*, as mentioned before, is a competent vector of *H. canis* and a likely vector of *H. procyonis* (Forlano et al. 2005; Rubini et al. 2009; Demoner et al. 2013; Silva et al., 2018). We detected DNA of *Hepatozoon* sp. closely related to *H. americanum* in *A. ovale* and thus, the role of this tick species as vector of *H. americanum* haplotypes detected in Brazil should be evaluated in the future.

In conclusion, this study indicate that *Hepatozoon* spp. and Piroplasmida may be circulating in ticks from *N. nasua* of urban and peri-urban regions of Botucatu municipality. Moreover, DNA of *H. americanum* haplotype was detected in *A. ovale* for the first time. Dogs shown to be highly suitable for the adult stage of *A. ovale* (Martins et al., 2012), and therefore, there is a risk of *H. americanum* haplotype infection in domestic animals.

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511

Table 1. PCR protocols used for detection of hemoprotozoa, in this study.

Protozoa	Target gene	Primers/Sequence (5' - 3')	Cycling conditions	Size (bp)	Reference
Piroplasms	18S rRNA	F-BAB2 143-167 / CCGTGCTAATTGTAGGGCTAATACA R-BAB2 694-667 / GCTTGAAACACTCTARTTTTCTCAAAG	1 cycle of 95 °C for 5 min, 58 °C for 1 min, and 72 °C for 2 min; 35 cycles of 94 °C for 30 sec, 58 °C for 20 sec, and 72 °C for 30 sec; Final extension of 72 ºC for 7 min.	500	Almeida et al., 2012
Piroplasms	18S rRNA	F-BT18SF1 / ACCTGGTTGATCCTGCCAGTAG R-BT18SR1 / GCAGGTTCACCTACGGAAACC	1º Round: 95 °C for 5 min; 40 cycles of 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 2 min; Final extension of 72 °C for 5 min.	1466	Paparini et al., 2012
		F-BT18SF2 / TTGTAGGGCTAATACAYGYTCG R-BT18SR2 / CACGGTCCGAATAATTCACC	2º Round: 95 °C for 5 min; 40 cycles of 94 °C for 30 sec, 50 °C for 30 sec and 72 °C for 1 min and 20 sec; Final extension of 72 °C for 5 min.		
Piroplasms	18S rRNA	F-BTF1 / GGCTCATTACAACAGTTATAG R-BTR1 / CCCAAAGACTTTGATTTCTCTC	1º Round: 1 cycle of 94 °C for 3 min, 58 °C for 1 min, and 72 °C for 2 min; 45 cycles of 94 °C for 30 sec, 58 °C for 20 sec, and 72 °C for 30 sec; Final extension of 72 ºC for 7 min.	800	Jefferies et al., 2007
		F-BTF2 / CCGTGCTAATTGTAGGGCTAATAC R-BTR2 / GGACTACGACGGTATCTGATCG	2º Round: 1 cycle of 94 °C for 3 min, 62 °C for 1 min, and 72 °C for 2 min; 45 cycles of 94 °C for 30 sec, 62 °C for 20 sec, and 72 °C for 30 sec; Final extension of 72 ºC for 7 min.		
Hepatozoon spp.	18S rRNA	F-HepF300 / GTTTCTGACCTATCAGCTTTCGACG R-HepR900 / CAAATCTAAGAATTTCACCTCTGAC	94 °C for 3 min; 37 cycles of 94 °C for 45 sec, 56 °C for 1 min and 72 °C for 1 min; Final extension of 72 °C for 7 min.	600	Ujvari et al., 2004
Hepatozoon spp.	18S rRNA	F-HAM1 / GCCAGTAGTCATATGCTTGTC R-HPF2 / GACTTCTCCTTCGTCTAA G	1º Round: 95 °C for 3 min; 40 cycles of 95 °C for 1 min, 56 °C for 1 min and 72 °C for 1 min and 30 sec; Final extension of 72 °C for 7 min.	1120	Criado-Fornelio et al., 2006
		F-4558 / GCTAATACATGAGCAAAATCTCAA R- 2733 / CGGAATTAACCAGACAAAT	2º Round: 95 °C for 3 min; 40 cycles of 94 °C for 1 min, 55 °C for 2 min and 72 °C for 2 min; Final extension of 72 °C for 10 min.		Mathew et al., 2000

F – Forward; R – Reverse.

Table 2. Tick species collected from *Nasua nasua* and tested for Hemoprotozoa.

Tick species	**Nº of tested samples**	**Nº of positive samples**	**Hemoparasite detected**
A. dubitatum	5 (4 Nymph, 1 Pool)	0	
A. ovale	25 (10 Female, 15 Male)	1 (Female) 1 (Male)	*H. procyonis* and *Hepatozoon* sp.
A. sculptum	17 (1 Female, 7 Nymph, 9 Pools)	2 (1 Pool and 1 nymph)	Piroplasmida
R. sanguineus s. l	1 (1 female)	0	

Nº - number; *A. dubitatum – Amblyomma dubitatum*; *A. ovale – Amblyomma ovale*; *A. sculptum – Amblyomma sculptum*; *R. sanguineus* - *Rhipicephalus sanguineus* sensu lato; *H. procyonis – Hepatozoon procyonis*; *H. americanum – Hepatozoon americanum*.

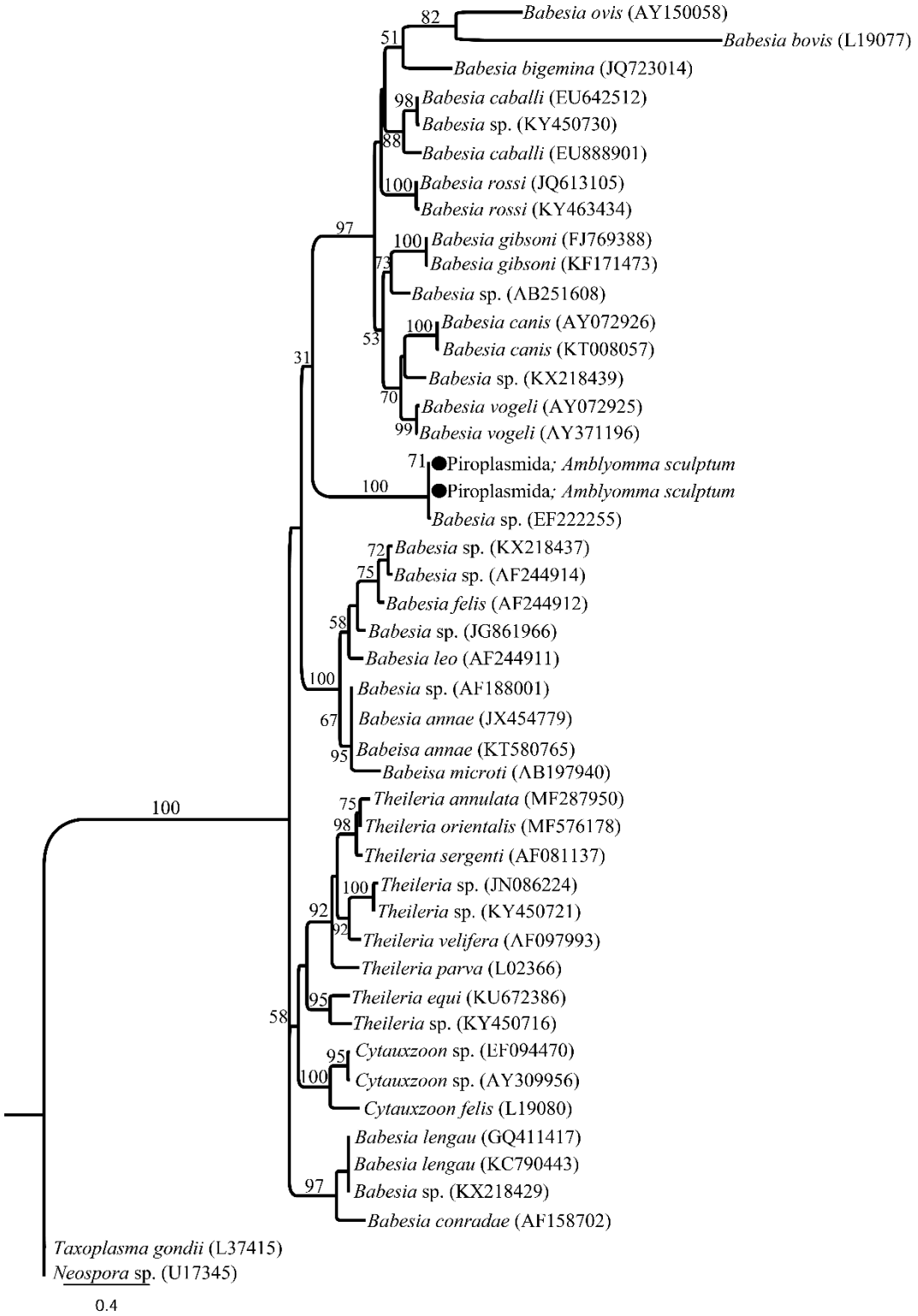

Fig. 1. Maximum likelihood tree inferred, on the partial sequences (709 bp) of the 18Sr RNA gene, among Piroplasmida sequences isolated in this study and sequences deposited in GenBank. Scale indicates an evolutionary distance of 0.04 nucleotides per

position. *Toxoplasma gondii* and *Neospora* sp. were used as outgoups. (●) sequences isolated in this study.

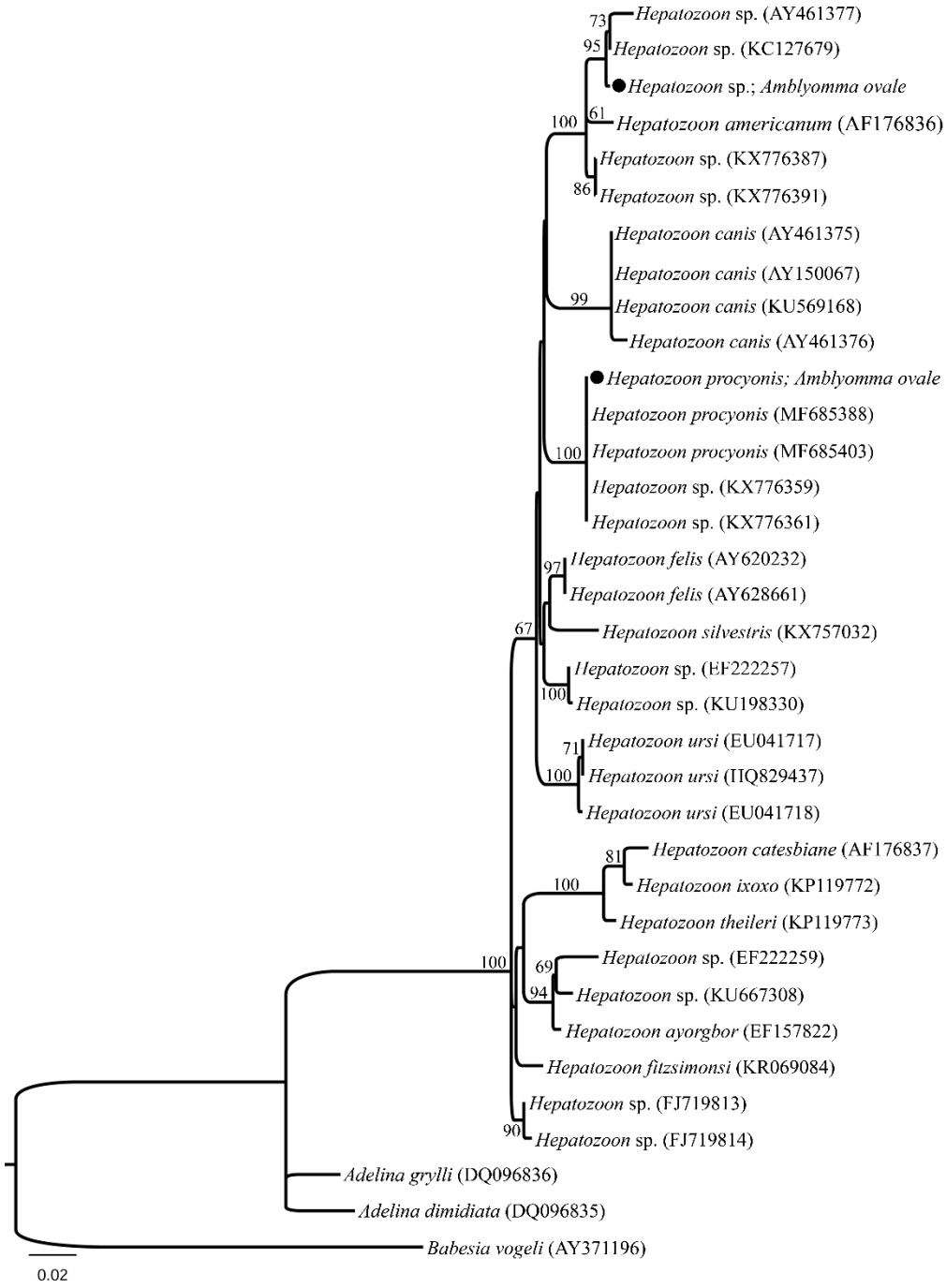

Fig. 2. Maximum likelihood tree inferred on the partial sequences (637 bp) of the 18Sr RNA gene of *Hepatozoon* spp. from *Amblyomma ovale* obtained in this study and sequences deposited in GenBank. Scale indicates an evolutionary distance of 0.02 nucleotides per position. *Babesia vogeli*, *Adelina dimidiata* and *Adelina grylli* were used as outgoups. (●) sequences isolated in this study.

7. CAPÍTULO 3

ARTIGO CIENTÍFICO:

Didelphis albiventris naturally infected with *Hepatozoon canis* in southeastern Brazil

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Short communication

Didelphis albiventris naturally infected with *Hepatozoon canis* in southeastern Brazil

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ABSTRACT

Hepatozoon species are vector-borne pathogens that infect domestic and wild animals. Marsupials of the species *Didelphis albiventris* are adapted to urban and peri-urban areas and act as reservoir hosts for several parasites. The present study evaluated the occurrence of infection by *Hepatozoon* species in synantropic *D. albiventris* from Botucatu, São Paulo, Brazil. Blood samples and ectoparasites from 19 *D. albiventris* were collected from urban and peri-urban areas. *Hepatozoon* spp. detection was performed by microscopy and molecular analysis. One opossum was positive for *Hepatozoon* spp. in microscopy analysis and PCR, while another animal was positive only in PCR. The obtained sequences were 100% identical to *Hepatozoon canis*. Six species of ticks and two species of fleas were detected on *D. albiventris*. This is the first report of *H. canis* in synantropic *D. albiventris*. In Brazil, *H. canis* transmission among dog populations is not well established, which highlights the importance of investigating the role that opossums might play in the epidemiology of this protozoan.

8. CAPÍTULO 4

ARTIGO CIENTÍFICO:

Morphological and molecular characterization of hemoparasites in *Didelphis* spp. from Brazil

.

Morphological and molecular characterization of hemoparasites in *Didelphis* spp. from Brazil

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ABSTRACT

Babesia spp., *Theileria* spp. and *Hepatozoon* spp. are tick-borne hemoparasites that infect domestic and wild animals. The aims of this study were to evaluate the presence of *Hepatozoon* and piroplasms in *Didelphis* spp. and to characterize the parasites found. Sixty-nine *D. albiventris* and 11 *D. aurita* were examined for piroplasm presence. For *Hepatozoon* detection, 50 blood samples from *D. albiventris* and 11 blood samples from *D. aurita* were used. Twenty-five tissue samples also were for presence of *Hepatozoon* spp. Hemoparasite detection was performed by molecular and morphological methods. All samples from *D. albiventris* were negative for *Hepatozoon* sp. One sample from *D. aurita* was positive in PCR for *Hepatozoon* DNA and the obtained sequence was closely related to *Hepatozoon* sp. from a Chilean marsupial. Morphologically, a small and a large piroplasm were observed in *D. albiventris*. In the molecular analysis four piroplasm haplotypes were detected. Piroplasm sequences obtained here were identical to *Babesia* sp. detected in *D. marsupialis* from Brazil and closely related to *Babesia* sp. from *Monodelphis domestica*. In conclusion, *D. albiventris* is likely an accidental host for *H. canis*. *Hepatozoon* sp from *D. aurita* and piroplasms from *D. albiventris* should be better characterize.

Keywords: *Didelphis albiventris*, *Didelphis aurita*, *Hepatozoon* sp., piroplasms, tick-borne protozoa.

1. Introduction

Tick-borne hemoparasites, which include apicomplexan microorganisms of the genera *Babesia* spp. *Theileria* spp. and *Hepatozoon* spp., are protozoa that can cause disease in livestock, domestic animals and humans (Baneth et al., 2003; Yabsley and Shock 2013; Alvarado-Rybak et al., 2016). Piroplasms are intraerythrocytic parasites transmitted by the bite of Ixodid ticks. These protozoa are frequently found infecting wild animals worldwide (Alvarado-Rybak et al., 2016). *Hepatozoon* spp. infect leucocytes of mammals and are transmitted to these animals commonly through the ingestion of ticks containing mature oocysts (Smith, 1996). Wild animals are considered reservoir for *Hepatozoon* species that affects dogs (Cardoso et al., 2014, Duscher et al., 2013, Duscher et al., 2015).

The genus *Didelphis* is composed of five species: *Didelphis albiventris*, *Didelphis aurita, Didelphis marsupialis*, *Didelphis virginiana* and *Didelphis imperfecta* (Cerqueira, 1985). These animals have a generalist diet, present synantropic habits and, live close to domestic animals and humans (Muller et al., 2005; Godim et al., 2017). In addition, *Didelphis* spp. play a role as reservoirs for *Trypanosoma cruzi* and *Leishmania infantum* (Yeo et al., 2005; Carreira et al., 2012; Humberg et al., 2012).

Hepatozoon spp. have been reported infecting *D. marsupials* and *D. albiventris* in Colombia and in French Guyana (Ayala et al., 1973; Thoisy et al., 2000). In Brazil, *Hepatozoon* infection was not detected in *D. albiventris* from South and Midwest regions (Criado-Fornelio et al., 2006; Wolf et al., 2016). In the Southeast region, Silva et al. (2017) related two *D. albiventris* infected by *Hepatozoon canis*. These authors emphasized the importance of investigating the role that *D. albiventris* might play in the epidemiology of *H. canis*.

Piroplasms are hemoparasites frequently detected in Australian marsupials and several species are described in these animals (Lee et al., 2009; Paparini et al., 2012; Dawood et al., 2013; Donahoe et al., 2015; Barbosa et al., 2017). In Brazilian marsupial of the gender *Didelphis* infection by piroplasm was firstly detected in *D. masupialis* from Northern region (Deane and Denae, 1961). This hemoparasite was identified as *Nuttallia brasiliensis*, a small piroplam synonym of *Babesia brasiliensis* (Deane and Deane, 1961; Sampaio and Massard, 2003). Posteriorly, Serra-Freire (1979) described a large piroplasm infecting *D. albiventris* and *D. marsupialis*, named *Babesia ernestoi*. This large piroplasm was also considered synonym of *B. brasiliensis* (Sampaio et al., 2001). In recent reports, via molecular methods, *Babesia* spp. were diagnosed in the marsupials *Monodelphis domestica* and *D. marsupialis* (Wolf et al., 2016; Soares et al., 2017). However, piroplasms especies that infect *Didelphis* spp. from Brazil remain unclear.

Information about hemoparasites from marsupials, in Brazil, are limited. Then, the aims of this study were to assess the occurrence of *Hepatozoon* spp. and piroplams in *D. albiventris* and *D. aurita* and to characterize the protozoa found.

2. Materials and Methods

Between September 2013 and September 2017, 69 *D. albiventris* and 11 *D. aurita* were sampled in Botucau (22° 53′ 25″ S, 48° 27′ 19″ W) and São Paulo (23° 29′ 29.0″ S, 46° 31′ 15.3″ W) municipalities, respectively. All *D. albiventris* were captured in the urban and peri-urban regions of Botucatu, and were sent to the Centro de Medicina e Pesquisa em Animais Selvagens (CEMPAS). In São Paulo, *D. aurita* were sampled in the “Centro de Triagem de Animais Silvestres (CETAS) of the Parque Ecológico do Tietê”, from the urban area of municipality. All *D. albiventris* and *D.*

aurita were examined for the presence of piroplasms. The diagnostic of *Hepatozoon* spp. and ectoparasites was only performed in 50 *D. albiventris* and 11 *D. aurita*, since 19 *D. albiventris* were previously screened by Silva et al. (2017).

A combination of ketamine and midazolam was used to anesthetize the *Didelphis* spp. and, posteriorly, blood samples were collected from the tail vein and stored in EDTA tubes at -20 °C until DNA extraction. Thin blood smear were prepared, fixed with methanol and stained with 10 % Giemsa for microscopic analysis.

Each *Didelphis* spp. was inspected for ectoparasites. Fleas and ticks were removed and stored in 70% ethanol. Morphological identification of arthropods was performed using taxonomic keys (Barros-Battesti et al., 2006; Martins et al., 2010; Linardi and Santos, 2012).

Histopathological analysis

Twenty-five *D. albiventris* that arrived at CEMPAS severely injured were euthanized, and tissue samples (spleen and liver) were collected for histopathological and molecular analyses. For histopathology, tissues were formalin-fixed, paraffin embedded, sliced at a thickness of 5 μm, and stained with haematoxylin and eosin (H&E).

Morphometric analysis

The parasites detected in the thin blood smears were measured via a computerized image analysis system, using the software QWin Lite 2.5 (Leica). When possible, twenty piroplasms, per animal, had their diameter measured.

125

126 **DNA extraction, amplification, cloning and sequencing**

127 Genomic DNA was isolated from 200 μL of *D. aurita* and *D. albiventris* blood
128 or 20–50 mg of tissues (liver and spleen) from *D. albiventris*, using the Illustra™ blood
129 genomicPrep Mini Spin Kit and the Illustra™ tissue and cells genomic Prep Mini Spin
130 Kit (GE Healthcare, Buckinghamshire, UK), respectively, following the manufacturer's
131 instructions. DNA concentrations (ng/μL) and quality (A260nm/A280nm) were
132 measured using spectrophotometry (NanoDrop® ND-2000 Spectrophotometer, Thermo
133 Scientific, Wilmington, DE, USA).

134 For molecular detection of piroplasms, PCR conventional was performed using
135 the primer pair BAB2 143-167 and BAB2 694-667, targeting a ≈ 500-bp fragment of the
136 18S rRNA gene of piroplasms (Almeida et al. 2012). All positive samples were
137 amplified and sequenced using the primers BT18SF1/BT18SR1 and
138 BT18SF2/BT18SR2 that amplified ≈ 1466 bp (Paparini et al., 2012). We also used the
139 primers BTF1/BTR1 and BTF2/BTR2 (Jefferies et al., 2007).

140 The primers Hep300 and Hep900 were used for molecular diagnostic of
141 *Hepatozoon* spp. (Ujvari et al., 2004). The PCR conditions of all reactions were
142 described previously (Silva et al., 2018). A negative (distilled sterile water) and a
143 positive control (*Hepatozoon canis* or *Babesia vogeli* DNA isolated from naturally
144 infected dogs were used in each reaction).

145 The amplicons were purified using Illustra ExoProStar Step (GE Healthcare,
146 Buckinghamshire, UK) in agreement with the manufacturer's instructions and were
147 sequenced using BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction Kit
148 (Applied Biosystems, Foster City, CA, USA) and an automated Applied Biosystems
149 ABI 3500 DNA genetic analyzer.

Cloning

One sample of *D. albiventris* that morphologically presented co-infection with a small and a large piroplasm and that was previously sequenced with the primers BT18SF2/R2 (Paparini et al., 2012), and the electropherogram presented double peak, had also its PCR product, amplified with Hep300 and Hep900 (Ujavari et al., 2004), purified and cloned. The PCR product was cloned using pGEM-T Easy Vector System II (Promega, USA). After transformation of competent cells, plasmid DNA was extracted and sequenced with specific primer pair.

Plylogenetic analysis

The obtained sequences, in our study, were edited using the BioEdit software v7.2.5 (Hall, 1999). The sequences were compared for similarity to the sequences available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Sequences in this study were aligned with sequences available in GenBank by the MUSCLE algorithm, in the software GENEIOUS v.7.1.3 (Biomatters, http://www.geneious.com). The best evolutionary model for maximum likelihood analysis was identified by the jModelTest v.2.1.10 (Darriba et al., 2012). The best models chosen based on the Akaike Information Criterion (AIC) were TVM + G and GTR + I + G for phylogenetic reconstruction of *Hepatozoon* spp. and piroplasms, respectively. A phylogenetic tree was inferred by the maximum likelihood methods using PhyML v.3.0 (Guindon et al., 2010), with 1000 bootstrap replicates. For displayed phylogenetic tree FigTree v.1.4.3 was used (http://tree.bio.ed.ac.uk/software/figtree/).

3. Results

The 50 *D. albiventris* and 11 *D. aurita* examined for presence of *Hepatozoon* spp. were negative for this parasite, in the thin blood smear. The liver and spleen of 25 *D. albiventris* were also negative in the histopathological and molecular analyses. In the PCR, for detection of *Hepatozoon* spp. DNA from blood samples, all *D. albiventris* were negative and one *D. aurita* was positive (Table 1). *Hepatozoon* sp. sequence obtained from *D. aurita* showed 98% similarity for *Hepatozoon* sp. (FJ719813), isolated from a marsupial in the Chile, and for *Hepatozoon felis* (AB771570). The phylogenetic tree inferred by *Hepatozoon* 18S rRNA gene partial sequences (659 bp) showed that *Hepatozoon* sp. sequence obtained in our study grouped in a clade with two sequences (FJ719813, FJ719814) isolated from *Dromiciops gliroides*, a Chilean marsupial (Fig. 1). The nucleotide difference among *Hepatozoon* sp. obtained in this study, and *Hepatozoon* sp. (FJ719813) and *Hepatozoon* sp. (FJ719814) from marsupial was of 2.03% and 2.45%, respectively.

All *D. aurita* were negative for piroplasms in the thin blood smear and PCR. Piroplasms were detected in the blood smear of 12 (17.39%) *D. albiventris*. Morphologically, large and a small of piroplasms were observed. Eight animals were only infected by large piroplams and one *D. albiventris* presented only small piroplasms. In three animals, co-infections with small and large piroplasms were observed. Large piroplasms showed spherical and elongated forms (Fig. 2), which had the diameter with mean of 3.39 ± 0.3 µm (n = 76) and 4.1 ± 0.6 µm (n = 16), respectively. Maltese cross formation and spherical forms (Fig. 3) were observed in thin blood smears from animals infected with small piroplasms. Spherical forms measured in mean 1.45 ± 0.47 µm (n = 13).

Piroplasm DNA was detected in 20 (28.98%) *D. albiventris* samples of the 69 examined (Table 1). Seventeen sequences with high quality were obtained. Three samples presented chromatograms with low quality. In two of the three chromatograms double peaks were observed. One sample was positive only for small piroplasm, and the other two were co-infected for larger and small piroplasms, in morphological analyses, were the samples that showed double peaks in the chromatograms. The seventeen sequences were identical and BLAST search revealed 95% similarity for *Theileria bicornis* (AF499604) and *Theileria* sp. (JQ682879) detected in marsupials from Australia and for *Babesia ardeae* (KY436057) and *Babesia* sp. (KC754965) from *Sula leucogaster* in Brazil. The maximum likelihood tree (Fig. 4), inferred from a short (408 bp) fragment of the 18S rRNA gene demonstrated that piroplasm sequence isolated from *D. albiventris* in this study grouped in the same clade with *Babesia* sp. (KY684002) from *D. marsupialis* of the northern region of Brazil and with *Babesia* sp. (KP757839) detected in the marsupial *Monodelphis domestica* captured in Brazilian Pantanal.

One sample that morphologically showed large and small piroplasm was cloned and three distinct sequences were obtained. The three cloned sequences were also different from the sequences obtained by convenctional PCR and sequencing. In the *p* distance analysis, the four haplotypes detected presented a low percentage of nucleotide divergence among them (Table 2). In addition, one haplotype was 100% similar to *Babesia* sp. (KY684002) detected from *D. marsupials* in the Amazônia (Soares et al., 2017).

Ticks *Ixodes loricatus*, *Amblyomma sculptum*, *Amblyomma dubitatum*, *Amblyomma ovale*, *Ornithodoros mimon* and *Amblyomma* sp. larvae, and fleas *Ctenocephalides felis* and *Polygenis* sp. were found infesting the 19 animals previously

assessed for presence of ectoparasites (Silva et al., 2017). In this study, ticks were found infesting six *D. albiventris* and one *D. aurita* (Table 1). Ticks were identified as *A. dubitatum*, *A. ovale*, *Amblyomma* sp. and *I. loricatus*. Fleas *Ctenocephalides canis*, *C. felis* and *Polygenis* spp. were identified parasitizing two *D. aurita* and seven *D. albiventris* (Table 1).

4. Discussion and Conclusion

In this study, we assessed the occurrence of *Hepatozoon* spp. and piroplasms in *D. albiventris* and *D. aurita* and reported the first molecular detection of *Hepatozoon* sp. in *D. aurita*. *Hepatozoon didelphydis* have been reported infecting *D. marsupialis* in Colombia and French Guyana (Ayala et al., 1973; Thoisy et al., 2000). However, these reports were only based in morphological analysis. *Dromiciops gliroides*, a marsupial present in Chile, was infected with *Hepatozoon* sp. by microscopic and molecular methods (Merino et al., 2009). *Hepatozoon* sp. sequence detected in our study grouped with *Hepatozoon* sequences from *D. gliroides* in the phylogenetic analysis and presented low nucleotide divergence among them indicating that *Hepatozoon* sp. from *D. aurita* is a haplotype of *Hepatozoon* sp. (FJ719813 and FJ719814) from *D. gliroides*.

Recently, Silva et al. (2017) identified two *D. albiventris* from peri-urban and urban areas of Botucatu municipality infected with *H. canis*. In our study, all *D. albiventris*, sampled in this same region, were negative for *Hepatozoon* spp. In the South and Midwest regions of Brazil, this marsupial species was also not infected by *Hepatozoon* spp. (Criado-Fornelio et al., 2006; Wolf et al., 2016). These results indicate that probably *D. albiventris* is an accidental host for *H. canis* and does not appear to be involved in the epidemiology of this hemoparasite.

Piroplasms are usually classified in to two groups based in their diameter: small piroplasms (>1–2.5 µm) and large piroplasms (≥2.5–5 µm) (Schnittger, 2012). Based in this classification, we detected larger and small piroplasmids, infecting erythrocytes of *D. albiventris.* A small piroplasm previously detected from *D. marsupialis*, in Brazil, was identified as *Babesia brasiliensis,* based on morphological analysis (Deane and Deane, 1961, Sampaio and Massard, 2003). *Didelphis albiventris* and *D. marsupials* were also infected by a large piroplasm named *B. ernestoi*, also based only on morphological methods (Serra-Freire, 1979). Although, *B. ernestoi* was morphologically large and has not demonstrated Maltese cross formation, characteristic of small piroplasms, this species was considered synonym of *B. brasiliensis* (small piroplasm) (Sampaio et al., 2001). Based on PCR, cloning and sequencing we identified four different piroplasm sequences, which presented low nucleotide divergence, indicating that are haplotypes of a same species. 18S rRNA gene is highly conserved (Paulino et al., 2018), and we sequenced a small fragment (600 pb) of cloned product, what may have hampered the identification of different species. In addition, some species of piroplasms are pleomorphic and may differ in form and size when infect different mammals hosts (Homer et al., 2000). Further studies are necessary to elucidate whether small and large piroplasms observed in *D. albiventris* represent a same pleomorphic species or whether are two distinct species.

Piroplasmids molecularly identified in this study grouped with sequences of *Babesia* spp. obtained from *D. marsupialis* in Amazonia and from *M. domestica* in Brazilian Pantanal (Wolf et al., 2016; Soares et al., 2017). Our sequence was identical to *Babesia* sp. from *D. marsupials* suggesting that these two sequences represents a same haplotype of a species. Furthermore, *Babesia* sp. isolated from *M. domestica,* probably, is a distinct haplotype of the same piroplasm species identified in *D. marsupialis*

(Soares et al., 2017) and in *D. albiventris*, in our study. However, new study using other genes are necessary to confim this hypothesis.

Wolf et al. (2016) observed that all *M. domestica* and *Thrichomys pachyurus* positive for *Babesia* spp., in Pantanal of Brazil, were also infested by *Ornithodoros guaporensis*. These authors suggested *O. guaporensis* could participate in the transmission of *Babesia* spp. for animals in this region. *Ornithodoros mimon* was detected previously on two *D. albiventris* (Silva et al., 2017), that were positive for piroplasm in this report. *Onithodoros moubata* is considered vector of *Babesia gibsoni* (Battsetseg et al., 2007). The potential of *O. mimon* as vector of piroplasm from *D. albiventris* should be investigated.

Other tick species that can be the vector of piroplasm from *D. albiventris* is *I. loricatus*, since this tick was the species most prevalent observed here. Moreover, several species of *Ixodes* as *Ixodes scapularis*, *Ixodes ricinus*, *Ixodes persulcatus* and *Ixodes dentatus* act as vectors of *Babesia* spp. (Yabsley and Shock, 2013).

In conclusion, *D. albiventris* is likely an accidental host for *H. canis* and seem not have epidemiological importance in the cycle this parasite. *Hepatozoon* sp. infect *D. aurita* and this protozoa should be better characterized in the future. Morphologically we observed large and small piroplasms infecting *D. albiventris* and by molecular analysis, four piroplasm haplotypes were detected. New studies are necessary to characterize and correctly identify the piroplasms present in these marsupials.

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Table 1. Prevalence of piroplasms and *Hepatozoon* spp. infection and ectoparasites species collected from *Didelphis albiventris* and *Didelphis aurita* in Botucatu and São Paulo municipalities.

Host	**Piroplasm**			***Hepatozoon* spp.**			**Ectoparasites**		**Nº of tick specimens collected**						**Nº of flea specimens collected**				
	Nº	Blood smear (n/%)	PCR (Blood) (n/%)	Nº	Blood smear (n/%)	PCR (Blood) (n/%)	Nº	Nº of animals infested		*A. dubitatum*	*A. ovale*		*Amblyomma* sp.		*I. loricatus*		*C. canis*	*C. felis*		*Polygenis* sp.	
								Ticks (n/%)	Fleas (n/%)	N	N	F	L	F	M	F	F	M	F	M
D. albiventris	69	12 (17.4)	20 (28.9)	50	0	0	50	6 (12)	7 (14)	2	4	1	2	5	6		9	4	2	2
D. aurita	11	0	0	11	0	1 (9)	11	1(9)	2 (18)					1		1	9	8		
Total	80	12 (15)	19 (23.7)	61	0	1 (1.6)	61	7 (11.5)	9 (14.7)	2	4	1	2	6	6	1	18	12	2	2

Nº - number; *D. albiventris – Didelphis albiventris*; *D. aurita – Didelphis aurita; A. dubitatum- Amblyomma dubitatum*; *A. ovale – Amblyomma ovale; I. loricatus - Ixodes loricatus; C. canis - Ctenocephalides canis; C. felis - Ctenocephalides felis;* N – Nymph; F- Female; L – Larvae; M – Male.

Table 2. Distance matrix among piroplasms sequences detected from one *D. albiventris* in this study and other marsupials species. Upper triangle shows the percentage of nucleotide difference, while the lower triangle shows the number of nucleotide difference.

Sequences	**1.** **(KP757839)**	**2** **(KY684002)**	**3** **Haplotype 1** **(PCR conventional)**	**4** **Haplotype 2** **(Clone I)**	**5** **Haplotype 3** **(Clone III)**	**6** **Haplotype 4** **(Clone IV)**
1. *Babesia* sp.		1.25%	1.25%	1.5%	1.5%	1.75%
2. *Babesia* sp.	5		0	0.25%	1.25%	0.5%
3. Piroplasm	5	0		0.25%	0.25%	0.5%
4. Piroplasm	6	1	1		0.25%	0.25%
5. Piroplasm	6	1	1	1		0.25%
6. Piroplasm	7	2	2	1	1	

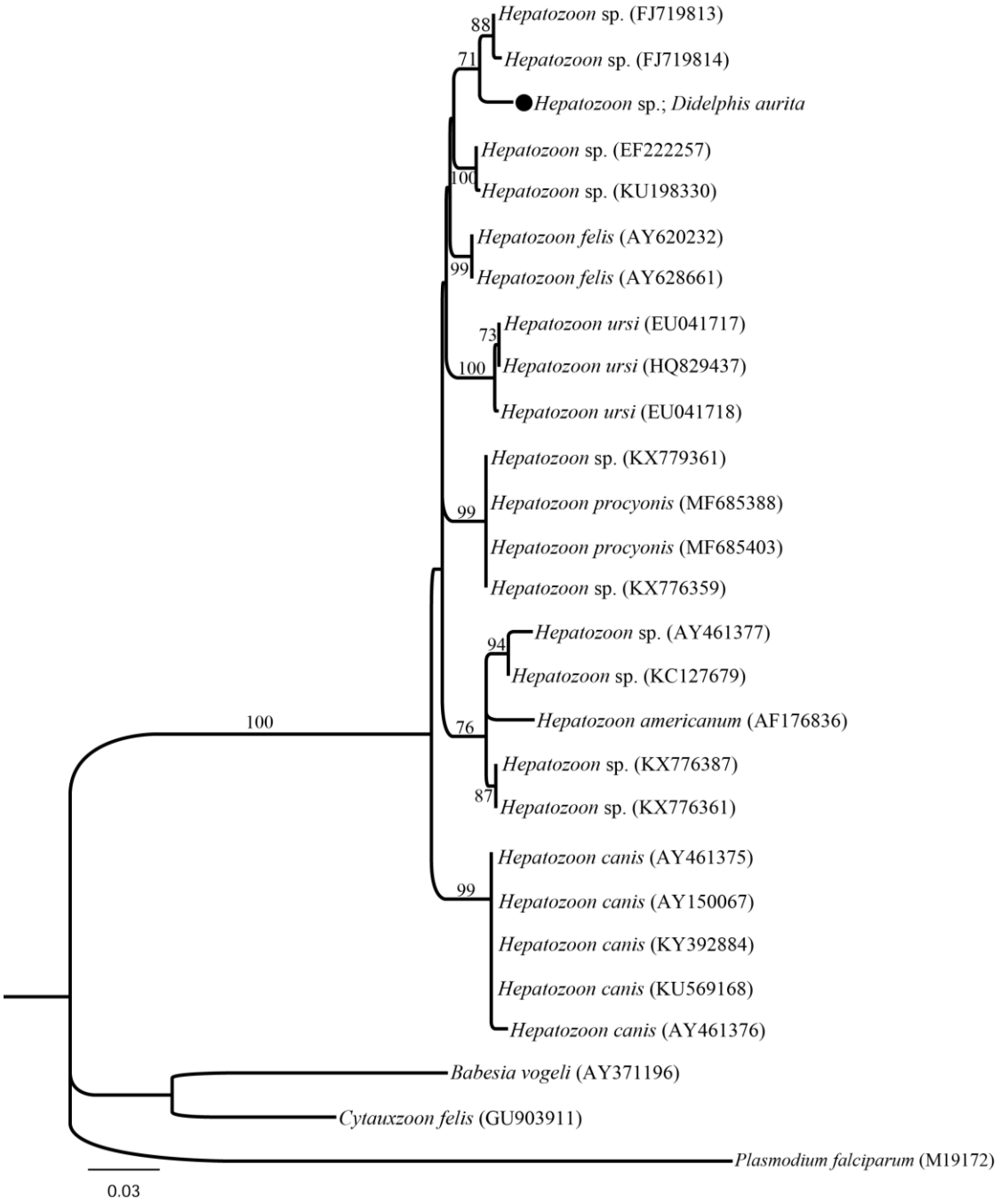

Fig. 1. Maximum likelihood tree of *Hepatozoon* sp. from *D. aurita*, isolated in this study with other *Hepatozoon* spp., based on 18S rRNA partial sequences (659 bp). Scale indicates an evolutionary distance of 0.03 nucleotides per position. *Babesia vogeli*, *Cytauxzoon felis* and *Plasmodium falciparum* were used as outgoups. (●) sequences isolated in this study.

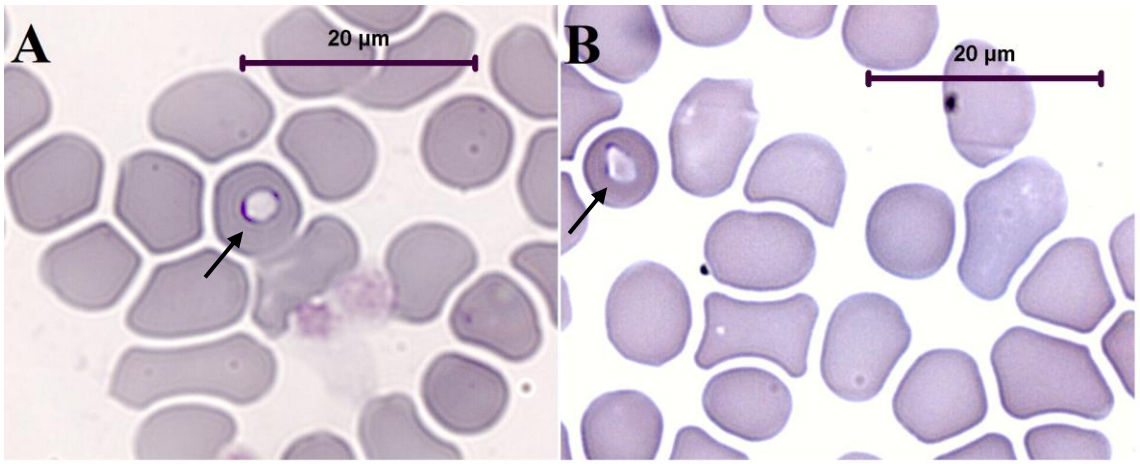

Fig 2. Larger piroplasms in the erythrocytes from *D. albiventris*. **A –** Spherical form. **B –** Elongated form.

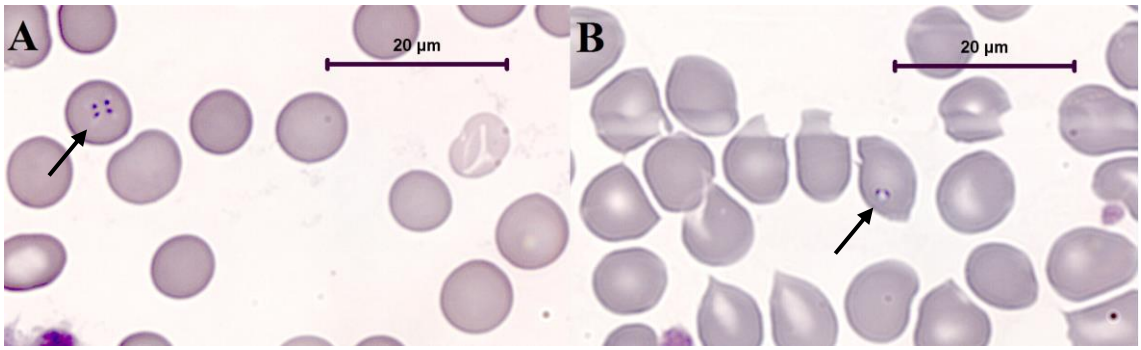

Fig 3. Small piroplasms in the erythrocytes from *D. albiventris*. **A** – Maltese cross formation. **B –** Spherical form.

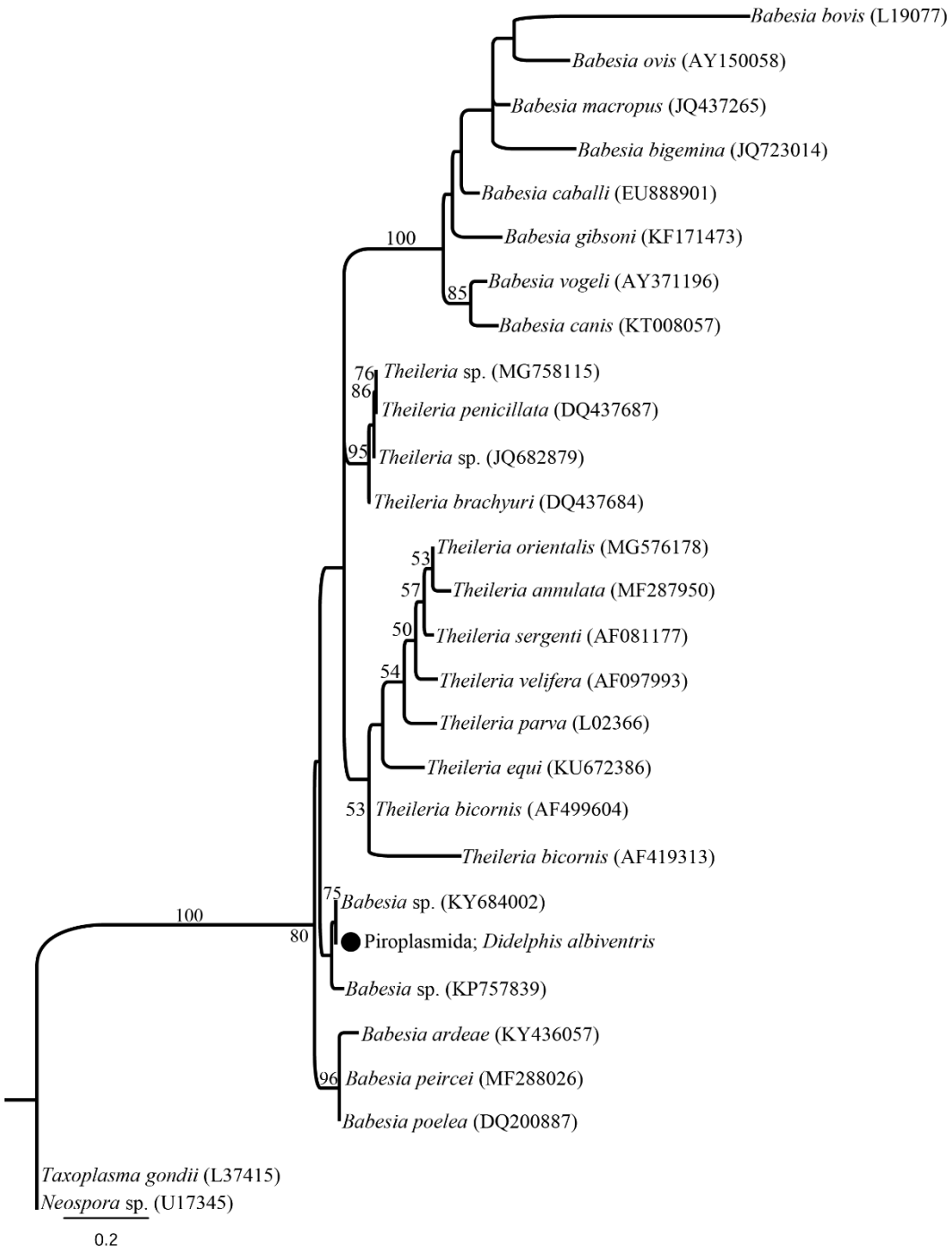

Fig. 4. Maximum likelihood tree o based on 18S rRNA partial sequences (408 bp) of piroplasmida from *D. albiventris* with other piroplasm species. Scale indicates an evolutionary distance of 0.2 nucleotides per position. *Toxoplasma gondii* and *Neospora* sp. were used as outgoups. (●) sequences isolated in our study.

9. Considerações Finais

- Neste estudo foi possível observar que *N. nasua* da região peri-urbana e urbana de Botucatu e São Paulo são infectados por *Hepatozoon procyonis.* Este parasita parece ser comum nestes animais pois, já foi detectado em outras regiões do país. Entretanto, os quatis capturados em Palmital não foram infectados por *H. procyonis,* provavelmente porque esses animais viviam restritos ao ambiente urbano e além disso, eles eram alimentados diariamente e se encontravam em boas condições nutricionais, o que pode ter interferido visto que, *Hepatozoon* spp. são parasitas oportunistas.
- *Didelphis albiventris* foi infectado por *Hepatozoon canis.* Porém, apenas dois animais de 69 analisados foram infectados, o que indicou que *D. albiventris* é apenas um hospedeiro acidental para este organismo. Além disso, *D. albiventris* também foi infectado por piroplasmas. Morfologicamente observamos um pequeno e um grande piroplasma. Novos estudos são necessários para determinar as (a) espécies (e) de piroplasma que acometem esses animais, bem como determinar o seu vetor e patogenia.
- *Didelphis aurita* capturado em São Paulo são infectados por *Hepatozoon* sp. filogeneticamente relacionado com *Hepatozoon* sp. (FJ719813 e FJ719814) detectado em *Dromiciops gliroides,* um marsupial amostrado no Chile. Novos estudos são necessários para caracterizar essa espécie de *Hepatozoon*.
- *Nasua nasua*, *D. aurita* e *D. albiventris* são infestados por ectoparasitas e várias espécies foram identificadas. DNA de *Hepatozoon* spp. circula em *Amblyomma ovale. Hepatozoon procyonis* e *Hepatozoon* sp. filogeneticamente próximo de *Hepatozoon americanum* foram detectados em *A. ovale*. DNA de *Babesia* sp.

filogeneticamente próximo de *Babesia* sp. capybara foi detectado em *Amblyomma sculptum*.

- Para finalizar, este estudo forneceu dados epidemiológicos sobre hemoparasitas e ectoparasitas em *N. nasua* e *Didelphis* spp., além de caracterizar filogeneticamente os hemoparasitas e redescrever *H. procyonis*.